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Phenstatin Analogues With Non-Aromatic Attachments In Place Of The B-Ring

Amanda Oswald
Department of Chemistry
The University of North Carolina at Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Herman Holt Jr.

Abstract

Cancer is a deadly disease caused by mutations in normal working cells; affecting millions of people each year.[1] Chemotherapy and surgeries are costly, necessitating the need for noninvasive and cost effective anti-cancer drugs.[2] Natural compounds like colchicine, combretastatin (CA-4), and phenstatin are examples of tubulin inhibiting drugs. These types of drugs attach to colchicine binding sites of microtubulin during the process of mitosis and halt cell division. The tubulin inhibiting drugs block nutrients and oxygen to the mutated cell therefore disrupting the cell's normal processes.[3] This research project aims to produce four new derivatives of phenstatin. The project focuses on the binding characteristics, like torsional and steric strain, of the new derivatives in the colchicine pocket. The phenstatin molecule will have a phenothiazine A-ring and a 3-, 4-, 7-, or 8- membered cyclic non-aromatic B-ring. The expectation is that the new derivatives will fit in the colchicine-binding site due to the hydrophobic interactions in the pocket by the phenothiazine and non-aromatic ring attachments. Phenothiazine based molecules were tested by another research group and found their phenstatin derivatives produced anti-cancer effects towards tumor cells. The challenge of synthesizing phenstatin analogues containing 3- and 4- membered non-aromatic rings will be due to their large torsional and steric strain. The case of synthesizing phenstatin analogues with 7- and 8- membered non-aromatic rings might be easier due to the low torsional strain. The synthesis for the desired phenstatin derivatives will be the focus of this paper.

Keywords: Phenstatin Analogues, Microtubulin-targeting agents, Anti-cancer Molecules

1. Introduction

Cancer is a disease that involves the production of mutated cells. These mutated cells divide at fast rates and attack major organs and tissues throughout the human body. According to the American Cancer Society, 1,685,210 patients will be diagnosed with some variation of cancer in 2016.[4] Although, treatments such as surgeries and therapies can be expensive, anticancer drugs have been studied to produce a noninvasive treatment for cancer patients. The purpose of this research is to create four phenstatin derivatives and determine if ring strain and size attribute to the affinity towards the colchicine binding site.

Anti-cancer molecules such as colchicine, combretastatin-A4 (CA-4), and phenstatin are known to deprive the mutated cell of nutrients and halt cancer cell division.[3] These anti-cancer molecules are also known as tubulin inhibiting drugs (Figure 1). The IC_{50} values for tubulin inhibitors are compared to each other (Table 1). The smaller the IC_{50} value is when half the drug is consumed, the more destructive the drug is to specific cancer cell lines. Colchicine is found in nature and is known as an irreversible inhibitor. An irreversible inhibitor is classified as a molecule that binds to a site or receptor and does not detach. Colchicine (Figure 1) contains two seven-membered rings and one six-membered ring. Ring A contains three methoxy groups, while the B-ring contains a carbonyl and a

single methoxy group. The IC_{50} value of colchicine of tubulin inhibition is 3.2±0.40 μM, which means that less of the drug is used to produce damage to specific cancer cell lines (Table 1). Although colchicine works to halt cancer cell division, it also attacks healthy cells making it dangerous for patients to consume. Combretastatin-A4 (CA-4), located in Figure 1, is naturally occurring in the *Combretum caffrum* tree, located in South Africa.[5] The structure of CA-4 is made up of two six membered rings held together by a double bond in the *cis* position. The *cis* conformation, in which both of the substituents are pointing in the same direction off the double bond, is important for this molecule because it is the most efficient way for CA-4 to fit into the colchicine pocket. The A-ring consists of three methoxy groups, which are assumed to be necessary for binding to the colchicine site of tubulin. Phenstatin is a naturally occurring derivative of CA-4. The phenstatin molecule has been heavily researched due to its favorable carbonyl bridge as well as the substituents off the A- and B-rings that are able to fit into the colchicine binding site of microtubulin. Both the IC_{50} values of phenstatin and CA-4 are not as low as colchicine, thus not as toxic. Colchicine is an irreversible inhibitor that is unable to detach from the colchicine binding site. The longer colchicine is attached to the site, the more toxic to cancerous and healthy cells. Both CA-4 and phenstatin act as reversible inhibitors, meaning that the drug will bind to a site and then detach. CA-4 and phenstatin are less toxic to the binding site because it is able to attach and detach, meaning that the toxicity only effects cancerous cells with minimal harm to healthy cells.

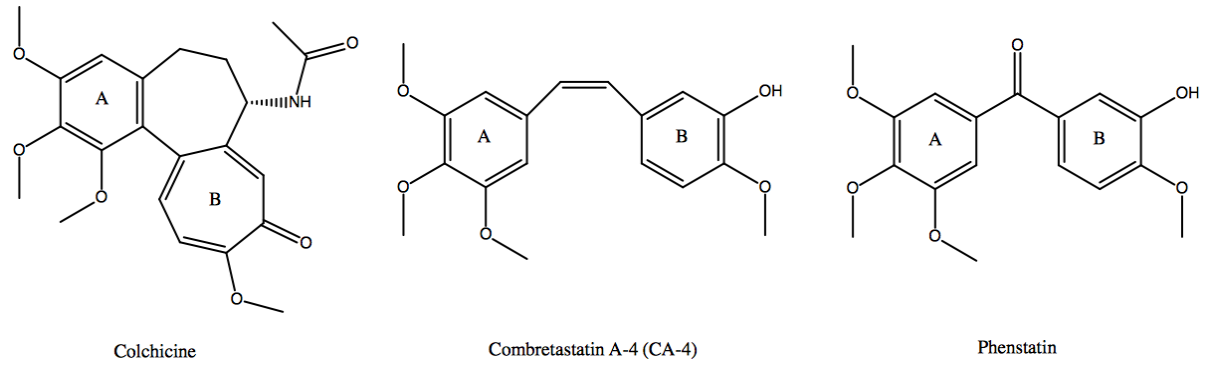


Figure 1: Three microtubulin targeting agents used in anti-cancer medicinal therapies.

Table 1: IC_{50} values for tubulin inhibition of various microtubulin-targeting agents and their derivatives. (units: micromolar)

Compound	**IC_{50} values (μM)**
Colchicine [6]	3.20±0.40
Combretastatin A-4 [7]	15.0±0.20
Phenstatin [5]	3.40±0.70
Abuhaie derivative 21 [5]	11.8±1.96

Phenstatin has grown in popularity due to the non-invasive drug therapy that microtubulin targeting agents have to offer. Microtubulin consists of alpha and beta proteins, a heterodimer, and is notably seen during the process of mitosis or cell division.[5] During mitosis, DNA will line up in the middle of a cell where microtubulin reaches out and begins to pull the DNA apart to begin producing two new daughter cells. The purpose of phenstatin is to deprive the cell of nutrients by attaching to colchicine binding sites located on microtubulin and shutting down the cell division process, thus blocking nutrients into the two new daughter cells.[3] Other research groups have studied many derivatives of phenstatin to understand how well a drug works in the binding pocket of microtubulin. The Abuhaie research group's derivatives were studied throughout this research to understand the importance and discovery of new derivatives that can potentially aid in cancer research. The Abuhaie group focused on synthesizing phenstatin derivatives with bulky A- and B-rings. The important aspect of their research was to identify whether the trimethoxy groups off the A-ring of the microtubulin targeting agent is necessary to bind to the colchicine binding pocket. The Abuhaie group replaced the A-ring of phenstatin with a tricyclic molecule, phenothiazine (Figure 2) and substituted the B-ring with various ring systems like indoles and aromatic rings with substituents. The most intriguing derivative, derivative 21, affected specific cancer cell lines and was able to fit into the colchicine binding pocket of microtubulin based on structure-

activity relationship (SAR) and docking studies (Figure 3).[5] SAR and docking studies help researchers understand how a molecule will interact with a specific environment. The carbonyl bridge of phenstatin remains on derivative 21 but both the A- and B-rings size and substituents have been altered to understand how these derivatives fit into the colchicine binding pocket. Results of the Abuhaie's research show that phenothiazine is able to fit into the pocket, but the trimethoxy groups are necessary to produce a greater binding affinity to the colchicine site.[5] However, derivative 21 was observed to halt cancer cell division of specific cancer cells better than phenstatin based on growth inhibition (GI_{50}) data.[5] GI_{50} is used to determine a drugs growth inhibition towards cancer cells.[5] The IC_{50} value of their derivatives were not as low as colchicine and phenstatin but demonstrated to the research community that molecules with more surface area are able to fit into the colchicine pocket of microtubulin (Table 1).

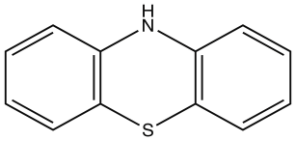


Figure 2: Phenothiazine; A-ring replacement.

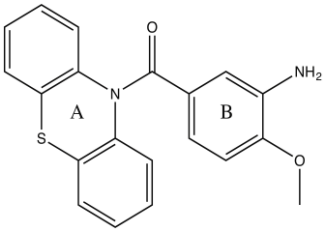


Figure 3: The Abuhaie research group's Derivative 21 of phenstatin.[5]

The focus of this research is to produce four new derivatives of phenstatin (Figure 4). The A-ring will consist of a phenothiazine molecule and the B-ring will utilize non-aromatic rings of various sizes. This research does not focus on the use of cyclopentane and cyclohexane because the emphasis of this research is to understand how size and torsional strain of the B-ring impacts the binding ability to the colchicine binding pocket. In addition, the use of aromatic rings in place of the B-ring were not tested because this research wants to investigate the advantages of non-aromatic rings interacting with the colchicine binding site. The idea is to use cyclopropanecarboxylic acid and cyclobutanecarboxylic acid to make an acid chloride and attach the phenothiazine molecule at the nitrogen atom for the 3 and 4-membered derivatives (Scheme 1). In addition, the 7 and 8-membered derivatives will be synthesized following a Grignard reaction by utilizing bromocylcoheptane and bromocyclooctane (Scheme 2). The goal is to have each of the new derivatives tested against cancer cells to determine IC_{50} values and observe if each molecule can fit into the colchicine binding site of microtubulin. This new information will aid in further research to give patients an option of a non-invasive cancer treatment.

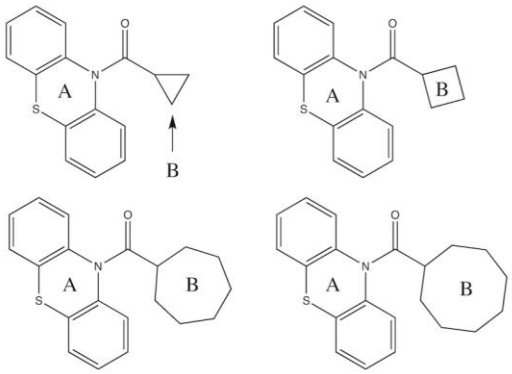
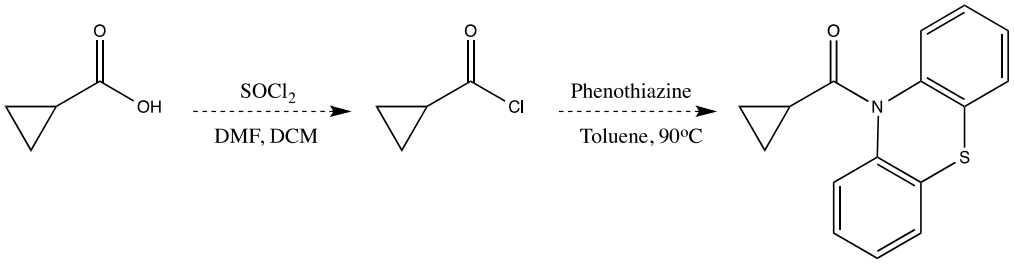
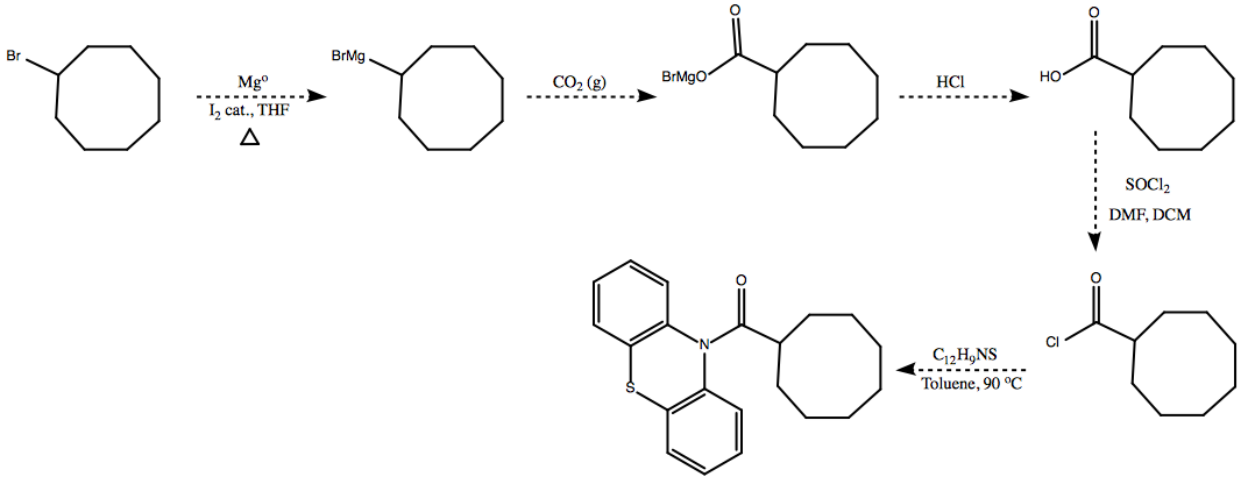


Figure 4: Four new target molecules.



Scheme 1: Proposed synthetic scheme of the acetylation of the phenstatin molecule. The phenstatin derivatives that contain the 3- and 4-membered non-aromatic rings will follow the same scheme.



Scheme 2: Proposed synthetic scheme to form 8-membered ring analog. Will be same for 7-membered ring analog.

2. Experimental

All products formed during this research were tested using an *Oxford 400 MHz ^{1}H-NMR* and a *Nicolet Thermo Scientific iS10 IR data.* Thin Layer Chromatography (TLC) glass plates with UV backing were used during this research to test products.

2.1. Representative Synthesis Of A Methyl Ester

Glassware was oven dried overnight to expel all traces of water. A two-necked 25 mL round bottom flask contained phenylacetic acid (201 mg, 1.48 mmol) and a stir bar. A nitrogen balloon was used to create an inert environment inside the flask. To the flask, thionyl chloride ($SOCl_2$) (0.2 mL, 2.76 mmol) was added along with 3-5 drops of N,N-dimethylformamide (DMF) and 3-5 drops of dichloromethane (DCM). The mixture was stirred for two hours and produced a yellow liquid. The reaction was monitored by TLC (70 Hexanes / 30 Ethyl Acetate) till all of the phenylacetic acid was reacted. Next, dry methanol (MeOH) (0.06 mL, 1.48 mmol) was added to the flask and remained under inert conditions overnight. The reaction was monitored by TLC (70 Hex/ 30 EA). The DCM and DMF were evaporated off the product. The crude product was dissolved in 20 mL of DCM and then washed twice with aqueous ammonium chloride (NH_4Cl). The organic layer was dried with K_2CO_3 and was filtered into a 50 mL one-necked round bottom flask through P8 filter paper. The DCM was evaporated away from the pure product till a colorless liquid formed. The pure product was characterized by IR and ^{1}H-NMR. The final product produced a 14% yield with a final mass of 31 mg. IR ν_{max} 3033, 1809, 750-699 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.64 (s, 2H), 3.71 (s, 3H), 7.28-7.38 (m, 5H).

2.2. Representative Synthesis Of An Acid Chloride

Glassware was oven dried for one hour to expel all traces of water. A three-necked 50 mL round bottom flask and stir bar were cooled to room temperature under nitrogen gas to produce an inert environment. Next, phenylacetic acid (500 mg, 3.67 mmol) was added to the flask along with $SOCl_2$ (0.4 mL, 5.51 mmol) (*Caution!*). In addition, 3 drops of DCM and DMF were added to the flask. The mixture was stirred for nine hours and monitored by TLC (70 Hex/ 30 EA then changed to 80 Hex/ 20 EA) until all of the starting material was reacted. Next, the crude product was evaporated till it produced an orange liquid. A separatory funnel was utilized to wash the crude product. To the orange liquid, 20 mL of DCM was added then transferred to the separatory funnel. To the separatory funnel, 10 mL of NH_4Cl (aq.) was used to wash the product. This step was done twice to eliminate water from the product. K_2CO_3 was added to the organic layer as a drying agent to absorb water. The product and drying agent were filtered with P8 filter paper through a funnel. The product was collected in a new flask and evaporated till an orange/brown oil was produced. The acid chloride was characterized by IR and ^{1}H-NMR. The pure acid chloride was obtained in a 13% yield with a final mass of 76 mg. IR ν_{max} 3031, 2954-2773, 1737, 1455, 1161 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.70 (s, 3H), 3.64 (s, 2H), 7.31 (m, 5H).

2.3. Synthesis Of The Phenothiazine Attachment

2.3.1 Continues from 2.2.

To the flask containing an acid chloride (2.2), phenothiazine (35 mL, 240 mmol) was dissolved in 10 mL of toluene and then refluxed at 90 °C. The reaction was monitored by TLC (70 Hex/ 30 EA). The crude product was transferred to a separatory funnel with ethyl acetate and washed with the following: 20% sodium bicarbonate ($NaHCO_3$), 5% HCl, and then 75% water. The organic layer was dried with magnesium sulfate ($MgSO_4$) and then filtered with P8 filter paper. The solvent was evaporated till a red solid was produced. TLC was used to observe two spots in the product. The product was characterized by IR and ^{1}H-NMR. Next, a gravity column with silica gel was used to separate the two compounds in the product (70 Hex/ 30 EA). The final product was a mixture and characterized by IR and ^{1}H-NMR. The product produced a 70% yield with a final mass of 55 mg. IR ν_{max} 2922-2852, 1682 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.78 (s, 2H), 7.07 (d, 1H), 7.07 (d, 1H), 7.22 (t, 2H), 7.25 (d, 4H), 7.31 (d, 1H), 7.33 (d, 1H), 7.35 (d, 1H), 7.39 (d, 1H), 7.41 (d, 1H).

2.4. Representative Synthesis Of A Grignard Reaction To Synthesize A Carboxylic Acid

Glassware was oven dried for one hour to expel all traces of water. In a two-necked 100 mL round bottom flask a stir bar and 403 mg of solid magnesium (Mg°) was added. The flask and the contents were flame dried and then cooled to room temperature with nitrogen gas. To the cooled flask, a crystal of iodine (I_2) and 4-bromoanisole (1.0 mL, 8.0 mmol) was added to the flask and stirred. In addition, 10 mL of tetrahydrofuran (THF) was added to the flask. The flask was then refluxed under nitrogen gas and monitored by TLC till all the starting material was gone. The flask was removed from the heat and cooled to room temperature while the flask was still under inert conditions. Another 10 mL of THF was added to the flask due to evaporation during the reflux process. The nitrogen balloon was removed and then carbon dioxide (CO_2) gas was bubbled into the flask through a needle for one hour with proper ventilation. A CO_2 (g) balloon was left in the flask for over sixteen hours. Next, 30 mL of 1M HCl was placed in an ice bath and chilled for about fifteen minutes. Slowly, the 30 mL of 1M HCl was added to the flask to dissolve the remaining Mg metal. After all the Mg metal was dissolved, 10 mL of ethyl acetate was added to the flask to dissolve solid product that formed. The product was pour into a separatory funnel with another 5 mL of ethyl acetate used to remove all of the solid product from the flask. The product was washed with additional ethyl acetate, (2 x 10 mL). The organic layer was dried (Na_2SO_4) and then filtered with P8 filter paper through a funnel. In a different one-necked round bottom flask, the pure product was collected and evaporated to produce an orange/white solid. The flask was then placed on the high-vac for one hour to evaporate all the solvent left in the flask. The pure product was characterized by IR and ^{1}H-NMR. The pure product, *p*-anisic acid, produced a 58% yield with a final mass of 705 mg. IR ν_{max} 2941-2545, 1683, 1578 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.89 (s, 3H), 6.98 (d, 2H), 8.09 (d, 2H).

3. Results and Discussion

The synthesis of the methyl ester was used as a verification method to determine if the acid chloride was synthesized. This method was used to ensure an acid chloride could be produced to move towards synthesizing an acid chloride with a non-aromatic ring. Dry methanol was used to substitute the chlorine atom to produce the methyl ester (Figure 5). The acid chloride and phenothiazine attachment were modeled after the Abuhaie research group's scheme.[5] The attempt to produce the acid chloride yielded 13% pure product. The acid chloride was then reacted with phenothiazine to produce the final product in 97% yield (Figure 6). The attachment of the phenothiazine molecule was verified by ^{1}H-NMR.

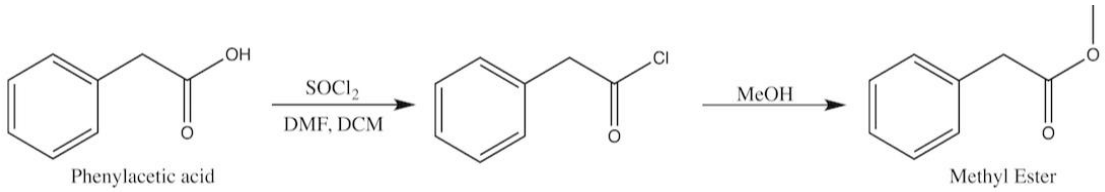


Figure 5: Synthesis and verification of a methyl ester.

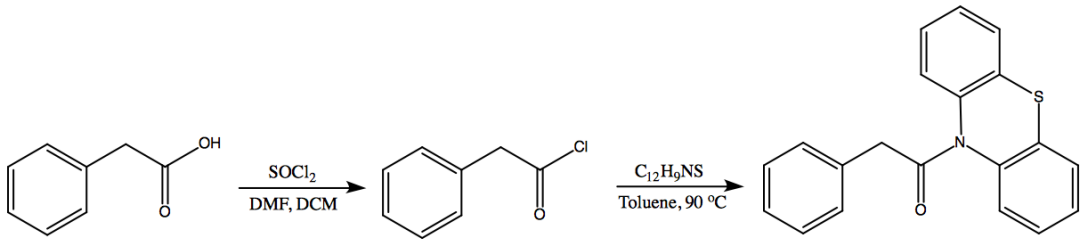


Figure 6: Synthesis of an acid chloride with a phenothiazine attachment.

The synthesis of *para*-anisic acid was used to prove that the Grignard reaction was capable of synthesizing a carboxylic acid. The Grignard reaction proved difficult, but achievable, to synthesize the final product of *p*-anisic acid. The 58% yield was the highest yield produced from the previous six experiments of *p*-anisic acid (Figure 7). The experiment was verified by ^{1}H-NMR, but the alcohol of the carboxylic acid was not noticeable. The absence of the carboxylic acid may be explained by a deuteron replacing the hydrogen of the carboxylic acid, thus making the carboxylic acid peak untraceable while using ^{1}H-NMR. An IR was used as verification to show that the carboxylic acid was produced.

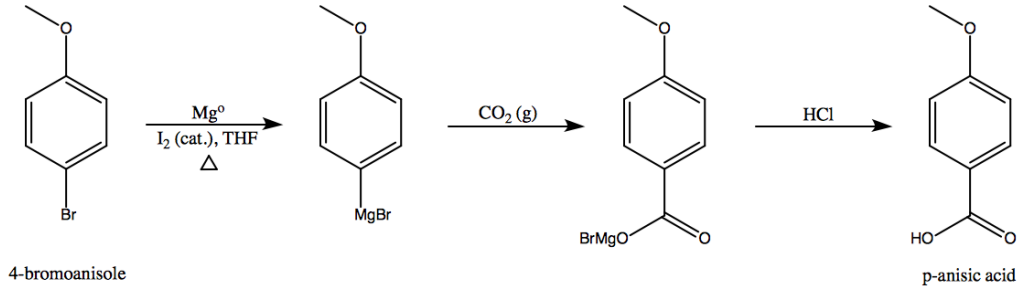


Figure 7: Grignard reaction to produce the product *p*-anisic acid.

The Grignard reaction synthesis examined with *p*-anisic acid will be utilized for the synthesis of cycloheptanecarboxylic acid and cyclooctanecarboxylic acid (Scheme 2). The source of carbon dioxide (CO_2), whether as a gas or solid, will be examined. In synthesizing *p*-anisic acid, CO_2 gas gave the best results. In addition, it was observed that heating Mg metal with bromobenzene was more effective than room temperature production of the Grignard reagent. These two modifications to the Grignard synthesis increased the yield from 12% to 58%. The Grignard synthesis will continually be improved in order to increase reaction yields of the final product.

Phenstatin derivatives are important to the cancer research community due to its versatility to alter substituents and still fit into the colchicine binding site. The need for microtubulin inhibiting drugs has increased in popularity due to its non-invasive therapeutic effects. More research is needed to further demonstrate and determine the effectiveness and efficiency for specific anti-cancer drug therapies. The four new molecules described in this research will aid in furthering the development of microtubulin inhibiting drugs (Figure 4).

4. Future Work

The next steps in this research will be to synthesize the starting carboxylic acids of the eight-membered ring using the Grignard reaction. In addition, the synthesis of the 3- and 4-membered carboxylic acids will be synthesized to an acid chloride. Following the acid chloride the phenothiazine molecule will be attached to form the A-ring of the target molecules (Scheme 1). The phenstatin derivatives that contain the 3- and 4-membered non-aromatic rings are predicted to be challenging to work with due to their high ring strain. Lastly, the Grignard reaction will be performed on the phenstatin derivatives that contain the 7- and 8-membered non-aromatic rings (Scheme 2). This research will aid in the understanding of how size and ring strain effect binding affinity to the colchicine binding site.

5. Acknowledgements

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