Tetrodotoxin's Effects on SCN1A in Relation to Epileptic Mutations: A Comparative Analysis of the Stability of the Inactivation State Sodium Channel Blockers

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Epilepsy is a disorder that affects millions of patients worldwide and is considered one of the most widespread neurological disorders. One of the molecular phenomena that can cause epilepsy is mutations in neuronal sodium channels, one of the most common ones being SCN1A. Current epilepsy therapeutics have limited strength and potency. Tetrodotoxin (TTX) is a naturally occurring SCB that is much stronger and more potent than currently available pharmaceutical treatments. This project uses a computational modeling approach to conduct an experiment that will compare TTX with other SCBs to examine if TTX can work as a potentially better therapeutic for sodium channel-induced epilepsy. To test this, two drug characteristics were measured as comparisons between TTX and current anticonvulsants: binding affinity and the stability of the inactivation state. Results indicate TTX has better functionalities within its induced stability of the inactivation state. Due to its higher functional impact, TTX may theoretically work as a better therapeutic for epilepsy than existing treatments.

Keywords: SCN1A; Epilepsy: Ranolazine; Carbamazepine; Oxcarbazepine; Phenytoin; GROMACS; VMD; AutoDock Vina; PyMol(r); RMSD; Stability of Inactivation State; Binding Affinity; Voltage-gated sodium channel

Introduction

Epilepsy is one of the most prevalent neurological disorders, affecting over 50 million people worldwide[1]. Epilepsy is described as the experience of recurring seizures due to abnormal neuronal activities causing an increase or decrease in electrical activity throughout the body. When the signals sent through tissues are amplified, the efflux of electrical activity causes a seizure. The seizures can differ heavily from patient to patient in aspects such as severity, frequency, and length of time[2]. Examples of the types of seizures include tonic-clonic seizures, petit mal, myoclonic, and partial seizures. Tonic-clonic seizures are usually characterized by jerking movements followed by rest. Petit mal seizures, as the name suggests, are more petite, generally causing the patient to lose consciousness for a few seconds to minutes.

Meanwhile, myoclonic seizures are mainly due to hereditary issues but have a variety of symptoms that are hard to diagnose. Finally, partial seizures are defined by jerking movements and emotional disturbance due to the center towards the temporal lobe of the brain[3]. Beyond these types of seizures, there are also many clinical symptoms, each characterized by different effects on the patient. Each patient diagnosed with epilepsy may undergo one of the various seizure types that may occur in the body to cause this disorder. The diverse ways that epilepsy can present itself in patients make it difficult to identify the molecular cause of and precisely treat epilepsy.

There are many different processes that, when disrupted, can cause epilepsy[4]. In one pathway, a voltage-gated sodium channel (VGSC) called SCN1A is an essential part of our body that can cause seizures if mutated. VGSCs are channels in the cell membrane that open and close to transport sodium ions based on the membrane potential[5,6]. There are nine different types of VGSCs. These channels are pore-forming transportation mechanisms that move ions based on the voltage of the intracellular and extracellular environments. All the channels play a role in the movement of sodium ions to eventually build up the electrical charge in the neuron needed to fire a signal. SCN1A is one of the essential VGSCs that can generate action potentials.

Action potentials are the processes in which an electrical charge is generated and propagated through neurons[5]. The signals are then passed out throughout the body to alert it of any actions that need to be performed. Throughout the action potential process, SCN1A has three different conformational stages[7]. The first stage is closed, in which the action potential is not occurring. Therefore, the channel is entirely nonconductive and essentially turned off to ensure no ions pass through. When an action potential begins, the sodium channel transitions from the closed state to the open state. In this state, an action potential occurs as the pore of SCN1A opens up and allows ions to pass

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through. The ions that pass through create the electrical charge buildup in the neuron. However, to ensure that the electrical signal does not grow too large in the neuron, there are breaks within the open state called the inactivation state. The inactivation state is when the sodium channel goes through brief rest periods and does not allow for ion conduction. The inactivation state can be split into two types: fast-inactivation state and slow-inactivation state[8,9]. The fast-inactivation state is used for quick repolarization, and the slow-inactivation spans tens of thousands of milliseconds to slowly reduce the overall number of conductive channels. The inactivation state then transitions back to the open state in a cycle until the action potential voltage is reached.

One of the main ways that epilepsy can manifest molecularly in patients is the mutations of sodium channels. Mutations or alterations can occur to the structure of the protein, leading to a change in function as well. When the function changes, based on the essential location of the mutation, action potential initiation is also affected, potentially starting a seizure[10]. There are hundreds of mutations, each identified by a different characteristic; for example, gain-of-function mutations increase the function of the channel, and loss-of-function mutations decrease the function of the channel, both of which can disrupt the balance of the voltage across the membrane of the neuron. With the countless mutations in these classes, each with a slight difference in their effect, it can prove to be even more challenging to treat[11]. Although sodium channels are essential for our body, mutations in these channels can cause detrimental effects on the patient.

Mutations can significantly affect the stability of the inactivation state of the SCN1A protein[12,13]. The stability of the inactivation can be defined as the length of the inactivation state. Mutations often disrupt the stability of the inactivation state and urge the transition toward the open state faster. This can lead to an increased buildup of an electrical charge, which, as mentioned before, has a high likelihood of causing seizures due to amplified signals[1]. The final job of the sodium channels is to allow for a viable and appropriate ion permeation[13]. Inactivation states limit the ion permeation in regular sodium channels; therefore, mutated sodium channels will ultimately have a higher ion permeation, which disrupts the whole function of the sodium channel.

It is difficult to identify the molecular cause of and treat epilepsy due to the significant amount of specific and unique deformations that can occur to the protein; however, there have been treatment approaches. One of the most important ways to treat sodium channel-related epilepsy that exists currently is sodium channel blockers (SCBs)[14]. SCBs are engineered explicitly pharmacologically to counteract the gain-of-function mutation effects in VGSCs. SCBs are a diverse range of molecules that can treat multiple sodium channel-related disorders. Neurons are not the only cell type that transfers signals to perform actions. One of the other main functions of sodium channels is to regulate heartbeat[8]. The action potentials in each cardiac cell regulates each heartbeat. Sodium channels are also crucial in this process as they regulate the buildup of the electrical signal in the cardiac cells for each action potential. For instance, if the mutation causes an increase in sodium ion influx, an SCB would lower the amount of sodium ions being transported to a lower or even base amount[15].

The SCBs that are specifically used to treat seizure disorders, mainly epilepsy, are called anticonvulsants. SCBs have been able to stabilize many patients with epilepsy; however, they have their limitations. Since SCBs are such diverse molecules, different mutations require different SCBs as their structure blocks the sodium channel in different ways[16]. Each class of SCBs has a specific mechanism of action. This is defined as how the drug binds to the target and performs its function. The anticonvulsants mostly have a similar mechanism of action[17–21]. Most anticonvulsants bind to the sodium channel on an extracellular portion of the sodium channel and block the pore formation portion. This is usually done during the inactivation states of the sodium channel to ensure that the stability of the inactivation state is increased. By increasing the stability of the inactivation state, the sodium channel opens less often, therefore not allowing for a significant buildup of an electrical signal. Although the same goal of reducing sodium ion permeation exists for cardiac SCBs, they have slightly different mechanisms of action. Some cardiac sodium channels can also bind to increase the stability of the inactivation state or even bind to the open state to block the pore; however, cardiac sodium channels are engineered to do a few additional functions as well[22,23]. Cardiac SCBs may also work to decrease intracellular calcium concentrations. This is done to keep the essential parts of the heartbeat continuing while reversing the effects of any mutations in the cardiac sodium channels to minimize any disturbance.

Molecules that block sodium channels can also naturally occur. Tetrodotoxin (TTX) is a highly harmful neurotoxin that can bind to sodium channels in our bodies, completely disrupting the sodium ion flow. When TTX enters the body and disrupts sodium ion flow, the generation of action potentials can no longer occur properly. As a result, the abnormal movement of signals in our body leads to seizures, which can cause paralysis and eventually death[24]. TTX can also bind to cardiac sodium channels, stopping sodium ion flow in cardiac tissue cells[25]. For this, one of the symptoms of TTX can be heart dysfunction, which can ultimately lead to death. TTX is found in a few animal species, one of the most common ones being pufferfish, and in 1774, the first case of poisoning was recorded when it was ingested as food. However, the degree of TTX's toxicity is highly dose-dependent, which leads to its potential within the clinical field as a possible therapeutic. Since TTX is a viable SCB, scientists have started considering its uses as a treatment for several neuromuscular disorders[25]. TTX has been researched

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as a possible treatment for neuropathic pain, which is prevalent in disorders such as cancer, arthritis, scoliosis, and other types of spinal problems[26]. Some mutations in SCN1A have been known to cause neuropathic pain related to migraines. These similarities raise the question of whether TTX can function as a viable treatment for SCN1A-induced epilepsy. However, the vitality of using TTX rather than current SCBs should be kept in mind, as TTX is a far more toxic molecule that can disrupt essential body functions. With serious considerations in place, it is important to note that since TTX is extreme, precautions such as its dosage and administration must be taken for specific patients[26].

Reasons have already been found for how TTX can serve as a better treatment than existing SCBs. TTX, on the other hand, is far more potent and can bind to different mutations for SCN1A and mutations of other sodium channels[25]. While TTX itself is harmful to our bodies, it is also important to note that SCBs have thousands of cases of being toxic to patients. In the end, even if they help reduce seizures, they are still blocking an essential part of bodily functions required to live[27]. TTX is also being used as a therapeutic for many neurological disorders such as any neural pain, brain trauma, spinal cord injury, anesthetics, and counters against drug abuse, all by targeting sodium channels[25]. Finally, TTX has a mechanism of action similar to that of anticonvulsants rather than cardiac SCBs[28,29]. TTX binds to the extracellular pore region of the sodium channel to block the sodium channel permeation. Since mutations relating to pain also disrupt the inactivation state, TTX must work to lower the frequency of potential firings of action. Epilepsy is one of the disorders heavily affected by sodium channel mutations, so there is a possibility that TTX could pose as a viable treatment.

Multiple factors, such as dosage administration, side effects, and other clinical aspects, must be considered when implementing TTX in the clinical field. While this project does not delve into those systemic effects, previous research has already introduced the idea for other disorders that can then be applied to Epilepsy as well. First, researchers found that when TTX was administered at 30µg, many neuropathic pain symptoms decreased in human patients, supporting TTX as a viable treatment27. Due to the toxicity issues of overdosage of TTX, 30µg is far below the administration of current anticonvulsants. The safety profile was well accepted as the pharmaceutical companies tried these therapies on the patients. The only side effect shown was irritation at the injected site, and the molecular effects of TTX on other bodily systems, such as the cardiac and muscular, were also insignificant. As for administration, clinical trials showed that TTX was administered by injection intramuscularly. Since there were few side effects and the effects are mainly dose-dependent, the safety profile of TTX suggests that it can be used under small concentrations to treat pain.

TTX will be tested as a potential therapeutic for sodium channel-induced mutations in this experiment compared to existing anticonvulsants. The study will use a computational methodology employing molecular dynamic simulations and binding measurements. It hypothesizes that TTX will better block sodium channels than existing anticonvulsants, decrease permeation, and better treat Epilepsy.

Results

	RAN	**CARB**	**PHEN**	**OXC**	**TTX**
Model 1	-6.1	-5.4	-5.8	-5.3	-5.4
Model 2	-5.8	-5.3	-5.6	-5.2	-5.2
Model 3	-5.7	-5.3	-5.6	-5.2	-5.2
Model 4	-5.6	-5.3	-5.4	-5.2	-5.2
Model 5	-5.6	-5.2	-5.2	-5.2	-5.1
Model 6	-5.5	-5.2	-5.2	-5.2	-5.0
Model 7	-5.5	-5.0	-5.1	-5.1	-5.0
Model 8	-5.5	-5.0	-5.1	-5.0	-5.0
Model 9	-5.5	-5.0	-5.1	-4.9	-4.9
Model 10	-5.4	-4.9	-5.0	-4.8	-4.9

RAN - Ranolazine
CARB - Carbamazepine
OXC - Oxcarbazepine
PHEN - Phenytoin

Table 1 Binding Affinity Comparisons for Sodium Channel Blockers (kcal/mol)

	RAN	CARB	PHEN	OXC	TTX
N (No. Models)	10	10	10	10	10
$\sum X$ (Sum of Values)	-56.2	-51.6	-53.1	-51.1	-50.9
Mean	-5.62	-5.16	-5.31	-5.11	-5.09
$\sum X^2$ (Sum of Values Squared)	316.22	266.52	282.63	261.35	259.31
Std. Dev.	0.2044	0.1713	0.2726	0.1595	0.1595

RAN - Ranolazine
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Table 2 Chi-Square Test for Statistical Significance in Binding Affinity Analysis. $F(4, 45) = 12.318$, $p < .01$

The first metric that was measured in this project was binding affinity. Researchers often use the binding affinity measurement to find the strength of the bonds between the protein and the drug. Since it is such an important metric that can play a role in how a drug works, measuring this value for TTX could also provide insights about whether or not TTX is a viable therapeutic. A more favorable binding affinity between the protein and the SCB represents a stronger bond between the two molecules. The stronger interaction suggests that the molecule can bind and carry out its mechanism of action better and potentially be a better therapeutic. When two molecules (in this project,

the molecules being the SCBs and the SCN1A protein) bind together, there are numerous positions they could be in with each other. Although some are more favorable than others, we do not know precisely how the two molecules will bind together. Therefore, when measuring binding affinity, ten different models of ten positions were considered for each SCB-SCN1A complex. The binding affinity was calculated for the best possible position between the SCB and the SCN1A protein. The ten models (Model 1 through Model 10) generated by the application are ordered from the most favorable to the least favorable model.

First, ten different models for binding affinities were measured for each SCB. These binding affinities were placed as a measurement for the binding affinities of TTX to SCN1A. Model 1 would be the most favorable biologically; model 2 would be the closest to the most favorable model, and so on. Ranolazine was the only cardiac SCB, and it had the lowest binding affinity compared to all the other medications in the most favorable binding position at -6.1. This pattern of having the lowest binding affinity persisted for models 2-10. Model 10 had the highest binding affinity out of the models at -5.4. The mean of the binding affinities of the ten models was the lowest at -5.62. Compared to the other SCBs, the range was 0.7, the second largest,t, and the median was the weakest at -5.55. The mode for Ranolazine was -5.5theee lowest out of all the SCBs.

Carbamazepine and Oxcarbazepine are both anticonvulsants that have very similar mechanisms of action. Both of their binding affinities for the models were very similar. For Carbamazepine, the most preferable model has a binding affinity of -5., fou,r, and the least favorable model has a binding affinity of -4.9. As for Oxcarbazepine, the most favorable model has a binding affinity of -5.3, and the least preferable model has a binding affinity of -4.8. The two anticonvulsants have similar meanings at -5.16 and -5.11 for Carbamazepine and Oxcarbazepine, respectively. Both have a range of 0.,5, the highest of all the medications, and a median of -5.2. The mode for the Carbamazepine is -5.3, and the mode for Oxcarbazepine is -5.2.

Phenytoin, although anticonvulsant, had different binding affinities to Oxcarbazepine, Carbamazepine, and Ranolazine. The most favorable model had a binding affinity of -5.8, and the least favorable model had a binding affinity of -5.0. The range was the largest for the Phenytoin binding affinities at 0.8. However, the mode for the Phenytoin was the highest at -5.1. The mean of the data for the binding affinities for Phenytoin was closer to the Carbamazepine and Oxcarbazepine at 5.31.

Next, the binding affinities of the TTX were measured to compare them to the different types of SCBs. TTX and SCN1A's most favorable model had a binding affinity of -5.4, and the least favorable model had a binding affinity of -4.9. The most and the least favorable binding affinities were the same as the Carbamazepine and very similar to the Oxcarbazepine. The mean was the highest of all the blockers at -5.09, similar to Carbamazepine and Oxcarbamazepine. The median of the binding affinities for TTX was also the highest at -5.05, the mode was -5.2, and the range was -0.5. Overall, the most favorable model, least favorable model, mean, mode, and range of TTX were similar to the anticonvulsants and differed heavily from the cardiac SCB.

Finally, for the binding affinity results, significance tests were then run to ensure that the results for the TTX treatments were different enough from those of the other treatment groups. An ANOVA test for significance was done. The p-value for this test was 0.00001, which is significant at $p<0.05$. This result for significance suggests that TTX is substantial from the other treatments.

Frame	RAN	CARB	PHEN	OXC	TTX	MP	NP
0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
1	1.583	1.936	1.887	1.912	1.755	2.784	1.772
2	0.718	0.386	0.358	0.431	0.141	2.155	0

Table 3 RMSD values in Angstroms for each SCB-SCN1A complex in intervals of 2 frames
RAN - Ranolazine
CARB - Carbamazepine
OXC - Oxcarbazepine
PHEN - Phenytoin
MP - Mutated Protein
NP - Normal Protein

An SCB's mechanism of action is how well the drug can stabilize the inactivation state to decrease the frequency of an action potential firing. For this reason, since the inactivation state is a state of the protein induced by a conformational change, Root Mean Square Deviations (RMSD) were used to measure the structural change. The structural change of the protein represents how much the protein shifts throughout the simulation. The more structural change there is, the more the protein has deviated and cannot return to its regular state from the inactivation state. Therefore, the more remarkable structural change derived from the higher RMSD signifies the higher instability within the protein. However, if the RMSD is lower, there is more stability in the compact structure of the protein. The average protein was measured first, and an excellent baseline was set up to compare. The first value was one of the lowest, showing the stability of the inactivation state. Then, the second value was 0, indicating that it completely stabilized the inactivation state.

On the other hand, the mutated protein showed extremely high levels of RMSD change, not turning back to 0 as quickly. This may illustrate that the mutated protein cannot stabilize the inactivation state as quickly. With these controls ready, the stability of the inactivation state was measured when the protein was attached to the SCBs.

This was done using calculations of the difference in structure in the protein through a few steps, for along with the SCB-sodium channel complex, the normal protein, or the nonmutated protein, and the mutated protein were also measured to be set as additional comparisons for the TTX measurements. First, for

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the normal protein, the first step had an RMSD difference of 1.772 from the zeroth step. However, the difference between the zeroth and second steps showed an RMSD of 0. As for the mutated protein, the difference between the zeroth step and the first step had the highest value of 2.784. The difference between the second and third steps remained high at 2.155 and stayed within the first value.

Once again, Ranolazine, the only cardiac SCB, had values different from those of the other SCB medications. The first RMSD difference value was the lowest, even lower than the normal protein, at 1.583. However, the second RMSD difference value did not significantly decrease from the first at 0.718. The values for the stability of the inactivation state for Carbamazepine, Oxcarbazepine, and Phenytoin were a little more similar. For Carbamazepine, the first difference value was slightly higher than the average protein at 1.936, and the second difference brought the RMSD difference to 0.386. Although not 0, the second difference was much lower than the second difference in Ranolazine. As for the Oxcarbazepine, the differences in RMSD were similar to the values for Carbamazepine. The first value was 1.912, and the second difference was down to 0.431. For Phenytoin, not much changed as the first difference was 1.887, and the second difference was 0.358. Finally, TTX was measured to compare the stability of the inactivation state with the other SCB medications and that of the standard and mutated protein. The first difference in RMSD was 1.755, the second lowest and the closest to the standard protein. Then, the second difference value for TTX was 0.141, the lowest out of all the SCB-sodium channel complexes and the nearest to 0.

Structure was also observed between the different SCBs, and the number of bonds was seen as a way to measure the size and arrangement of the SCBs. Once again, the Ranolazine had a far more different number of bonds than all other SCBs at 64. This was compared to around half the bonds found in the other SCBs. The Carbamazepine had 30, the Oxcarbazepine had 31, and the Phenytoin had 27. Then, the number of bonds for the TTX molecule was measured and compared to that of the other SCBs. It had 29 bonds, much closer to the anticonvulsants, and around half the bonds as Ranolazine.

Discussion

In this project, a computational methodology was used to compare TTX as a potential therapeutic for epilepsy to current anticonvulsants. First, binding affinities were measured to observe how strongly each medication was bound to SCN1A. Since researchers use binding affinity to design drugs, it is essential to measure it as it can provide valuable information about how selectively the drug is bound to the protein[30]. These values can be fundamental when trying to gain insight into interactions between ligands and receptors. The first piece of information that stood out was the highly negative binding affinity for Ranolazine. The relationship between binding affinity values and binding strength is a negative correlation, so this would suggest high binding strength between Ranolazine and SCN1A. This could be because Ranolazine is a cardiac sodium channel, and due to differences in the mechanism of action, the binding affinity also differs with the anticonvulsants of the study[23,24].

Carbamazepine and Oxcarbazepine were then found to have very similar binding affinities. This similarity goes past the simple fact that both are anticonvulsants. Phenytoin is also an epilepsy medication but was not seen as having binding affinities with this level of exactitude. Oxcarbazepine and Carbamazepine are designed to be very similar in structure and function[17,31?]. Their similarity in mechanism of action already suggests this, but oxcarbazepine, when released, was a derivative of Carbamazepine. Phenytoin also has a similar mechanism of action and has a mean closer to Oxcarbazepine and Carbamazepine, indicating its anticonvulsant abilities. However, when the binding affinity between TTX and SCN1A was measured, it was highly similar to Carbamazepine and Oxcarbazepine. Although this helps establish a connection between TTX and anticonvulsants, the closeness within the values demonstrates how similarly TTX could work as therapeutics compared to Carbamazepine and Oxcarbazepine. Structurally, while Oxcarbazepine and Carbamazepine look almost identical, TTX looks much different[32–34]. The weight of Oxcarbazepine and Carbamazepine are 252.268 and 236.269, respectively; their chemical formulas are $C_{15}H_{12}N_2O_2$ and $C_{15}H_{12}N_2,O$, respectively. However, TTX's molecular weight is 319.2,68, and the chemical formula is $C_{11}H_{17}N_3O_8$. Despite the difference in structure, the similarity in binding affinity values shows that TTX can have functions similar to anticonvulsant molecules that are derivatives of each other.

The first noticeable difference in the data was that the Ranolazine-SCN1A complex was inconsistent with the other anticonvulsants. This would make sense due to the differences in mechanisms of action. The stability of the inactivation state for Ranolazine went from the lowest first value at 1.583 to the highest second value (excluding the mutated protein) at 0.718. This could be due to cardiac SCBs performing differently in cardiac cells' sodium channels. The sodium channels in the cardiac cells have different mechanisms for inactivation and gating than those in neurons[?]. This may be why we see different behaviors in cardiac SCB than in anticonvulsants.

As expected, the anticonvulsants all showed similar RMSD change values. Since they have similar mechanisms of action and function, such as blocking ion permeation, they have the same effect on the stability of the inactivation state. While, based on the data, Ranolazine tried to stabilize the SCN1A protein over time to ensure it does not block ion permeation completely, the opposite was suggested for anticonvulsants. Since all the epileptic medications started from a higher RMSD and went closer to 0, it can be inferred that stabilization needs to occur for

neuronal sodium channels to counteract the effects of mutations that can cause epilepsy. The stability of the inactivation state for TTX was then measured. TTX had a similar pattern for the RMSD differences as the anticonvulsants. However, the first value of the RMSD was closer to the first value of the standard protein than any other SCB. The second value of the RMSD difference was also closer to 0 than any other SCB.

RMSD represents the stability of the inactivation state, and the higher the structural change, the more instability exists within the protein. Since TTX can bring down the RMSD the closest to 0 fastest compared to other anticonvulsant treatments, this indicates low structural change. This assumption is also supported as the control group of the regular protein has an RMSD of 0 in the second step, suggesting that there is barely any structural change and high levels of stability. Therefore, the fact that TTX can bring the RMSD difference the closest to 0 conveys its molecular significance and ability to stabilize the inactivation state completely. The molecular effect can resonate throughout the body. Therefore, the TTX treatment could significantly decrease the symptomatic effects and carry out its mechanism of action in a way that more closely returns the SCN1A to its regular state.

These results convey that TTX follows the same pattern of RMSD change as anticonvulsants and can also work better than existing medications. Since the RMSD value was able to get much closer to the normal protein than the current medications without surpassing the values, TTX could theoretically work as a therapeutic for epilepsy and potentially be better than existing treatments.

Implications

Based on the results of this experiment, it can be concluded that TTX could be used as a viable treatment for epilepsy. However, there would need to be further research to provide substantial evidence to support its clinical use due to the fact it is lethal in large doses. Theoretically, based on the data, TTX works similarly to existing anticonvulsants and carries out a mechanism of action that is better than the currently available pharmaceutical medications. Due to its more potent effects, there should be more research into how TTX can be implemented and what conditions, such as dosage, administration, and scheduling, need to be applied.

Binding affinity measures how strongly the SCB can bind to its protein target. Having a high binding affinity also represents that medication is more targeted and better at carrying out its mechanism of action. However, since TTX has a similar binding affinity to current treatments, suggesting that it works the same way as other treatments. If TTX can work in the same way as the current drugs, it signifies that TTX can already function as a viable treatment by binding to the SCN1A and blocking the channel. In addition, TTX's ability to stabilize the inactivation state better than existing treatments adds to its claim of use.

Limitations

For this experiment, using a computational methodology has some possible limitations. First, the SCN1A protein structure itself has some issues. A predicted structure was used instead since no perfect 3D model of SCN1A is available. The AlphaFold Database uses the DeepMind AI to follow molecular interactions to form a prediction of how the protein would look like[35,36]. Because this predicted structure could have some flaws, any interaction between SCN1A and the SCBs could be slightly off.

In the same way, there are issues with the 3D structures of the SCBs and TTX[37]. There is a CH penalty value for the bonds of a molecule, which measures how reliable the conformation is and how ready it is for any simulations. For Ranolazine, the CH penalty was extremely low at 3.6,4, and there was not much to worry about. Carbamazepine and Oxcarbazepine were also acceptable at 29.10 and 30.19. However, Phenytoin and TTX were slightly higher at 69.04 and 58.6,0, respectively. Since these were a bit high, there could be skewed results on any GROMACS simulations.

Although TTX could functionally work better as an SCB, according to the data, this does not ignore all the possible side effects that can occur. Measurements of binding affinity and the stability of the inactivation state do not consider the molecule's toxicity. With a computational methodology, this is hard to determine; it is necessary to be informed about the side effects of TTX as a therapeutic and accordingly proceed with any further research.

Finally, this project's computational methodology used an iMac with 8 GB memory and a 3.1 GHz processor. This project's application is CPU-intensive, taking much time to produce data. Even if the data were small, it would take up a lot of memory. Given these technological limitations, generating small amounts of data to make conclusions was only possible.

While TTX has been suggested as a viable therapeutic for Epilepsy, the molecular scope of this project does not take into account that its mechanism of action is similar to that of drugs for other disorders. This is due to TTX's wide range of functions, and this project only considers TTX's effects on SCN1A mutations, which mainly result in Epilepsy. TTX has been used for multiple disorders affecting different sodium channels[26]. In addition, TTX has been shown to have a mechanism of action similar to that of heart SCBs, as demonstrated by the results of Ranolazine[8,22,23]. Since TTX can have many systematic effects, one of the limitations of the results of this project includes the drug-drug and drug-molecule interactions that may arise as a result of using TTX as a treatment. To address this in future studies, more computational methodologies must be conducted, and the pharmacokinetic properties of TTX must be looked at

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systematically.

Very few research studies have examined the molecular effects of TTX on the SCN1A channel in Epilepsy. While TTX has been researched for other disorders, this has been based on its toxic nature and its ability to block sodium channels. Therefore, one of the limitations of this project is that previous research has mixed results in clinical support of TTX. Thus, this project can apply to many aspects of future directions. To gain a deeper understanding of the precise effects of TTX, its side effects, and its potential to treat epilepsy better than existing anticonvulsants, animal models can be employed. Mouse models are currently being used to examine how TTX can be used as a therapeutic for neuropathic pain[26]. A push toward using epileptic mouse models to test TTX's effects would add support to TTX's therapeutic capabilities. The results from these mouse models could serve as adequate evidence to support the conclusions stated in this paper and provide the basis for why it should be used in future studies.

Future Directions

Many parts of this research could be turned into a far more large-scale experiment. The first step is replicating the same methodology but outputting more data to support conclusions. The measurements of RMSD difference for the stability of the inactivation state were only observed through three steps for this project. These three steps are a small snapshot of a much larger biological process. With more extensive processing power and higher memory storage, RMSD calculations could be measured across thousands of steps to measure multiple inactivation states over time. With a much larger snapshot, we can say much more confidently whether TTX can be used as a therapeutic or not.

Another way to increase the project's data and generalize the results to more populations is to test multiple mutations. In this project, only one mutation was tested as it was held as a constant, p.S228P. However, in the future, various mutations can be used to see if the pattern of effects of the SCBs is consistent. In addition, instead of just the four SCBs that are used to compare to TTX, more SCBs with multiple different functions could be tested. Adding more control groups to test with TTX could help solidify the position that TTX could be used as a therapeutic.

Since this experiment employed a full computational methodology, much of these results are theoretical. To observe more about the exact effects of TTX, its side effects, and whether or not it can treat epilepsy better than existing anticonvulsants, animal models can be used. Mouse models are already currently being used to find how TTX can be used as a therapeutic for neuropathic pain[26]. Therefore, more push toward using epileptic mouse models to test TTX's effects on other anticonvulsants would be beneficial to see if TTX can and should be used as an anticonvulsant.

Conclusion

TTX, a potent neurotoxin, has been used in research for quite some time, and considerations have been taken to implement it as a potential therapeutic for some disorders, including neuromuscular conditions. However, epilepsy, a neurodegenerative disorder, has not been considered as a possible condition that could be alleviated by utilizing TTX as a medication despite its similarities to existing anticonvulsants. This raises the critical question of whether or not TTX can be used in replacement of current SCBs for the treatment of epilepsy. The results of this computational experiment suggest that TTX has similar binding affinities to anticonvulsants and stabilizes the inactivation state of the mutated protein better than the anticonvulsants. Although this project's computational methodology yields purely theoretical results, it may be possible to test TTX in further applications to strengthen the claim that TTX should be used as therapeutics.

Materials & Methods

Materials

Multiple applications were used for the computational methodology. These included PyMOL, Autodock Vina, GROMACS, and VMD. Excel was also used to record data. PyMOL is a visualization software showing multiple processes such as micromoles, drugs, protein-ligand complexes, etc[38]. For this project P,yMOL was used to visualize the SCN1A protein. PyMOL was also used to edit the protein's residues to simulate a mutation that can occur in patients. Autodock Vina is an application capable of performing binding simulations between proteins and drugs and can calculate binding affinities by predicting molecular interactions[39,40]. PyMOL and Autodock Vina can be used together to visualize potential binding sites for ligands and bind the ligand to those coordinates. Molecular dynamic (MD) simulations were a significant part of this project, and GROMACS was used extensively for this portion of the experiment. GROMACS is one of the most popular tools for running and analyzing MD simulations for complex systems, including those with protein-ligand complexes[41]. GROMACS is highly used and cited in thousands of research papers due to its convenience and efficacy. The final application was another visualization tool called VMD (Visual Molecular Dynamics). VMD and GROMACS are often used together since they complement each other[42,43]. GROMACS can create MD simulations, and VMD specializes in visualizing them, so any analysis can be done using the many tools VMD offers.

Other than the applications, a couple of databases were also used to find predetermined 3D structures of the molecules used in this experiment. First, the AlphaFold Database was used to find a predicted model of the SCN1A protein[35,36]. The AlphaFold Database is highly ranked for predicting structures as

it uses DeepMind AI to predict conformations of proteins with high accuracy. Second, the DrugBank Online database has pharmaceutical information about countless drugs for any type of disorder[44]. The DrugBank database has been used for multiple purposes involving therapeutics, including drug interactions, designs, metabolism, screening, etc. The DrugBank was used to obtain 3D structure and other information about the SCBs used in this experiment: Ranolazine, Carbamazepine, Phenytoin, Oxcarbazepine, and TTX[32–34,45–47].

Methods

The first step in this experiment was to mutate the protein to replicate the version found in epileptic patients. Since numerous mutations in SCN1A can produce similar effects, the challenge is to identify a common mutation. To achieve this, multiple clinical trial papers were analyzed to pinpoint common mutations among patients[4,47–50]. A list of some of the most common mutations was recorded. PyMOL was used to find the effect of the mutation in SCN1A used in this project. Among the mutations studied, p. S228P was chosen because it exhibited the protein's biggest structural and functional change, making it a perfect candidate for this experiment[4,47–50]. Since the mutation was held constant in this experiment, one mutation that had a high effect had to be chosen. Based on multiple previous papers, p.S228P was one of the most prevalent found in patients and difficult to treat with current treatments. Therefore, proving TTX's clinical significance in this mutation would add to the basis of reasoning for TTX's use.

The AlphaFold Database, which uses DeepMind AI to predict structures of molecules, was used to obtain a file of the 3D structure of the protein so that it could be used in certain applications[35,36]. The first of these applications was PyMol, a molecular visualization tool that allows for rendering of the 3D structure of molecules. Through PyMol, the goal was to mutate the protein. The steps to mutate the protein begin with importing it to visualize its 3D structure on PyMol. Next, PyMol gives access to the SEQ option to visualize every residue in the protein. Accessing the amino acid sequence enables the identification of the residue that will be mutated. Residue number 228 is chosen and is mutated from a serine to a proline. This point mutation applied to the protein serves as the experimental model for the next step.

Now that the experimental model was created, the drugs used to counteract the effects of the mutation had to be chosen. A few specific SCBs from previous research and clinical trials were used to test the effects of TTX. Ranolazine, Oxcarbazepine, Carbamazepine, and Phenytoin were all chosen as they are commonly prescribed medications with a good record of reducing patient symptoms[30,51–53]. Oxcarbazepine, Carbamazepine, and Phenytoin are mainly epileptic medications. Ranolazine, while capable of treating epilepsy, is used primarily in heart disorders. Ranolazine was selected to compare TTX not only with epilepsy medications but also with those used for heart-related disorders. The SCBs chosen for this research are some of the most common in research papers, suggesting they are commonly used in epilepsy treatment. The DrugBank database was used to obtain a 3D model of each SCB. Files of 3D models of each SCB were taken and stored to attach to the protein.

This experimental model can physically bind each SCB to a specific protein region. The protein region for which the SCBs would bind remained constant throughout the experiment. The particular region selected for the SCB to bind in was the pore of the protein where the sodium ion transportation occurs. This particular region was chosen because it is where most of SCN1A's function happens. Since mutations increase the sodium ion transportation in the protein, blocking the pore can counteract the effects exerted by the mutation. Most SCBs operate through a specific mechanism of action that is carried through a multi-step process. Binding affinity, or the strength of the bonds between the receptor and the ligand, is exhibited when each SCB attaches to the protein. Binding affinity is a critical measurement because it can provide insight into whether or not the SCBs can bind to the protein. The stronger the binding affinity, the more likely the SCB can carry out its function on the protein.

Next, Autodock Vina measured the binding affinity between the protein and the SCB. However, two things must be done before the binding: the protein and the drug must be prepared, and the binding parameters must be set. The protein and the drug can be prepared using a set of commands so that Autodock Vina can accept them55. These commands would ensure the atom positions are correct and find the optimal conditions for the protein and the drug. As for the conditions, the coordinates of the binding, the exhaustiveness, and the energy range all need to be defined. After these conditions are set, the binding affinities of a binding position between the SCB and the protein can be outputted. However, instead of using only the best-recorded binding position, we analyzed the top 10 binding positions. This was done to compare multiple binding affinities between TTX and the other SCBs.

As mentioned before, the stability of the inactivation state can be measured by the RMSD or the structural change of the protein. An MD simulation needs to be created. This is required to calculate this computationally and change a motionless 3D structure into a moving image that predicts the interactions of the molecules over time. For this, an application called GROMACS was used. GROMACS is a tool that can run MD simulations to mimic the conditions happening in the natural body. To set these conditions, multiple files must be created to define the parameters of the environmental conditions. These parameters are essential to the simulation as they include real-world situations such as temperature, pressure, velocity of molecules, bond parameters, etc. These files and a series of commands were used

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to prepare the protein-drug complex and run the MD simulation. However, another application must be used to visualize the MD simulation and measure the RMSD. The MD files from GROMACS are transferred to an application called VMD.

Uploading a molecule to VMD allows visualization of the structure and dynamics in a three-dimensional space, providing insights into its conformation and interactions. For the MD simulation, data must be loaded into the molecule to start the movement of the molecules. The RMSD calculation is the next step with the protein packed into VMD. In VMD, the trajectory tool is used to measure the RMSD of the protein in the simulation. The scope of the RMSD trajectory tool needs to be focused on the protein of interest, isolating it from other molecular components. Next, the trajectory tool aligns and calculates the RMSD. Finally, saving the data ensures that the analyzed results are preserved for further interpretation, sharing, or publication, contributing significantly to our understanding of protein dynamics and function in a biological context. The RMSD calculation is crucial for understanding the protein's stability and conformational shifts during the simulation.

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