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Guvacoline in betel nuts directly binds to and inhibits AChE activity, lowering ACh levels released by neurons

Dejia Zhou

Episcopal High School, Alexandria, VA 22302, United States

vickydjzhou@outlook.com

Abstract. This paper investigates guvacoline, a compound found in betel nut, and its activity on acetylcholine level. Betel nuts are the chewable seeds of plant used in Chinese traditional medicine. It is commonly used all over southern Asia and the east African seaboard, being the fourth most commonly used drug in the world. It is known for being carcinogenic for throat cancer. Immediate symptoms of betel nut chewing indicate activation of the parasympathetic system, in which acetylcholine and acetylcholinesterase is involved. This paper predicts that guvacoline, found in areca nuts, increases free acetylcholine levels released from neurons by allosterically binding to acetylcholinesterase inhibiting it.

Keywords: Traditional Chinese Medicine, Betel Nuts, Acetylcholine

1. Introduction

Betel nuts, also called areca nuts, are the chewable seeds of *Areca catechu*, a plant commonly found in southeast Asia [1]. Betel nuts are the fourth most commonly used drug in the world with more than 600 million users. It is comprised of the seeds of *Areca catechu* wrapped in leaves of the betel tree, often with flavoring and tobacco added [2]. Chewing betel nuts is known to be a carcinogen for throat cancer. The short-term effects of chewing the betel nut includes slowed heart rate, lower blood pressure, pupil contraction, and excessive salivation. Toxic compounds such as arecoline are abundant in betel nuts [1]. Betel nuts induce dependency. Withdrawal symptoms include lethargy, anxiety, irritability, and insomnia [2]. There has been some investigation into the mechanism of betel nuts, but it is not nearly as well-researched and well-understood as other drugs. Due to the widespread use of betel nuts, effective cessation therapies have not been developed or put in use [2].

Natural psychotropic agents such as betel nuts have been used by animals everywhere for hundreds of thousands of years. Dolphins intentionally get stung by pufferfish to be anesthetized by the pufferfish toxin. Naturally, humans sought out drugs as well.

In traditional Chinese medicine, betel nuts are used as an anesthetic, insect repellant, and a remedy for indigestion and gas. It is used both externally and as an oral drug. It is commonly used with *Dichora febrifuga Lour.* to treat malaria [3]. The reason that the betel nut is effective in inducing gut movement is because betel nut extract activates the parasympathetic nervous system.

The parasympathetic nervous system controls “rest and digest,” including gut function, slowing the heart rate, lowering blood pressure, etc. Drugs like nicotine activate the parasympathetic system by activating acetylcholine (ACh) receptors [1]. This activation results in a higher level of acetylcholine in

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the brain. Due to the similarity of effects of nicotine and betel nut, acetylcholine levels have been researched and are found to be increase immediately after betel nut chewing [2]. Substances like arecoline, found in betel nuts, are known to have an effect on this process.

Acetylcholine is the first discovered neurotransmitter. In the peripheral nervous system, acetylcholine regulates skeletal muscular movement. In the central nervous system, it is involved in memory, sleep, and mood. It is produced by cholinergic neurons. Notably, its activity is inhibited in patients of Parkinson's disease. It is broken down and recycled by acetylcholinesterase (AChE) in neural cells. There are two main types of G-protein coupled acetylcholine receptors: muscarinic and nicotinic [1]. Nicotine activates the nicotinic receptor which activates the parasympathetic system. Examples of nicotinic ACh receptor agonists include nicotine and cytisine. Examples of muscarinic ACh receptor agonists include darifenacin, solifenacin, and other approved drugs.

Many components of the betel nut have known effects but no known mechanism of action. Even though betel nut extract has been shown to inhibit activity of acetylcholinesterase, no isolated, individual substance has been found to achieve the same effect [4]. Arecoline, named after the areca nut, is known to act on the parasympathetic system, which controls "rest and digest." Structurally, it is somewhat similar to nicotine, which may explain the similar effects [5]. Guvacoline, on the other hand, is a less commonly researched compound also found in betel nuts. It is known as a muscarinic agonist [1].

Hypothesis: This paper predicts that guvacoline, found in areca nuts, increases free acetylcholine levels released from neurons by allosterically binding to acetylcholinesterase inhibiting it. Acetylcholine will be measured with the kit and esterase activity in vitro and direct inhibition will be proved by affinity chromatography.

2. Methods

2.1. GPCR-activation-based ACh ($GRAB_{ACh}$) sensor

$GRAB_{ACh}$ converts the structural change that ACh induces on type 3 muscarinic ACh receptor into fluorescence. Parts of the sensor are inserted into muscarinic ACh receptors to detect the structural change from ACh binding [6]. Thus, it fluoresces. By measuring the intensity of fluorescence, ACh levels in synapses can be observed. Other random neurotransmitters like nicotine, glycine, serotonin, epinephrine, coline, GABA, glutamate, dopamine, histamine, norepinephrine, and adenosine do not activate the sensor. Overall, the sensor is pretty reliable. Additionally, it has been improved to also respond to nicotinic ACh receptors [6].

2.2. Acetylcholinesterase Kit

The AChE kit detects AChE activity by making the hydrogen peroxide fluoresce. AChE, choline oxidase, and the absence of horseradish peroxide enables this. The functional part of the kit is Amplex red reagent that reacts with hydrogen peroxide when there is no horseradish peroxide [7]. Basically, ACh goes through AChE and becomes choline, which goes through choline oxidase to become betaine and hydrogen peroxide. When there is no horseradish peroxide, which is supposed to react to the hydrogen peroxide, hydrogen peroxide reacts with the reagent instead to make resorufin, which fluoresces, and whose fluorescence can be measured [7].

2.3. Affinity Chromatography

Guvacoline is used in AChE affinity chromatography. In this method a combination of AChE, other proteins, and guvacoline is washed to leave whatever guvacoline binds to. Affinity chromatography immobilizes guvacoline into a chromatographic support [8]. First, an application buffer with the proteins is washed through the contraption which creates the optimal conditions for guvacoline to bind to AChE. Then, an elution buffer washes extra protein away to leave only guvacoline and whatever guvacoline binds to, which may be AChE [8].

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2.4. Titration and Concentration

We will start with 10 micromolar then 100, 1000 micromolar and 1 millimolar. This will tell us the concentration at which guvacoline starts and stops working. Treatment duration: The concentrations will be tested immediately, after 10 minutes, 2 hours, 4 hours, 8 hours, and one day.

2.5. Results

Table 1. Combination of possible results

Combination of possible results	Acetylcholine levels increase by sensor	acetylcholinesterase activity *in vitro* decreases by kit	Direct binding of guvacoline to AChE by affinity chromatography	Supports hypothesis?
CR1	-	-	-	no
CR2	-	-	+	Partially
CR3	-	+	+	Partially
CR4	+	-	+	Partially
CR5	+	-	-	Partially
CR6	-	+	-	Partially
CR7	+	+	+	Yes
CR8	+	+	-	Partially

Note: + indicates result is similar to the direction stated in the table 1, consistent with positive control (pyridostigmine, a known AChE inhibitor), and supporting the hypothesis. – indicates results deviant from direction stated in the table, consistent with negative control (saline solution), and opposing the hypothesis.

CR1: This result does not support the hypothesis. ACh sensor indicates that ACh levels produced by neurons do not increase after addition of guvacoline. AChE kit indicates that AChE activity does not decrease after addition of guvacoline. Affinity chromatography indicates that guvacoline does not only bind to AChE.

CR2; This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons do not increase after addition of guvacoline. AChE kit indicates that AChE activity does not decrease after addition of guvacoline. Affinity chromatography indicates that guvacoline only binds to AChE.

CR3: This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons do not increase after addition of guvacoline. AChE kit indicates that AChE activity decreases after addition of guvacoline. Affinity chromatography indicates that guvacoline only binds to AChE.

CR4: This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons increase after addition of guvacoline. AChE kit indicates that AChE activity does not decrease after addition of guvacoline. Affinity chromatography indicates that guvacoline only binds to AChE.

CR5: This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons increase after addition of guvacoline. AChE kit indicates that AChE activity does not decrease after addition of guvacoline. Affinity chromatography indicates that guvacoline does not only bind to AChE.

CR6: This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons do not increase after addition of guvacoline. AChE kit indicates that AChE activity decreases after addition of guvacoline. Affinity chromatography indicates that guvacoline does not only bind to AChE.

CR7: This result fully supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons increase after addition of guvacoline. AChE kit indicates that AChE activity decreases after addition of guvacoline. Affinity chromatography indicates that guvacoline only binds to AChE.

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CR8: This result partially supports the hypothesis. ACh sensor indicates that ACh levels produced by neurons increase after addition of guvacoline. AChE kit indicates that AChE activity decreases after addition of guvacoline. Affinity chromatography indicates that guvacoline does not only bind to AChE.

2.6. Titration and Concentration

At a certain concentration, guvacoline's impact on AChE and ACh becomes observable. At one point more guvacoline stops having further any impact on AChE. There are two scenarios for titration: Positive relationship: as concentration of guvacoline increases, AChE activity decreases and ACh levels increase. Negative relationship: as concentration of guvacoline increases, AChE activity also increases and ACh levels decrease.

Treatment duration: the effect of guvacoline on ACh and AChE will either be short term (surfacing immediately or in hours after consumption) or long term (persisting or surfacing in a few days.)

3. Discussion

CR1: This result does not support the hypothesis. Guvacoline is not involved in acetylcholine levels at all in the neurons. One explanation can be that it acts on a different neurotransmitter. This experiment can be repeated using different esterase and neurotransmitters: for example, serotonin. The neurotoxicity of guvacoline should also be investigated. If guvacoline is proven to be neurotoxic, it can be extracted and used in biochemical weapons. If guvacoline has no impact on the nervous system at all, it may have applications in agriculture. Perhaps guvacoline is used as a natural pest repellant or otherwise by areca plants.

CR2: This result partially supports the hypothesis. Guvacoline does not bind to any functional part of AChE, but it does directly bind to AChE. An x-ray crystallography should be conducted to investigate which part of AChE it binds to and to what extent it changes the structure of AChE. Then, fluorescent structures can be designed using guvacoline as a reference to track AChE concentration and fluctuation. This has the potential to aid future AChE research.

CR3: This result partially supports the hypothesis. After binding to guvacoline, AChE sends out a signal for the cell to maintain level of ACh, through increased reuptake or decreased release, in order to compensate for reduction in AChE activity. More experiments should be conducted *in vivo* to investigate this unknown pathway. Activity of ACh reuptakers, vesicles, and the genome of the neuron after addition of guvacoline should be observed through western blot, florescent kits, and sequencing. If guvacoline is an ACh reuptake inhibitor, it may have application in Parkinson's.

CR4: This result partially supports the hypothesis. Upon binding to guvacoline, AChE sends a signal for the cell to increase level of ACh. How exactly the cell increases the level of ACh and what signaling pathway is used warrants further investigation. Known signaling pathways should be monitored using fluorescence and narrowed down until a direct relationship is found between guvacoline addition and a signaling pathway. This would open up more possibilities for AChE as a potential treatment target, and of course, more knowledge of a signaling pathway is always a good thing.

CR5: This result partially supports the hypothesis. Guvacoline activates a different pathway that increases levels of ACh released by the neuron. The affinity chromatography should give insight into which protein guvacoline binds to, which makes it easier to identify a signaling pathway. Drugs targeting this signaling pathway can then be developed using structure-based methods to treat Parkinson's.

CR6: This result partially supports the hypothesis. Guvacoline activates a different pathway that decreases AChE activity but also increases ACh reuptake or limits the level of ACh released by the cell. How ACh level is decreased must be investigated. There are two directions one can go with this: perhaps guvacoline activates a genetic switch that switches pathways of ACh inhibition. In this case the genome, as well as epigenome, transcription and translation process should be carefully observed. If epigenetic changes are observed as a result of guvacoline addition, this would give insight into the epigenetic effects of betel nut chewing. Perhaps Guvacoline binds to a cell-surface receptor and triggers a signaling cascade. One can use the affinity chromatography aforementioned to identify this signaling cascade.

CR7: This result fully supports the hypothesis. Guvacoline directly binds to AChE and reduces its activity, which is how it increases ACh levels released by the neurons. This is wonderful and opens up new insights into guvacoline's impact on the parasympathetic nervous system. Maybe it can be used in developing a rehabilitation therapy for betel nut chewing dependence and addiction. Maybe it can be a substitute for nicotine addiction or some other substance abuse disorder that involves ACh. There has been research into arecoline and the cortex, and a similar experiment with guvacoline can be conducted [2].

CR8: This result partially supports the hypothesis. Guvacoline activates a different pathway that does not act upon AChE but inhibits reuptake, increases release, or in some other way increases levels of ACh in synapses. Some aforementioned experiments should be repeated. Affinity chromatography should reveal what protein receptor or enzyme guvacoline binds to, which would reveal the mechanism of action of guvacoline. For example, if guvacoline binds to nicotinic or muscarinic receptors.

3.1. Titration and Concentration

There requires a certain concentration of guvacoline to have an effect on the nervous system. This threshold may be low or high and will advise us on the dosage of betel nuts. At some point it becomes too high which will also advise us on the dosage of betel nuts. If at some point it becomes toxic, this will open up new insight into harm reduction.

As far as the relationship between concentration of guvacoline and AChE activity/ACh levels: In the scenario that the relationship is positive, the hypothesis would be supported. Guvacoline is an inhibitor of AChE that raises the level of ACh released by neurons. In the scenario that the relationship is negative, the hypothesis would not be supported. This would indicate that guvacoline is actually an AChE agonist. For treatment duration, if the effect on ACh and AChE is immediate or relatively recent after guvacoline addition, that would mean the guvacoline is implicated in the initial betel nut high. If it is not as recent, perhaps after a day or two, then it should be implicated in dependence and would be of more aid in cessation therapy development.

4. Conclusion

Overall, if the hypothesis is completely supported, this would open up new insight into rehabilitation therapies for betel nut chewing dependence and addiction as well as the parasympathetic system. If it is somewhat supported, it indicates some other pathway and should be investigated further. If it is not supported at all, that warrants more investigation into guvacoline, and researchers researching only ACh levels do not have to waste time on guvacoline.

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