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Nanodisc assisted terbinafine transport into *Saccharomyces cerevisiae*

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Abstract

Terbinafine is a common antifungal medication that is used to stop the growth of fungal infections such as athlete's foot; it is most commonly used as a topical ointment with a prolonged application time (upwards of seven weeks). Nanodiscs are a supramolecular complex made up of a lipid bilayer and a membrane scaffold protein. In this experiment, we sought to test if we could improve the effectiveness of Terbinafine against *Saccharomyces cerevisiae* through the use of nanodiscs. We prepared our Brewer's yeast in a YM broth liquid medium concurrently with a DMPC lipid bilayer wrapped in an ApoA1 scaffold protein containing our terbinafine medication. We then separated our yeast broth into 1 ml samples to be treated, and the treated samples were allowed to sit incubating overnight. The measurement of cell death was achieved by staining our samples with Methylene Blue before measuring absorbance and fluorescence on a SpectraMax i3 microplate reader (i3). Through our experimentation, we were able to determine a extinction coefficient for terbinafine suspended in ethanol and TBS, which we calculated to be 20.056 $(mg/ml)^{-1}cm^{-1}$; however, we did not observe any significant increases in cell death on account of the nanodisc loaded with terbinafine, and any samples that did appear to support our assumptions contained so much associated error as to make it impossible to claim anything definitively. While our results do not present compelling evidence to support our hypothesis, we do feel this experiment presents a novel look at the medicinal transport capabilities of nanodiscs in fighting fungal infections, and might be a stepping stone for future researchers in this very field. Further, not only were we able to determine an extinction coefficient for terbinafine, but this was also a new experimental method for using methylene blue to measure yeast death that we had been unable to find prior research using.

Introduction

Fungus

Saccharomyces cerevisiae is a species of yeast commonly referred to as Brewer's Yeast. It is a common household ingredient used in things like bread making, brewing, baking, and wine making. It is a significant model organism in the scientific community, and has been studied and used in studies countless times; it has a short generation time and can be easily cultured.

Antifungals (Terbinafine)

Antifungals are medicines that prevent or kill fungi, which are the cause of fungal infections. Antifungals come in many shapes and sizes, but in general they can be separated into 4 ways that they attack the fungal cell: (1) they target the ergosterol biosynthesis pathway, (2) they inhibit nucleic acid synthesis, (3) they inhibit the **β** (1,3)-D-glucan synthase and disrupt the cell wall, and (4) they bind to ergosterol and disrupt the cell membrane. For the purposes of this study, we will be focusing on the antifungal Terbinafine, which operates by disrupting the cell membrane, making holes in the membrane which causes the cell contents to leak out. Under the assumption that Terbinafine is an effective antifungal, especially in direct contact with fungus, this experiment hopes to determine whether it can be made more effective through the use of nanodisc by testing it on Brewer's yeast.

Nanodisc (DMPC and ApoA1)

Nanodiscs are a disk shaped lipid bilayer that are stabilized and made soluble by membrane scaffold protein which encircle the layers.[1] Nanodiscs can form rapidly and in high quantities and show fast acting membrane integration; they have proved to be a useful tool in direct transport, especially in the medical field. Studies have shown that nanodiscs improved the efficacy of the anti-cancer drug Cabazitaxel as well as in a study where chemotherapeutic agents were transported via nanodisc into tumors, causing them to become sensitized to immune activation, subsequently increasing the achieving potent anti-tumor effects.[2,3]

In this experiment, we used DMPC as the lipid bilayer to develop our nanodiscs because of its ease of use and proven effectiveness. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is a synthetic phospholipid used in liposomes and lipid bilayers for the study of biological membranes; it is a phospholipid consisting of two C_{14} myristic acid groups attached to a phosphatidylcholine head-group. Using these fatty acids formed the lipid bilayer which was encircled in apolipoproteins coded by APOA1. Apolipoproteins are proteins that bind lipids to form lipoproteins, and the APOA1 gene provides the instructions for making the protein apolipoprotein A-I. ApoA-I is a component of high-density lipoprotein (HDL), which is a molecule that transports cholesterol and certain fats called phospholipids through the bloodstream from the body's tissues to the liver. They transport lipids (and fat soluble vitamins) in blood, cerebrospinal fluid and lymph.

Methylene Blue

After developing our nanodisc, we needed to be able to observe fungal cell death; to do this, we employed the use of a die that visually stains dead yeast cells called methylene blue. Methylene blue is a thiazine dye that works by converting the ferric iron in hemoglobin to ferrous iron. It readily permeates yeast cells, but it's reduced to a colorless compound in living cells; methylene blue acts as a better electron donor (hydrogen acceptor) than ADP/ATP, so living yeast favor methylene blue and utilize this substrate, preventing it from turning blue. However, in dead cells it appears dark blue (stained with methylene blue) because these are cells unable to process it, where living cells are colorless and remain unaffected.

Summary of research

The purpose of conducting this research is not only to confirm the effectiveness of nanodiscs as a medication transport vessel, but to potentially find a more effective way to administer antifungals. Up till now, antifungals are medications that are applied topically to affected areas or consumed orally slowly overtime, sometimes for weeks or months at a time. And medications like terbinafine can be hard for the body to process, broken down by the stomach, and acutely toxic to the liver before being able to treat the affected regions. So the development of safe methods that minimize unwanted cellular damage is a worthwhile pursuit that may be solved through the use of nanodiscs. However, in order to ascertain their effectiveness, we must first test their ability to transport medication into fungi. We first needed to assemble the nanodiscs with medication inside them before administering them to colonies of our brewers yeast grown in carefully cultivated environments through direct application. While this research cannot speak to the effectiveness as medicinal transport in human subjects, we believe it represents an important first step in research in that direction.

Methods

- **Preparing YM Broth:**
  - 1.5 g of yeast extract, 1.5 g of malt extract, 2.5 g of peptone, and 5 g of dextrose was added to 500 mL of milli Q distilled water
  - Mixture heated on hot plate and magnetic stir bar until solids dissolved
  - Mixture was poured into an autoclave bottles and autoclaved for 15 minutes with 15 lbs of pressure at 121 ℃
- **Preparing Antifungal Stock Solution (and Dilution):**
  - 0.50 g of Terbinafine hydrochloride 99% was suspended in 20 mL 95% ethanol (5% water)
  - First dilution made by placing 0.01 ml (10 µl) of the stock solution into 0.99 ml (990 µl) of TBS (50 mM Tris, 300 mM sodium chloride pH 7.4)
  - Second dilution made by placing 0.04 mL (40 µl) of this diluted solution into 1 mL TBS.
- **DMPC Generating Nanodiscs:**
  - Lipid film of DMPC prepared by adding 17.2 mg of solid DMPC and 0.5 mL chloroform in Reacti-Vial. Vial was capped and septa pierced by 2 needles to dry under nitrogen stream
  - 2 mL of TBS was added to dried DMPC then split into two 1 mL samples
  - Each vial was sonicated for ~30 mins until clear, then 155 µl apoA-I (1.29 mg/mL) was added to both vials and 40 µl diluted Terbinafine added to one vial. Vials were sonicated again for 45 mins and then incubated at 23.8 ℃ for 24 hrs
- **Cultivating yeast colonies:**
  - 5g of yeast was added to YM broth in sterile field, then divided into 6 flasks (each ~50 mL) and incubated at 37 ℃ until next lab period
- **UV-vis Spectroscopy:**
  - The absorbance of the growth + broth from each flask following incubation was measured on UV-vis spectrophotometer at 600 nm, using pure broth as a blank
- **Adding Medication to Samples:**
  - 15 µl of empty nanodiscs and nanodiscs containing terbinafine were added to separate 1 mL samples taken from samples that had their absorbance measured
  - Samples containing nanodiscs were vortexed then left overnight at 2 ℃
  - 6 drops of diluted and stock medication solutions were added to two separate agar plates showing growth
- **Creating and Collecting the Absorbance/Fluorescence of the Samples from the Six Broth Flasks:**
  - Five new samples per the six growth flasks were made, each containing 1 mL of yeast/broth mixture. Different treatments were used on each tube within the groups of five, this being: nothing added, 15 µL 10 µL/mL Terbinafine hydrochloride in ethanol (95%), 15 µL empty nanodiscs, 15 µL previous Terbinafine mixture in nanodiscs, and 15 µL of ethanol (95%)
  - One drop of methylene blue (100% ethanol) was added to tubes, then vortexed. 100 µL from each tube was pipetted into black Greiner 96 well plate with a clear bottom
  - Plate was placed in SpectraMax i3 microplate reader. Absorbance was read at 665 nm and fluorescence at 665 nm and 700 nm with excitation value of 9 nm and emission value of 15 nm
- **Creating and Collecting the Absorbance/Fluorescence of the Treated Samples of Fresh Yeast:**
  - 0.5 g dried yeast was placed into 50 mL YM broth. Then three sets of five tubes with 100 µL of broth with yeast growth and different treatments like nothing, 20 µL 10 µL/mL Terbinafine hydrochloride in ethanol (95%), 15 µL empty nanodiscs, 17.09 µL previous Terbinafine mixture in nanodiscs, and 3 drops ethanol (95%) was made. Three samples of 100 µL YM broth with no yeast was also included. Samples incubated at 37 ℃ for 1 hour.
  - One drop of methylene blue (100% ethanol) was added to tubes, then vortexed and then centrifuged for 1 minute at 2.0 x gravity x 1000. 100 µL from each tube was pipetted into black Greiner 96 well plate with a clear bottom
  - Samples were read the same way as previous samples
- **Creating and Collecting the Absorbance/Fluorescence of the Diluted Treated Samples of Fresh Yeast:**
  - 1:5 and 1:10 dilution samples of previous new samples was made. For 1:5, 20 µL of sample and 80 µL of Milli-Q water was mixed. For 1:10, 10 µL of sample and 90 µL of Milli-Q water was mixed. 100 µL of each sample was pipetted into 96 well plate and read as previously done.
- **Creating and Collecting the Absorbance/Fluorescence of the Treated Samples of Fresh Yeast and Smaller Amount of Methylene Blue:**
  - Performed the same as when "Creating and Collecting the Absorbance/Fluorescence of the Treated Samples of Fresh Yeast" except the samples incubated for 40 minutes at 37 ℃ and 0.5 µL methylene blue (100% ethanol) was added instead of a drop

Results

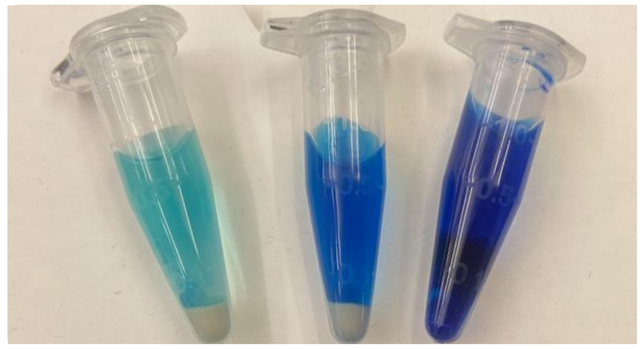


Figure 1. Initial test samples of terbinafine (TB) and nanodiscs (ND) with methylene blue (MB). In order, the tubes contain: broth, yeast, MB, and TB; broth, yeast, MB, and TB in ND; broth and MB. The darker the blue, the more MB present- lighter color indicates more active yeast.

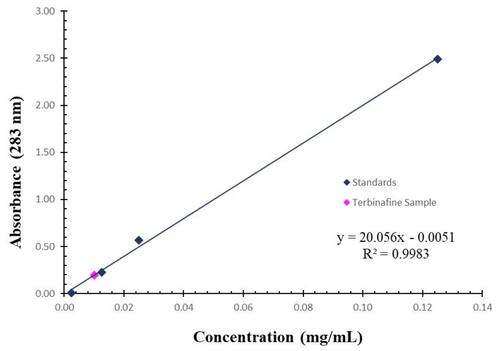


Figure 2. Standard curve of terbinafine concentration with added unknown sample with calculated concentration based on its absorbance. Blue being the standards and pink being Terbinafine.

Table 1. Concentrations and Absorbances of Serial Dilutions Used to Calculate the Concentration of Unknown Terbinafine Sample

Sample	Concentration (mg/mL)	Absorbance
1	0.1250	2.4910
2	0.0250	0.5650
3	0.0125	0.2260
4	0.0025	0.0071
Terbinafine	0.0101	0.1967

Results

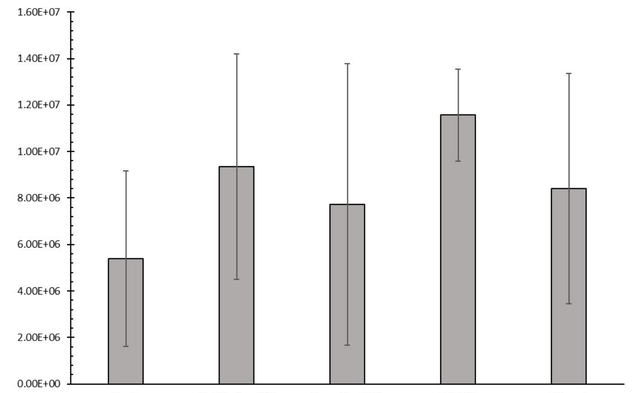
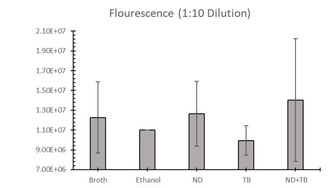


Figure 3. Histogram of treatments and resulting fluorescence. Data presented in SEM + standard deviation.

Figure 4. Histogram summary of treatments and resulting absorbance/fluorescence. Sampling was taken of samples with no dilution, 1:5 dilution, and 1:10 dilution. Data presented in SEM ± standard deviation



Our initial results (Figure 1) were a short test to determine if our treatments might work as expected, or if there were changes that needed to be made. Three tubes were generated: the first with terbinafine (15 µl) and yeast, the second with nanodiscs containing terbinafine (15 µl) and yeast, and the last with just broth. These samples were allowed to sit for about 72 hours, at which point they were treated with methylene blue. As expected, the broth does not react at all, and methylene blue merely becomes diluted. However, we saw that there was still significant activity in the terbinafine tube; the lighter color indicates yeast is alive and processing the methyl blue, removing it from the broth. However, the nanodisc tube is much darker, indicating that at least some yeast has died as compared to the terbinafine sample. These results appear to confirm our expectations; that in the same amount of time, nanodiscs containing terbinafine were able to kill more yeast than direct application of terbinafine. However, only the tubes in the picture were created, and no absorbances and fluorescence was measured, so these observations were hardly conclusive.

We then generated a standard curve (Figure 2) with our serial dilutions to confirm the concentration of our terbinafine dilution. Using our terbinafine samples absorbance (Table 1), we calculated the concentration to be 0.0101 mg/ml, or 10.1 µg/mL. This is exactly what we expect it to be, and in the process we were able to determine an extinction coefficient for terbinafine suspended in TBS: 20.056 $(mg/ml)^{-1}cm^{-1}$.

Our initial round of sampling involving all 6 of our yeast flasks were all treated with methylene blue and were measured for absorbance and fluorescence. However, the absorbances of this sampling was higher than what was within the measuring capacities of the instrument (Abs > 3), so this data has been excluded. The fluorescence measurements are within the limits of the instrument, and have been summarized (Table 2). From these results, we do seem to confirm our suspicions that nanodiscs improve the potency of the terbinafine, being that the nanodisc + terbinafine samples have the highest fluorescence; however, accounting for standard deviation our results have a significant amount of associated error, so much so that it may make our results questionable. For this reason, we have decided to do another round of sampling, except that this is now a triplicate of the same yeast sample.

The resulting triplicate samples from a single yeast source appeared to have the absorbance and the fluorescence data observed, which confirmed our hypothesis, save for the undiluted sample for its values were likely out of the range of the machine. However, the nanodiscs containing Terbinafine sample did show the highest overall absorbance and fluorescence values but it also had the highest margin of error.

Conclusions

After taking a triplicate of a single yeast sample, we feel far less confident with our assertions as we did with our last sample data. It would appear that both absorbance data and fluorescence data would appear to confirm our assumptions, similar to the last sample data; this accepting of course the non diluted fluorescence data, which we believe appears the way it does because it is outside the usable measuring limits of the instrument, and merely included to indicate why dilutions were necessary for our samples. However, it is clear that while the nanodisc + terbinafine sample does have the highest overall absorbance and fluorescence values, it also has the highest associated error. More than any of our controls. This is concerning, and led us to attempt to exclude what we felt was the outlier point in our data, which also happened to be the sample that most confirmed our hypothesis. In so doing it indicated that our nanodisc + terbinafine samples were in fact not the samples with the highest overall absorbance/fluorescence. Thus, we must further assume that our data does not sufficiently present compelling enough evidence to confirm our hypothesis.

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