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Synthesizing Substituted Pyrazolines as Tubulin Inhibitors Targeting Hypoxia

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Abstract

Chalcones and pyrazolines are reported to exhibit anti-cancer properties. To continue this research, successful synthesis schemes starting with chalcone derivatives were discovered that would lead to synthesis of pyrazoline prodrugs. Prodrugs are non-toxic until they are activated once the compound enters the oxygen depleted environment of a hypoxic tumor cell. Activated by the cleaving of the promoiety, a sulfate group, of the molecule and resulting in the death of the cancer cell by inhibiting the production of tubulin. To achieve the synthesis of the desired pyrazoline derivative, 3-(4-fluorophenyl)-1-(4-methoxyphenyl)prop-2-en-1-one was synthesized as a necessary precursor using a base-promoted reaction of 4-fluorobenzaldehyde and 4-methoxyacetophenone. It was determined that synthesizing a pyrazoline in a one-pot reaction with the chalcone was not favorable and resulted in hydrazone. Therefore, 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one intermediate was synthesized along with 3-(benzylamino)-3-(2,5-dimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl)propan-1-one to determine that the reaction would be successful with another chalcone derivative. 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one was used to synthesis 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one oxime. Which then will be reacted to close the nitrogen ring to produce the pyrazoline. This methodology allows for pyrazolines derivatives to be synthesized from varying chalcones derivatives. Then to be synthesized further into a prodrug that is designed to target hypoxic tumor cells to cause apoptosis.

1. Introduction

Cancer is a devastating disease that claims the lives of millions each year. Globally in 2015 there were 17.5 million cancer cases and 8.7 million cancer related deaths[1].

Cancer is the abnormal, rapid reproduction of cell division in the cell cycle caused by adaptation that promotes proliferation and invasion in the body. There are many different types of cancer, but all cancers can spread and compromise nearby healthy cells. If a cancer cell deviates from its original location, it can travel through the bloodstream or lymph system. The final location of these cancer cells can cause new cancer masses in this new area of the body[2].

Cancer cells differ from healthy cells in many ways. The most significant difference is that cancer cells do not die, stop growing, or stop dividing. These cells do not repair damaged DNA. All healthy cells have a mechanism to self destruct, apoptosis, if they become too damaged or come to the end of their cell life. Cancer cells are a result of a mutation of the cell cycle in which the cell has a mutated protein, p53[1]. This protein recognizes when the cell has come to the end of its natural life[2]. Since this protein is mutated it cannot detect that the cell is damaged or that it has damaged DNA. This same protein is responsible for signaling when the cell needs to repair its DNA. So, with no cell death and damaged DNA the cell continues to divide with no repairs and the DNA can continue to be copied incorrectly which leads to what we recognize as cancer. The cancer cells replicated are immature, carry out no function, and do not receive communication from surrounding cells. The body does have a built-in defense against cancer cells when

they are in their early stages. Healthy cells identify cells that refuse to be apoptotic and they are killed off or blood supply is restricted to that area. Unknowingly, many humans live with cancer cells in their bodies and never have any symptoms because they have cancer cells that the body can defend against, but not the cancer disease[2].

There are treatments on the market for most cancers in the form of chemotherapy, radiation, surgical removal of cancerous tissue, immunotherapy, and biotherapy. However, these treatments may not always work and are often incredibly invasive, often toxic to healthy cells, and can cause patient death because of the cancer treatment and not the cancer itself[3].

Cancerous tumors can progress so that they outgrow and cut off their local blood supply. This causes the tumor mass to be oxygen depleted, a condition known as hypoxia[4]. In a large tumor, the cells adapt and thrive in these microenvironments and become a problem when the patient seeks treatment. Hypoxic tumor growth increases because the anaerobic environment stimulates more growth, glycolysis, angiogenesis factor activity, metastasis, more mutations, and greater inhibition of apoptosis. This makes treating hypoxia more difficult than non-hypoxic cancer cells and this condition is often found in matured tumors[2,4]. Hypoxic, solid tumors have been found to be difficult to treat with chemotherapy drugs currently available. Because of the lack of blood supply the drugs have difficulty permeating the tumor mass[3,5].

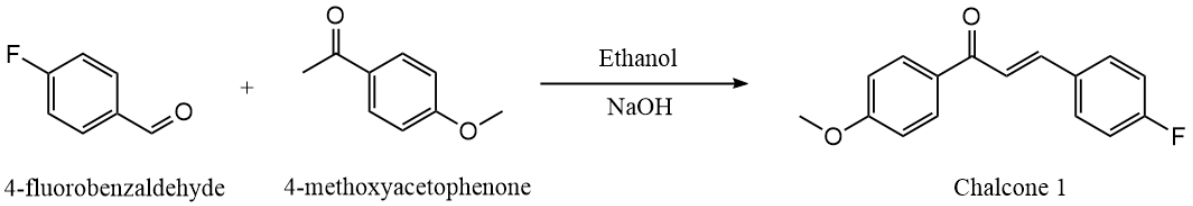
Prior research in the Holt research group examined the synthesis of pyrazoline derivatives because of their known anticancer, antibiotic, and anti-inflammatory properties [6,7]. Combretastatins are also researched and synthesized in Holt's research group for their interactions with tubulin. Tubulin is a key protein in the cell division process. During metaphase of a cell, tubulin creates spindles that connect across cells to create mitotic spindles. These spindles attach to components of the cell that separate into two cells. If tubulin is not produced or not functional, the spindles cannot form and the cell would not be able to divide, leading to cell death. Danielle Davis[7] synthesized pyrazoline derivatives from cyclization of chalcones to inhibit tubulin in cancer cells. Davis found that four of her pyrazoline derivatives showed tubulin inhibition in bacteria cells and concluded in her research that methoxy groups enhance bioactivity[7].

Dr. Jalisa Ferguson researches hypoxia and pyrazoline prodrugs to inhibit the formation of microtubules in cancer cells. Cancerous tumors can survive in a harsh environment, like hypoxia, by altering their metabolic pathway[3]. Ferguson's research targets this transcriptional factor, hypoxia-inducible factor, HIF-1□, in hypoxia. One of HIF-1□ uses is to use tubulin to enter into cancer cells and alter it so they can withstand hypoxic conditions. When a tumor mass is large enough and becomes hypoxic anaerobic respiration occurs. This causes HIF-1□ to become upregulated and before the cells can no longer withstand a hypoxic environment they migrate to the nucleus. In the nucleus HIF-1□ binds to HIF-1□ and they produce proteins and enzymes to help withstand anaerobic respiration caused by a hypoxic environment. This causes the cancerous tumor to continue to grow and avoid apoptosis[9,10]. Ferguson has been synthesizing prodrugs to target the microtubule and inhibit it from functioning in hypoxia so that the cancer cells cannot divide or be able to withstand hypoxic conditions and die.

Prodrugs are inactive, conditionally stable compounds. Prodrugs become active when they get metabolized in the microenvironment that contains the targeted binding site. The promoiety that caused inactivity of the drug is selectively reduced and cleaved from the compound in the target environment[11]. Ferguson's prodrugs are pyrazolines synthesized from chalcones, and are only metabolized in hypoxia so that the drug stays inactive around healthy cells and prevents apoptosis from occurring in healthy cells. Often, chemotherapy is used instead of surgery to physically remove cancerous tumors because they can be close to vital and sensitive organs. Therefore, if a drug that is not a prodrug is used it could damage the surrounding healthy tissue anyway. It is believed that once the substituent, the promoiety, causing the prodrug to be inactive is cleaved from the compound it is inert and is metabolized out of the body.

Based on Davis' research, chalocones can be synthesized using a base-catalyzed Aldol condensation in a 40% ethanol solution, using sodium hydroxide as a catalyst. From there, pyrazolines, which are heterocyclic nitrogenous compounds, can be synthesized into multiple derivatives from chalcones by a way of cyclization. This scheme below consists of reacting chalcone with hydrazine hydrate and acetic acid in the presence of ethanol, producing a 2-pyrazoline derivative[8].

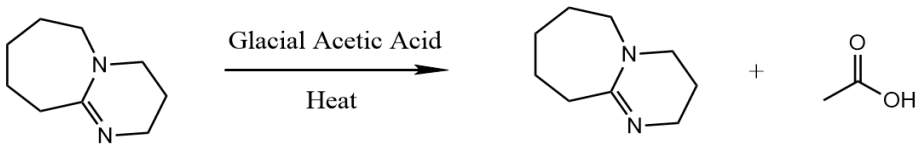
Scheme 1. Chalcone synthesis derived from Davis



4-fluorobenzaldehyde 4-methoxyacetophenone Chalcone 1

2. Results and Discussion

Scheme 2. Synthesis of [DBU][Ac] ionic solvent



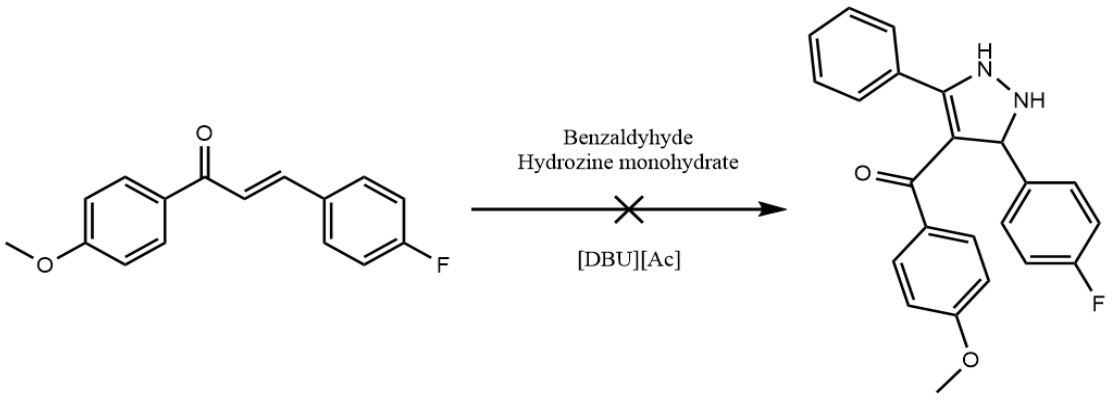
[DBU] [DBU][Ac]

First [DBU][Ac] was synthesized to be used as a solvent free ionic liquid catalyst. This is a green solvent that can be recycled and decreases the risk of fire and explosion during a reaction, along with requiring only a small amount to dissolve the products.

The first steps in synthesizing the chalcone, scheme 1, was a success with high yields of 78% after crystal purification. The ^{1}H-NMR of the product supports this hypothesis along with TLC results revealing only one compound in the product.

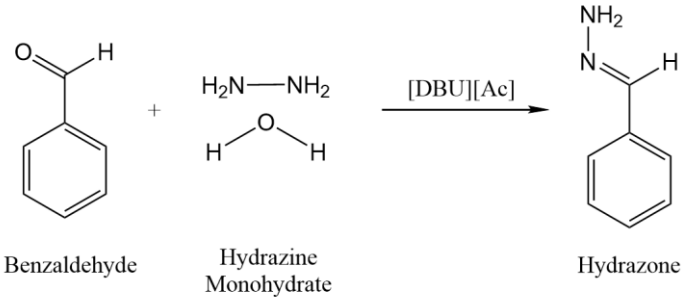
The pyrazoline synthesis in scheme 3, using pure chalcone from scheme 1, benzaldehyde, hydrazine, [DBU][Ac], in a one-pot reaction to form the pyrazoline. This reaction was found to be unsuccessful in forming the desired pyrazoline. It was found that the hydrazone derived from hydrazine and benzaldehyde was the major product shown in scheme 4.

Scheme 3. Pyrazoline synthesis derived from Davis



Chalcone 1 Pyrazoline derivative

Scheme 4. The reaction that was discovered to be occurring when attempting scheme 3 reaction. Benzaldehyde appeared to react exclusively with hydrazine to produce hydrazone.

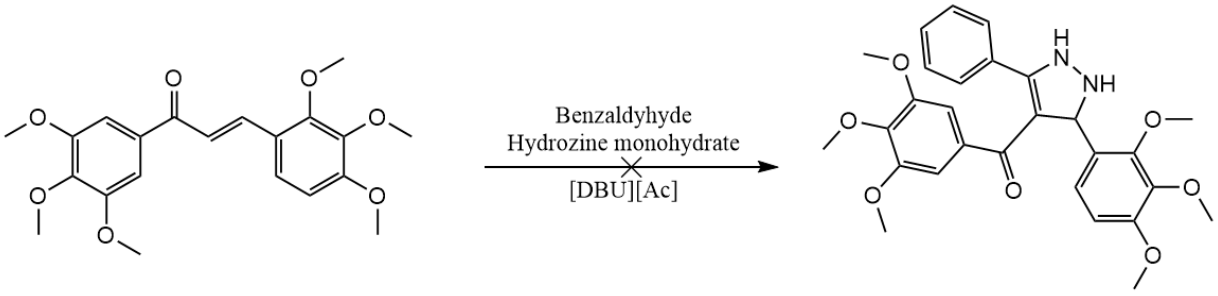


Benzaldehyde | Hydrazine Monohydrate | Hydrazone

Hydrazone was discovered to be the primary product of the initial reaction in scheme 3 with leftover starting materials in the reaction. The hydrazone was reacted with more chalcone, using [DBU][Ac] as a solvent free ionic liquid as a catalyst to produce the desired pyrazoline. This reaction was refluxed at a high temperature for multiple hours. The reaction was monitored by TLC to then determine that no reaction occurred and the reagents burnt.

Scheme 5 shows the re-attempt to synthesize a pyrazoline from a different chalcone derivative that was available. The difference in the substituted chalcones should not affect the success of the reaction as the carbonyl and the alkene on a chalcone are what will be reacting during the synthesis. Scheme 5 increased the molar ratio of chalcone and benzaldehyde to 1:1. This reaction also resulted in hydrazone and was unsuccessful in forming the desired pyrazoline.

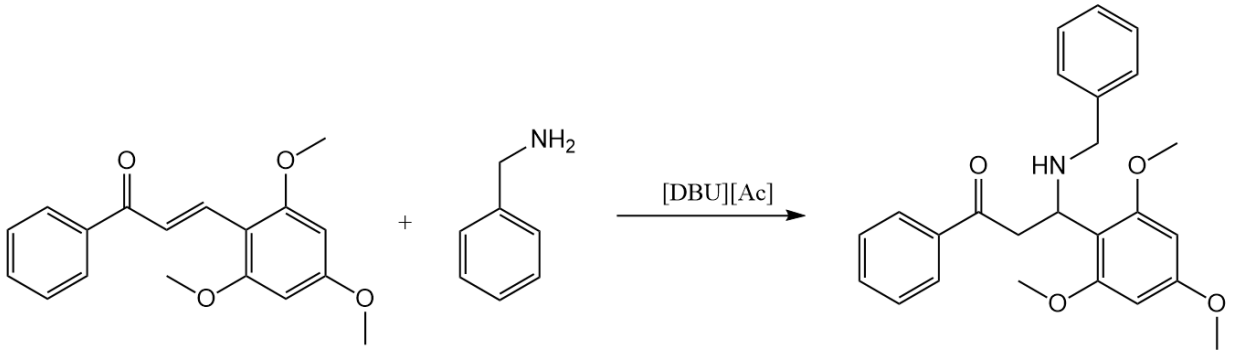
Scheme 5. Second chalcone reaction to synthesize pyrazoline



Chalcone 2 | Pyrazoline Derivative

With the unsuccessful attempts at synthesizing a pyrazoline in schemes 3-5, a different approach was taken to synthesize a pyrazoline beginning with scheme 6. This new scheme pathway synthesizes the pyrazoline by adding the two nitrogens that form the pyrazoline separately, instead of adding the two nitrogens to the compound already bound together[13]. A different chalcone derivative was used, chalcone 3, again the different chalcone derivative while producing a different pyrazoline compound, should not affect the synthesis pathway in producing a pyrazoline. This chalcone was reacted with benzylamine by aza-Michael addition. This reaction appears to be successful, further purification is to be done to determine the final yield.

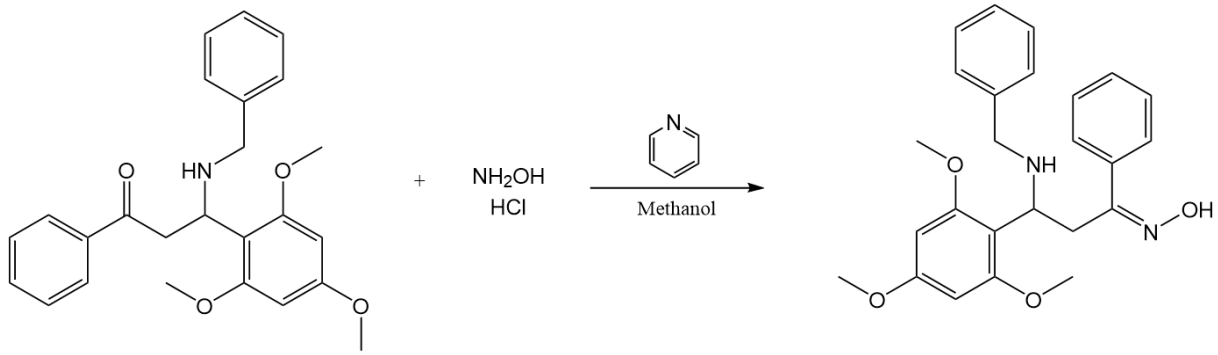
Scheme 6. First chalcone aza-Michael addition of benzylamine



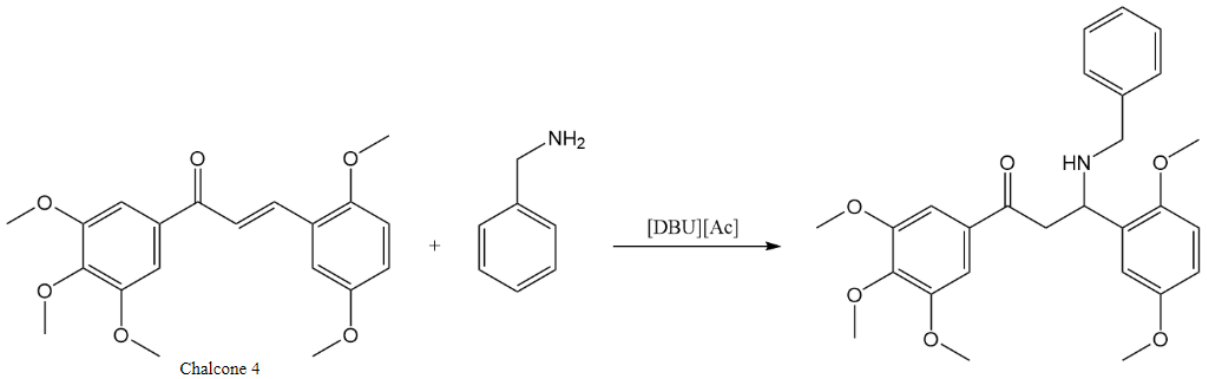
Chalcone 3

Scheme 7 shows a Beckmann rearrangement using hydroxylamine hydrochloride with pyridine and methanol as the solvent system to yield a product with an oxime. This adds the second nitrogen that will be apart of the pyrazoline ring.

Scheme 7. Addition of oxime



Scheme 8. Second chalcone aza-Michael addition of benzylamine with chalcone 4

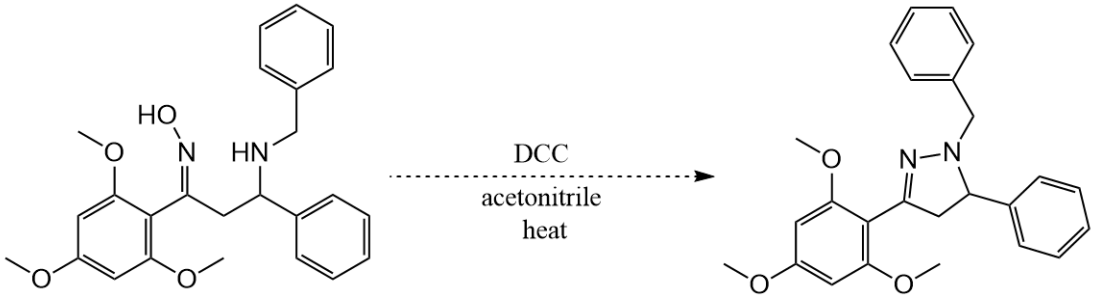


Chalcone 4

Scheme 8 was the beginning of synthesizing another compound to attempt the nitrogen coupling to close the 5 membered ring to produce a pyrazoline. The next synthesis with this compound would have been to react it with the

same reagents as scheme 7 for the addition of the oxime. This would have insured that this pyrazoline synthesis is successful with other chalcone derivatives and if the nitrogen coupling reaction were to be unsuccessful, there would be a compound ready to attempt a different approach to coupling the nitrogens.

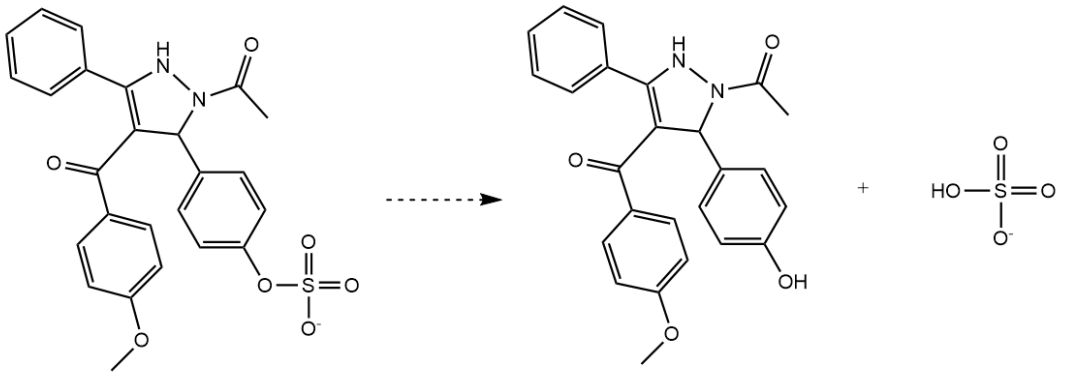
Scheme 9. The proposed synthesis to couple the nitrogens to yield a pyrazoline product.



Scheme 9 shows what the next proposed synthesis would be. N,N'-dicyclohexylcarbodiimide (DCC) is a carbodiimide compound used as a coupling agent. In this reaction it would be used to couple the nitrogens from the oxime and the benzylamine to produce the pyrazoline ring. Acetonitrile would be used as a polar aprotic solvent under heat[14].

Once a successful pyrazoline synthesis scheme is determined, then it will be substituted to produce the final prodrug product as shown in scheme 10. An amide will be attached by an acylation reaction with acetyl chloride. The amide group will make the base of the pyrazoline derived from prior prodrug research. The 4-methoxybenzaldehyde attached at position 4 on the pyrazoline shown in scheme 10 would have to be added in a separate step. Initially this structure would have been present from the chalcone if scheme 4 had been successful. The sulfate promoiety group, shown in scheme 10 below, will be substituted in the place of the original fluorine on the chalcone shown in scheme 1. Sulfate groups are theorized to improve the function of prodrugs because they are a weak base and make for a good leaving group and have been used to select for hypoxic environments [7,12]. The prodrug compound will be analyzed for its binding activity in the hypoxia DNA and tested on cancer cells and healthy cells to observe its IC_{50}, the half maximal inhibitory concentration for a drug to inhibit a function of a cell. This will be determined using a MTT assay to observe how many cells survive the active drug and the inactive drug.

Scheme 10. The activation of the inactive prodrug.



Pyrazoline
Inactive Prodrug

Pyrazoline
Active Drug

hydrogen sulfate
Cleaved promoiety

3. Conclusion

Prodrugs are commonly used because of their ability to target specific microenvironments found in unhealthy cells being targeted for treatment. Since a prodrug can be used to target binding sites to inhibit vital function for any cell it

is important for this drug to be inactive in healthy tissue. Synthesizing pyrazoline prodrugs to inhibit tubulin in hypoxic, cancerous tumors can reveal a way to treat large, developed tumors that are resistant to chemotherapy and surgical options. The purpose of this research was to determine a successful scheme to synthesize pyrazolines so that further research can expand upon the possibilities of pyrazoline based cancer drugs. In this research the chalcone derivatives used for the synthesis likely may not be ideal in the final reaction scheme to synthesizing a pyrazoline prodrug, but starting material similar the chalcones used upon further research could follow the same reaction schemes to synthesize a pyrazoline. Once a synthesis is found to be successful in producing the final prodrug compound, further tests such as an MTT assay can be done to determine how effective this approach to a prodrug is at inhibiting tubulin and causing cell death in hypoxic cells. Therefore, the sulfate promoiety can be analyzed for enhancing the ability of the prodrug to be inactive in healthy cells and become active in hypoxic environments to inhibit tubulin production.

4. Experimental

4.1 [DBU][Ac] (1,8-diazabicyclo-undec7-en-8-ium acetate)

In a 100 mL round bottomed flask (RBF), 0.001 mol of DBU was added and cooled on an ice bath. Then 0.001 mol of glacial acetic acid was then slowly added to the RBF and stirred. The RBF was removed from the ice bath and was stirred and heated on low with a condenser for 24 hours. Then the RBF was under vacuum for 24 hours. This process produced an amber, viscous ionic "liquid." This ionic liquid was used without further purification or characterization.

4.2 3-(4-Fluorophenyl)-1-(4-methoxyphenyl) prop-2-en-1-one

In a 100 mL RBF, 0.0016 mol of 4-fluorobenzaldehyde and 0.0013 mol of 4-methoxyacetophenone were added and dissolved in 7.5 mL EtOH. A solution was made of 0.0080 mol of NaOH and 5.0 mL deionized H_20 and added to the RBF. Upon base addition the solution turned yellow and was stirred for 24 hours. The solution was golden-yellow and clumpy and was fritted filtered and washed with deionized H_2O. The chalcone was purified by recrystallization with 70:30 solution of methanol and deionized H_20 and heated. Then the white crystals were filtered out and characterized by ^{1}H-NMR with yield of 77.6%.

4.3 (3-(4-Flurophenyl)-5-phenyl-2, 3-dihydro-1H-pyrazol-4-yl)(4-methoxyphenyl) methanone

In a 25mL RBF, 0.077 g of the chalcone, 3-(4-fluorophenyl)-1-(4-methoxyphenyl) prop-2-en-1-one, and a couple drops of [DBU][Ac] was added. Then 0.5 mL hydrazine hydrate and 0.6 mL benzaldehyde were added. The mixture was stirred for 24 hours at room temperature. Then the amber mixture was separated via separatory funnel with 50.0 mL ethyl acetate and washed with saturated $NaHCO_3$ three times. The organic layer was saved in a 250 mL Erlenmeyer flask and dried with $MgSO_4$. Then the liquid was filtered through a fritted funnel and transferred into a 100 mL RBF and rotovapped. The amber-yellow viscous "liquid" was dried under vacuum for 24 hours. The orange crystal-oil mixture was characterized via ^{1}H-NMR, and determined that there was an excess of hydrazone in the product. The product was washed with hexane to try to purify the pyrazoline in the product, but was unsuccessful. In a 25 mL RBF, 0.35 g of the hydrazone mixture and 0.23 g of pure chalcone (scheme 1) were combined with [DBU][Ac] as the solvent and heated on high heat for 4 hours resulting in a thick black-brown product. The product was dissolved in ethyl acetate. Via separatory funnel with an excess of ethyl acetate and brine, the organic layer was washed and separated. The product was dried with $MgSO_4$ and filtered. After drying via rotovap the product was identified with TLC comparison and ^{1}H-NMR and determined that there was no reaction and the reagents had burnt.

4.4 (5-phenyl-3-(2,3,4-trimethoxyphenyl)-2,3-dihydro-1H-pyrazol-4-yl)(3,4,5-trimethoxyphenyl)methanone

In a 50 ml RBF, 0.91 g of chalcone (scheme 4) (E)-3-(2,3,4-trimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one, and one drop of [DBU][Ac] was added. Then 0.5 ml hydrazine monohydrate and 0.6 ml *p*-anisaldehyde was added. The reaction was stirred for over 24 hours at room temperature. The reaction resulted in a pale yellow, smooth ball. This was dissolved in 50 ml ethyl acetate and washed with $NaHCO_3$ 3 times in a 250 ml separatory funnel. The organic layer was dried with $MgSO_4$. Than filtered the organic layer with a fritted funnel and rotovapped. The solid was determined to be hydrazone.

4.5 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one

In a 50 mL RBF 0.45 g of chalcone (scheme 6) (E)-1-phenyl-3-(2,4,6-trimethoxyphenyl)prop-2-en-1-one was added with a drop of [DBU][Ac] and 1.75 ml benzylamine. Reaction stirred at room temperature for 24 hours. Dissolved the product in 50 ml of ethyl acetate and in a 250 ml separatory funnel washed the product with $NaHCO_3$ saturated H_2O 3 times. Dried the organic layer with $MgSO_4$. Filtered with a fritted funnel and rotovapped and was left with a pale yellow oil.

4.6 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one oxime

In a 250 mL RBF 0.63g of 3-(benzylamino)-1-phenyl-3-(2,4,6-trimethoxyphenyl)propan-1-one was added with 7.8 mL MeOH and 1.04 g $NH_2OH{\cdot}HCl$. Then 2-3 drops of pyridine were added to the solution and the reaction was refluxed for 12 hours. Methanol was evaporated out of solution and the product was dissolved in CH_2Cl_2 and washed with brine 3 times. The organic layer was separated and dried and filtered. The product was dark orange viscous oil.

4.7 3-(benzylamino)-3-(2,5-dimethoxyphenyl)-1-(3,4,5-trimethoxyphenyl)propan-1-one

In a 50 mL RBF 0.75 g of chalcone (scheme 8) (E)-1-phenyl-3-(2,4,6-trimethoxyphenyl)prop-2-en-1-one was added with a drop of [DBU][Ac] and 1.75 ml benzylamine. Reaction stirred at room temperature for 24 hours. Dissolved the product in 50 ml of ethyl acetate and in a 250 ml separatory funnel washed the product with $NaHCO_3$ saturated H_2O 3 times. Dried the organic layer with $MgSO_4$. Filtered with a fritted funnel and rotovapped and was left with a pale orange fluid oil.

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