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Camila Garcia
Florida International University, cgarc622@fiu.edu

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Sequence Selectivity in the Binding of Melatonin to PhiX174RF DNA

Camila Garcia, Florida International University, US, cgarc622@gmail.com
Faculty Advisor **Stephen Winkle**, Florida International University, US, winkles@fiu.edu

Melatonin is an endogenous neurotransmitter that controls the circadian rhythm. When consumed through medication, it is proven to serve as a treatment for sleep disorders and improve sleep quality. Stable levels of melatonin in the human organism are shown to be beneficial as it provides a protective layer to DNA strands that ensures their longevity; however, its excessive consumption may have damaging consequences on the long run. Here we present a study on the sequence selectivity in the binding of melatonin to DNA, intending to show that melatonin targets specific DNA sites. Binding tophiX174RF DNA was assayed using restriction enzyme activity assays employing nine restriction enzymes with differing target sequences and melatonin/DNA base pair ratios ranging from 0.5 – 20. Products of restriction enzyme digests were separated using agarose gel electrophoresis and the relative amounts of digestion product analyzed optically. The results indicated that melatonin selectively binds to DNA. The studies also revealed that upon binding, melatonin nicks DNA strands indicating potential strand damage in the long run.

Keywords: *melatonin*, *DNA*, *selective binding*, *inhibitory activity*, *neurotransmitter*, *DNA damage*

Melatonin, the neurotransmitter that mediates sleep, controls the circadian rhythm through a light/darkness trigger system (Vitaterna, 2001) that synchronizes our sleep-wake cycle with night and day (Drawson, 1993). Exogenous melatonin, artificially engineered to treat sleep difficulties and disorders (Suni et al., 2021), is also used to treat other conditions such as ADHD, Alzheimer's, and depression due to its sedative properties; as well as to regulate vascular flow and inflammation (Turek, 2004). Studies show that when observing DNA in *vitro* conditions, melatonin protected DNA by significantly reducing cleavage-induced deterioration (Takada, 2012). Other studies have also revealed that melatonin stimulates oxidative damage repair in DNA by scavenging reactive oxygen species of their primary sources (Sliwinski, 2007). In addition, when observing DNA-melatonin reactive activities, studies showed that in the presence of copper, melatonin protected DNA from free radical damage, presenting anti- carcinogenic, anti-aging, and anti-tumor properties (Nida, 2016).

While most studies corroborate the antioxidant action of melatonin, it has exhibited pro-oxidant properties in some instances (Nida, 2016). Research revealed that melatonin protected normal and cancer cells against genotoxic treatment and apoptosis induced by idarubicin (Majsterek, 2005). Therefore, it was concluded that it could be damaging for patients with leukemia if not used with caution (Majsterek, 2005). Other studies have suggested that while the pharmacologic melatonin dose improves sleep, it can also induce hypothermia and cause plasma melatonin to remain elevated during the day (Zhdanova, et al., 2001).

While previous studies have indicated that short-term melatonin use appeared safe for consumers for up to three months (Carter, 2012), its long-term safety remains unknown. The inconcrete understanding of the effects of melatonin, when in contact with the human genome, inspired a need to understand this molecule. A study that evaluated the melatonin-DNA binding activity and molecular interaction was a fundamental step to understanding the potential threats of melatonin.

Materials and Methods

Assays to Determine the Binding Selectivity of Melatonin to DNA

PhiX174RF DNA, a mixture of supercoiled and relaxed DNA, was used through all trials of the experiment (Winkle, 2013 & 2015). The melatonin-DNA base pair ratios ranged from 0.5– 20, and this range was maintained through all trials and experiments performed. DNA was assayed employing nine restriction enzymes with different target sequences that were diluted into a solution prepared with a 1:1:8 ratio of stock enzyme, compatible buffer solution, and glycerol water (**Figure 1**). The restriction enzymes were compared to search for similarities of the arrangement of their base pairs.

Figure 1

PhiX Cleavage Sites with Flanking Sequences and Location of Cleavage

Restriction Enzyme	Location	Restriction sequence with flanking sequences (5'-3')
Nar-I	1019	TTATGTCTAATATTCAAACT**GGCGCC**GAGCGTATGCCGCATGACCT
	2976	TTGCTTCTGCTCTTGCTGGT**GGCGCC**ATGTCTAAATTGTTTGGAG
Alw-44	4779	GCCGTCTTCATTTCCATGCG**GTGCAC**TTTTATGCGGACACTTCCTA
Ava-II	5042	GTTAACAAAAAGTCAGATAT**GGACC**TGCTAAAGGTCTAGGAGCTA
BssH-II	5348	ACAAATCTGCTCAAATTTAT**GCGCGC**TTCGATAAAAATGATTGGCG
Dra-I	327	AGAGATTCTCTTGTTGACATT**TTTAAA**AGAGCGTGGATTACTATCTG
	1406	ATTTGATATCTATAGCGTAT**TTTAAA**GCGCCGTGGATGCCTGACCG
Mlu-I	221	AACGATTCTGTCAAAAACTG**ACGCGT**TGGATGAGGACTGGCTTAAT
	2146	CCTTCTGGTGATTTGCAAGA**ACGCGT**ACTTTCGCAACGATGATTAT
Nci-I	2800	TTGACGTCGTTCCCCGTACG**CCCGGG**CAATAACGTCTACGTTGGTT
Nru-I	2260	TATCGCAATCTGCCGACCACT**CGCGA**TTCAATCATGACTTCGTGAA
	4424	GAACGCCCTCTTAAGGATAT**TCGCGA**TGAGTATAATTACCCCAAAA
Pst-I	5382	AAATGATTGGCGTATCCAAC**CTGCAG**AGTTTTATCGCTTCCATGAC
Stu-I	4486	TGAGTGTTCAAGATTGCTGG**AGGCCT**CCACTAAGATATCGCGTAGA
Xho-I	162	TTCGACCTATCCTTGCGCAG**CTCGAG**AAGCTCTTACTTTGCGACCT

Note: This figure was used to determine potential factors of binding promotion through the comparison of bases of each flanking sequence.

Different buffers independently compatible with the restriction enzymes were also employed along with their complementary enzyme (**Table 1**) (Winkle, 2013 & 2015).

Table 1

Restriction Enzymes and their Corresponding Buffers

Enzyme	**Ava II**	**Dra I**	**Mlu I**	**Pst I**	**Stu I**	**XhoI I**	**Alw II**	**Nar I**	**Bssh II**	**Nci I**	**Nru I**
Buffer	C	B	D	H	B	D	C	G	H	B	K

The DNA samples were incubated with their indicated ligand for 10 minutes at 37 degrees Celsius. Then, the samples were digested for 30 minutes at the same temperature after three microliters of diluted restriction enzyme were added to Samples 2 through 6, as Sample 1 constitutes the control group. Lastly, the reaction was stopped by adding sodium dodecyl sulfate (detergent) with a concentration of 0.3% to all samples and heating them to 65 degrees Celsius for five minutes (Winkle, 2013 & 2015). Once the preparation was completed, all the samples were frozen until the separation of products through gel electrophoresis at a voltage of 100 V. The relative amounts of digestion products were analyzed optically under a UV lamp.

Assays to Determine the Impact of Melatonin on DNA Strands

No Cleaving Agent

When preparing the assays, the concentrations of buffer and DNA resembled those of the first experiment and were kept constant through all the samples. Buffer Mung Bean was employed for this trial due to its universal compatibility with several cleaving agents. The concentration of melatonin was incremented in each sample by one microliter. In this experiment, the DNA samples were also incubated for 10 minutes at

37 degrees Celsius. However, the digestion process was omitted due to the absence of a restriction enzyme (Winkle, 2013 & 2015). Lastly, the reaction was stopped through denaturation by 0.3% SDS (detergent) and exposure to 65 degrees Celsius for five minutes (Winkle, 2013 & 2015). All the samples were frozen and then separated through gel electrophoresis at a voltage of 100 V to later be analyzed optically.

Nuclease as a Cleaving Agent

For this experiment, the concentrations of buffer and DNA resembled those of the previous experiments and were kept constant through all the samples. For this assay, Mung Bean Nuclease was used as a cleaving agent. One microliter of 0.1 m $ZnCl_2$ solution was added in every sample. The melatonin concentration was also increased through the samples by 0.01 ml increments (starting with 0.01ml in Sample 3 and reaching 0.04 ml by Sample 6). MultiCore 10X, also known as universal buffer, was specifically used for this experiment due to its high compatibility with multiple restriction enzyme and nuclease solutions. The nuclease was diluted by adding a 1:1:8 ratio of stock enzyme, compatible buffer solution, and glycerol water. The samples were incubated for 15 minutes at 37 degrees Celsius and then digested after the addition of the three microliters of diluted nuclease solution to Samples 2 through 6 for 30 minutes at 37 degrees Celsius. Finally, the solution was stopped by adding three microliters of 0.3% sodium dodecyl sulfate and heating the solution to 65 degrees Celsius for five minutes (Winkle, 2013 &2015) to then be frozen, separated by gel electrophoresis, and analyzed optically.

Gel Electrophoresis

In all these experiments, the products of digestion were separated by electrophoresis of 1% agarose gels. To prepare the agarose, a solution of one gram of agarose, 10 milliliters of 10X Tris/Borate/ EDTA (TBE) buffer, and 90 milliliters of deionized water were stirred into a homogeneous mixture that was then heated for approximately two to three minutes. The hot agarose solution was left to decrease its temperature to 60-70 degrees Celsius and was then poured in the mold for the wells. Once solidified, the mold was placed in the electrophoresis apparatus. The side with the wells was positioned towards the negatively charged side of the apparatus to allow the negatively charged DNA pieces to travel down the gel towards the positively charged side. The voltage of the apparatus for each trial was 100V.

With a bath of ethidium staining, the process of optical analysis was facilitated as this technique aids the determination of the sizes of the DNA pieces (Winkle, 2013 & 2015). Finally, the gels were observed with the aid of an ultraviolet lamp that allowed for a clearer visualization of the DNA pieces' travel through the gel.

Results

Binding Selectivity of Melatonin to DNA

To determine if the result implied the binding of melatonin to DNA, Lanes 2 and 6 were compared due to their reflection of a gradient-like increase of melatonin concentration (**Figure 2A**). The different restriction enzymes can elicit different binding interactions between melatonin and DNA. Enhancement is the negative evidence of binding and will be reflected through a decrease of starting material and increase in the product band with the increase of melatonin concentration; indicating that the restriction enzyme

prevents the binding of melatonin to DNA. In this experiment, however, none of the used restriction enzymes led to these types of results.

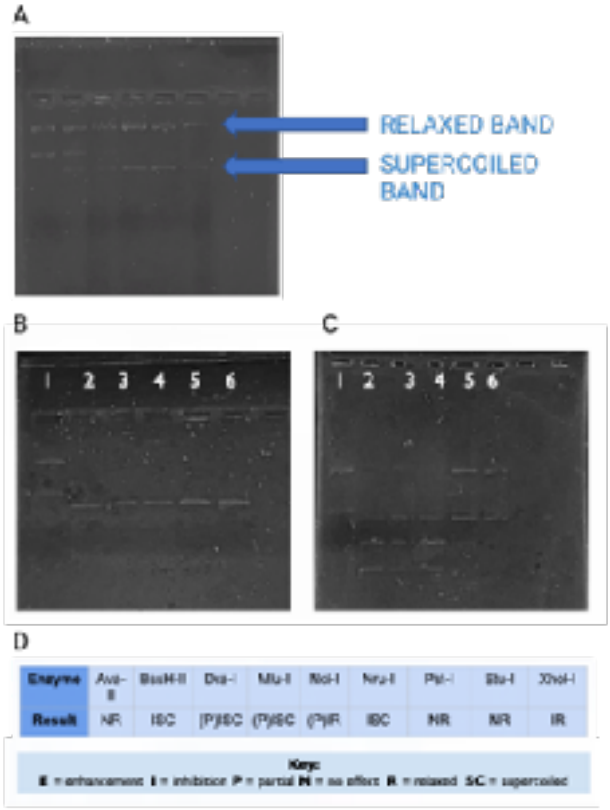
Inhibition implies positive evidence of binding and is evident when there is an increase in starting material and a decrease in product DNA bands as melatonin concentration increases (**Figure 2C**). An indication that the restriction enzyme enables the binding of melatonin to DNA. Both results can present themselves at different levels of intensity depending on the prominence of the products seen on the gels and are classified as partial inhibition or partial enhancement.

No results could also be apparent when the products from all lanes have traveled the same distance down the agarose gel and show the same level of prominence, implying that the restriction enzyme does not enable nor prevent the binding of melatonin to DNA (**Figure 2B**).

The diversity of the obtained results demonstrates selectivity in the binding of melatonin to DNA as this implies that binding can be facilitated or unaffected depending on the presence of certain enzymes.

Figure 2

Separated Products of Enzyme ActivityAssays



Note: A) Shows the different bands present in the separated products that must be examined to determine the binding behavior present in each trial B) Assay that contained PST-I as a restriction enzyme showing no effect. C) Assay containing NRU-I showing inhibitory activity and positive evidence of binding.

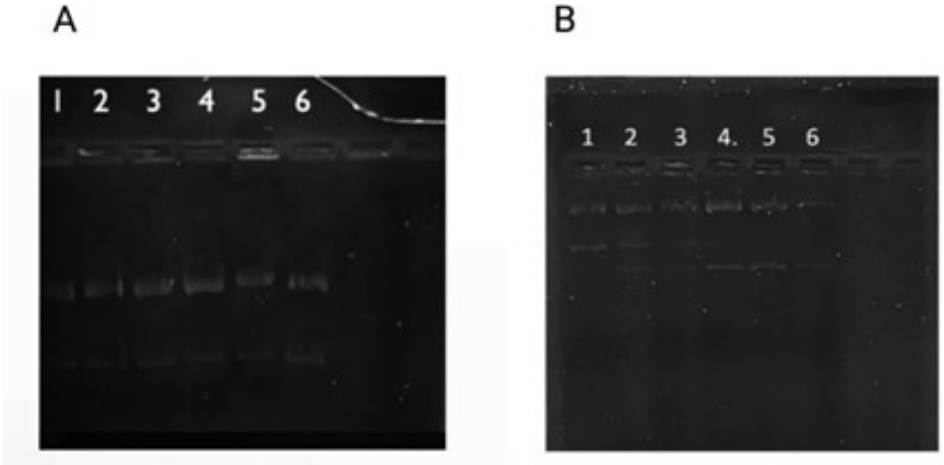
Below, the content of each lane's sample is provided:(Lane 1: DNA only, Lane 2: DNA +Restriction Enzyme, Lane 3: [MEL] / [Enzyme] = 5, Lane 4: [MEL] / [Enzyme] = 10, Lane 5: [MEL] / [Enzyme] = 15, Lane 6: [MEL] / [Enzyme] = 20 D) Results of each assay containing different restriction enzyme.

Melatonin's Impact on DNA Strands

To understand the direct effect of melatonin upon contact with DNA, the gel was interpreted through optical analysis to reveal a structural change. Such change becomes evident when the supercoiled band turns into the relaxed band after the Mung Bean nuclease cleaves the DNA. In both the presence of Mung Bean Buffer + no enzyme (**Figure 3A**) and Mung Bean Nuclease (**Figure 3B**), the formation of linear DNA at higher levels of melatonin concentration can be appreciated. The nicking of the supercoiled DNA is clearly evidenced through the fading of the supercoiled band as melatonin concentration increases while the relaxed band remains intact. In conclusion, at higher concentrations of melatonin, there will be sufficient alterations caused by melatonin to DNA that will cause Mung Bean to produce a double strand break that creates linear DNA (**Figure 3B**). Since potential damage is predictable, these results are strong enough rationale to bring about an interest in the investigation on the effects of long-term melatonin intake.

Figure 3

Separated Products of Enzyme Activity Assays to Determine if Melatonin Directly Afffected DNA



Note: A) Assay that contained no cleaving agent to determine the effects of melatonin in DNA. B) Assay that substituted restriction enzyme with Mung Bean nuclease as a cleaving agent to further confirm the results from panel A. Below, the content of each lane's sample is provided: (Lane 1: DNA only, Lane 2:

DNA +Restriction Enzyme, Lane 3: [MEL] / [Enzyme] = 5, Lane 4: [MEL] / [Enzyme] = 10, Lane 5: [MEL] / [Enzyme] = 15, Lane 6: [MEL] / [Enzyme] = 20).

Discussion

As the consumption of melatonin has become widely normalized, it is essential to understand the effects of its presence in our organism. Melatonin's lipid and water solubility allow it to easily cross the cell membrane and come in contact with our genomic information. Therefore, our study's purpose was to discover if melatonin could bind to DNA and what factors made it more feasible while shedding light on its effects on the DNA strands.

Melatonin Selectively Binds to DNA

Our results suggest that melatonin binds to DNA selectively as three types of binding were evidenced during the optical analysis: Inhibition, partial inhibition, and no results. From these, it was also possible to infer that the used restriction enzymes do not hinder the binding of melatonin to DNA.

Flanking Sequences

To indagate what caused this preference for some enzymes over others, the flanking sequences of each restriction enzyme were examined and compared based on AT and/or GC revealing structural information about the strands. The gels that presented inhibitory activity frequently had regions that were AT predominant. For example, restriction enzyme Dra-I has a cleavage site TTTAAA and other AT predominant regions. Simultaneously, enzymes such as BssH-I and Nru-I had AT-rich flanking sequences (**Figure 1**). As a result, it could be assumed that AT contributes to melatonin binding to DNA and therefore, melatonin presents sequence selectivity. A possible contribution to the facilitation of melatonin binding to DNA could be the potential presence of a TATA box or element due to its binding facilitatory functions.

In the case of restriction enzymes like Stu-I and Ava-II, no significant effects were evidenced in the gels, while in Mlu-I and Nci-I, evidence of partial inhibition was demonstrated (**Fig. 2C**); which could be attributed to a decreased abundance of AT in the flanking sequences. However, it is important to note that some of the restriction enzymes that contained CG rich sequences also showed inhibitory activity. Therefore, it is more appropriate to conclude that AT richness contributes to the binding of melatonin to DNA, but it does not constitute a mandatory component to allow for it.

Presence of Supercoiled Band

According to the results, those restriction enzymes that resulted in supercoiled fragments were more likely to show inhibitory activity. When two bands can be counted on the first lane, it indicates that the strand has been cleaved twice. These two new bands are the relaxed and supercoiled bands. The latter will be the one that has traveled the furthest down the gel (towards the positively charged end) due to its higher compatibility level relative to the relaxed band (Gibson, 2020). Some enzymes that portray this effect are Bssh-I, Dra-I and Mlu-I. While most of the restriction enzymes that showed inhibitory activity showcased a

supercoiled band, some of the restriction enzymes that showed inhibitory activity only had a relaxed band: Xhol-I and Nci-I. This would lead to the conclusion that although supercoiled bands are predominant in gels that show inhibitory activity, this band is not necessary to allow for binding.

Melatonin Nicks DNA Strands

Since these studies would only evaluate melatonin's interaction with DNA at their mere contact, deterioration caused by long term consumption could not be evaluated. To make up for this limitation, strand nicking was the considered factor to evaluate as it could represent potential damage to the strands after long term exposure. To test for nicking, the DNA structure must show sufficient alterations to produce a double strand break that creates linear DNA. Our results indicated the nicking of the strands when in the presence of melatonin, implying that the supercoiled band was affected when in contact with melatonin. While the result from this study suggests potential damage to the DNA strands, further studies will be necessary to determine if these effects might negatively evolve over time. Although nicking does not necessarily imply the deterioration of the strands, it is initiated by their breakage to form linear DNA. Therefore, the potential of future deterioration should not be discarded.

Conclusion

For future replications of this study, it is important to note that there are factors that will facilitate data collection and allow for higher quality of results if followed. The reproduction of the gel electrophoresis run with one specific restriction enzyme is strongly recommended as it allows for a wider variety of pictures for selection to display as results. It is also imperative to find buffers that are strongly compatible with an enzyme, as it would allow for clearer results. To follow through with this recommendation, it is suggested to opt for buffers and restriction enzymes coming from the same company and/or distributor. Avoiding old solutions of diluted restriction enzymes can prevent unclear results in the gel electrophoresis. It is recommended that once prepared, the enzyme solution should be preferably used within 48 hours after preparation, as its effectiveness might dwindle overtime.

If the effects of melatonin in other types of DNA become of interest, it is recommended that the previous experimental design is followed. This would allow for a deeper understanding of the differing components in the strands that may be the cause of the potentially fluctuating consequences when in the presence of melatonin. Also, the use of a different type of DNA might be helpful to have a wider understanding of the relationship of melatonin with different flanking sequences.

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