A Comparison of Minimal Inhibitory Concentration (MIC) and Agar Diffusion Assays for Clinical use in Determining Minimal Effective Drug Concentrations

Janki Patel, General Biology
Mentors: Jill P. Adler-Moore & Jon A. Olson
Kellogg Honors College Capstone Project

Abstract

The incidence of life threatening fungal infections has increased since the development of improved medical interventions, such as organ transplantations and the wider use of anticancer chemotherapy. Identifying the etiologic fungal agent is important to more effectively treat the patient and to identify a potential lack of drug sensitivity (i.e., resistance). The use of a minimal inhibitory concentration (MIC) assay and an agar diffusion assay is useful to aid the clinician in developing a more effective therapeutic regimen to eliminate the fungal infection. In this report, we examined these assays using the yeast Candida albicans tested against the antifungal agents Amphotericin B (Ambisome) and Anidulafungin. Using these assays, we identified the minimal drug levels necessary to be achieved in the patient to effectively treat a potential systemic candidiasis infection.

Introduction

A number of fungal species have been identified as the etiological agents of human infections ranging from superficial, non-lethal colonization to systemic, life-threatening infections since the first fungal infections were first reported in the 1800's. Some of these species are Aspergillus niger, Candida albicans, Pneumocystitis carinii (Revankar & Sobel, 2014). In the past 50 years, the incidence of serious fungal infections has increased and corresponds to the increase use of chemical (drug) treatments that adversely affect the immune system, such as cancer chemotherapy and transplantation drugs.

Amphotericin B, isolated from the bacterium Streptomyces nodosum and marketed under the trade name Fungizone®, was identified in the mid-1950's as having antifungal properties for effectively treating fungal infections. This chemical has a high binding affinity to sterols; one of the structural components of fungal cell membranes is the sterol ergosterol, not present in mammalian cells (Kagan et. al 2014). However, this amphotericin B was also has significant undersierable side effects including damage to the distant tubules of the kidneys. To help reduce these adverse reactions in humans, amphotericin B was incorporated into the bilayer of liposomes (lipid nanospheres) containing cholesterol (Kagan et. al 2014)l.

When amphotericin B is administered IV it is not easily able to enter the blood stream, making it less useful for combating certain yeast and fungal infections (Olson et. al, 2011). When administered intravenously it tends to have many side effects including kidney damage that can be severe and/or irreversible (Olson et. al, 2011). When amphotericin B is administered in a liposomal formulation the drug is encapsulated in a lipid membrane which disrupts when it reaches the cell wall of fungi; this allows for the drug to be administered in higher doses and with a lesser severity of the side effects (Olson et. al, 2011).

AmBisome® is a liposomal formulation of amphotericin B approved by the FDA to treat systemic fungal infections. AmBisome has been shown in both pre-clinical and clinical use to retain the antifungal activity of amphotericin B with a reduction in the negative side effects even when given at higher doses than the conventional amphotericin B (Ambisome, 2008). AmBisome was also found to have a longer half-life compared to Fungizone which allows AmBisome to be dosed less frequently. Although there are other lipid formulations used to deliver amphotericin B, it is the structure of the liposome, and not just the lipids which confers the beneficial characteristic of AmBisome (Ambisome, 2008).

Due to increasing toxicity effects of amphotericin B treatments and the development of resistance to Azoles and 5-fluorocytossine the development of new antifungal medications has been imperative. Anidulafungin a type of echinocandin antifungal drug, which was first discovered in the 1970's gained FDA approval in 2006 (Emri et. al, 2013). It is a cyclic-lipohexapeptide that has been found to be helpful in treating invasive esophageal Candida infections. It functions by targeting fungal cell walls by non-competitively inhibiting β-1,3 glucan synthase. Anidulafungin, similar to Amphotericin B, differs from most other antifungal drugs as it does not rely on hepatic or renal excretion or enzymatic degradation (Emri et. al, 2013). It is chemically degraded to an inactive form at body pH making it safer for patients with varying degrees of hepatic or renal impairments; however, as it is a fairly new drug testing is still underway to determine the efficacy of Anidulafungin for various fungal infections (Emri et. al, 2013).

There are many several different methods for testing the effectiveness of antifungal medications including the minimum inhibitory concentration (MIC) assay and the agar diffusion test (Andrews, 2002). MIC assays are useful for determining the lowest concentration at which an antifungal will inhibit visible growth (Andrews, 2002). They are important as a diagnostic technique as they can help confirm the resistance of microorganisms to an antimicrobial agent (Andrews, 2002). They also allow one to monitor the activity or effectiveness of the antimicrobial agents.

Agar diffusion testing, such as Kirby-Bauer antibiotic testing, is another valuable biological technique that can be useful for determining whether or not microbes are affected by antibiotic (Bonev et. al, 2008). The effectiveness of the antifungal agents can visibly be seen on the agar plates (Bonev et. al, 2008). By testing varying concentrations of antifungal drugs, the minimal amount of antifungal required for a visible decrease in fungal growth can be determined (Bonev et. al, 2008).

Materials and Methods

MIC Assay

- Subculture the yeast in Sabouraud's daily for 3 consecutive days, wash with PBS and adjust the concentration to $2.0x10^5$ yeast cells per mL of RPMI
- A series of drug dilutions were prepared in RPMI medium;
- 100µL of each drug dilution was dispensed in a 96 well plate, in triplicate. Yeast cells were dispensed in wells 2-11, the negative control was dispensed in well 1, and the positive control was dispensed in well 12. 100µL of yeast, 100µL of RPMI and 20µL of alamarBlue® were dispensed into the positive control well. 100µL of the drug, 100µL of RPMI and 20µL of alamarBlue® were dispensed in the negative control.
- The plate was placed in an incubating box with a moistening towel and incubated at 35˚C for 20 hours. The plate was read using Spectromax 340 plate reader and the numbers generated were the result of reading the absorbance at 570nm minus the absorbance at 600nm.
- The yeast was subcultured in Sabouraud's broth daily for 3 consecutive days; then washed with

Agar Diffusion

- 13.2 grams of AM19 medium was hydrated with 220mL of DI water and 75 µL of saturated NaOH.
- The solution was sterilized by autoclaving for 15 minutes at 1 atm.
- The solution was then placed in a 48˚C water bath for a minimum of 30 minutes.
- The medium was allowed to cool briefly prior to inoculating with 2.2mL of $2.2x10^5$ yeast cells per mL of solution. The solution was then stirred briefly with a stir bar for approximately 1 minute
- The solution was carefully poured into an agar plate on a level surface and allowed to sit for approximately 30 minutes to allow the agar to solidify
- A sterile template cutter was then sued to punch wells and the plugs were then vacuumed out.
- The wells were filled with varying concentrations of Ambisome and the plate was incubated for 20 hours at 35˚C
- Three measurements were obtained per well for the zones of inhibition.

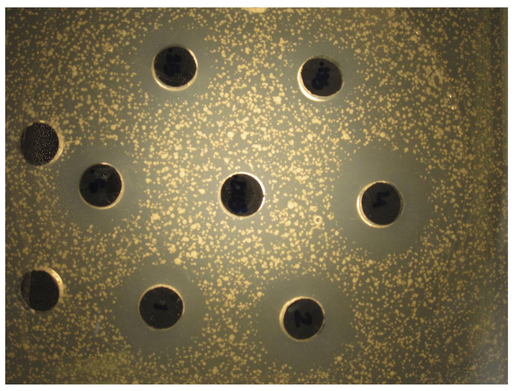


Figure 1: Agar diffusion assay of varying drug concentrations of Ambisome

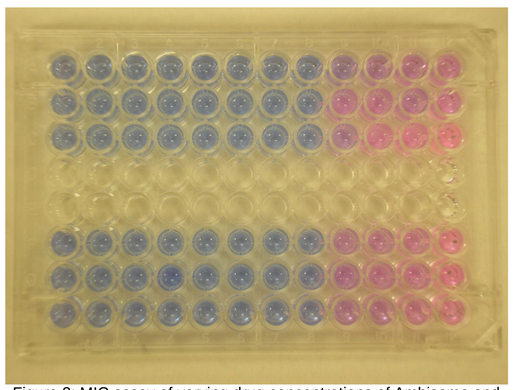
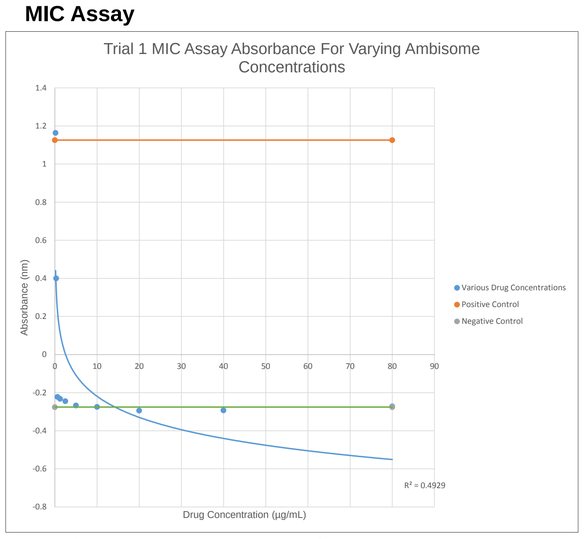


Figure 2: MIC assay of varying drug concentrations of Ambisome and Anidulafungin

Results

MIC Assay



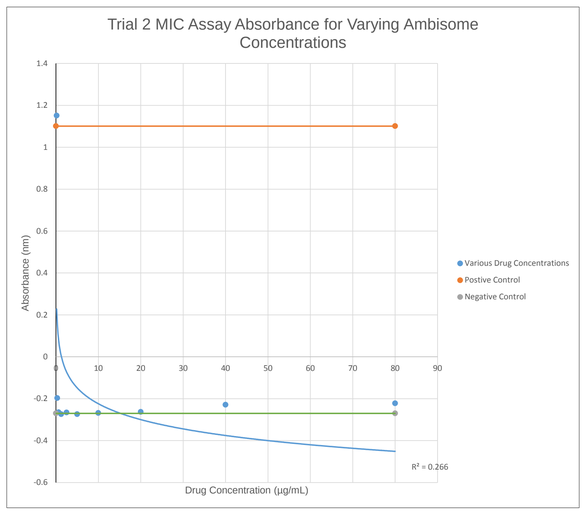
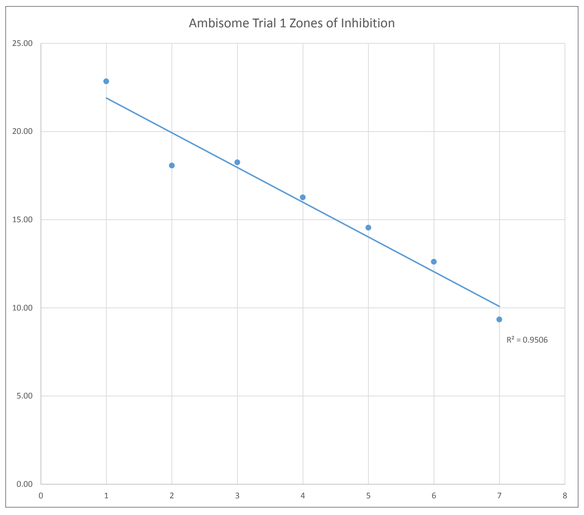


Chart 1 & 2: Spectrophotometric results of Ambisome dilutions were plotted and demonstrate the minimal inhibitory drug concentrations needed to prevent the growth of C. albicans

Agar Diffusion



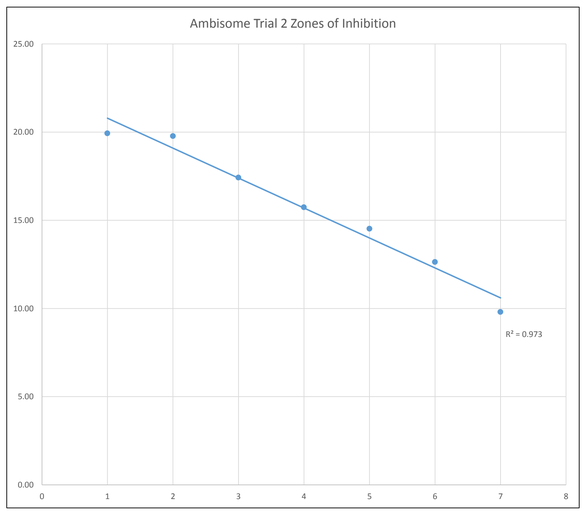
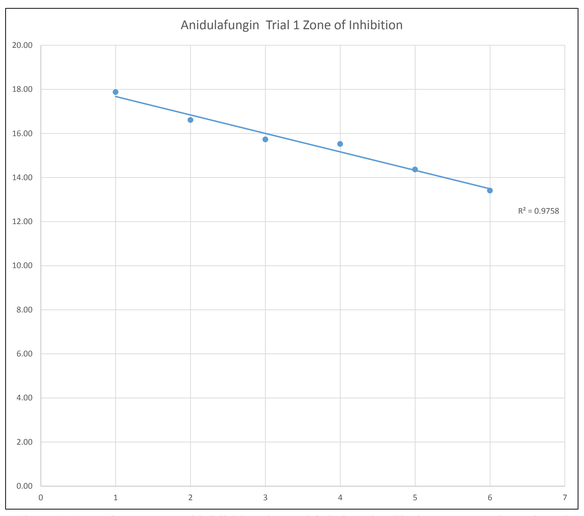


Chart 3 & 4: The zones of inhibition for Ambisome dilutions were plotted to develop a standard curve to which clinical samples can be compared to determine drug levels following to determine drug levels following drug administration



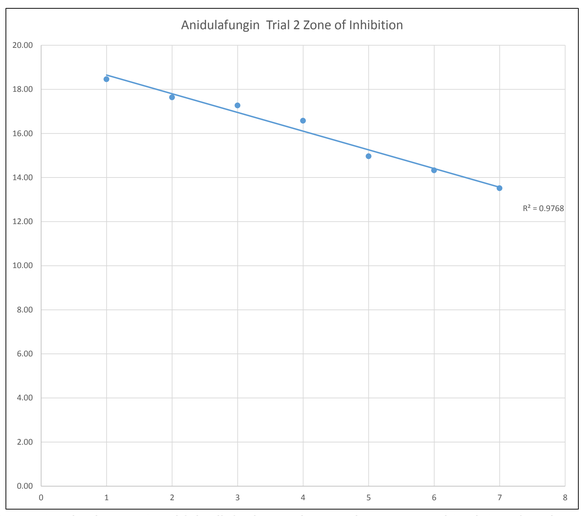


Chart 5 & 6: The zones of inhibition for Anidulafungin dilutions were plotted to develop a standard curve to which clinical samples can be compared to determine drug levels following drug administration

Discussion

Standard MIC assays are easily performed with minimal space requirements when compared to agar diffusion plates which require greater amounts of space. Agar diffusions and MIC assays can be performed with equal amounts of ease; however, Agar diffusion assays require more work and are slightly less precise as the zones of inhibition are open for interpretation, thus allowing more human error. MIC assays can be visually interpreted by examining color changes and electronically with spectrophotometry for more precision; however MIC assays are most effective at an identified drug concentration as the results plateau after certain drug concentrations.

Both MIC Assays and agar diffusion assays can be useful in clinical practice for developing effective therapeutic regimens for the treatment of fungal infections. The spectrophotometric results of varying drug concentrations from MIC assays can be plotted to determine the minimal effective drug concentration, and the zones of inhibition of agar diffusion assays can be plotted to develop a standard curve to determine effective drug concentrations for administration.

References

1. AmBisome® (2008): n. pag. Web. <http://www.accessdata.fda.gov/drugsatfda_docs/label/2008/050740s016lbl.pdf>.
2. Andrews, J. M. "Determination of Minimum Inhibitory Concentrations." Journal of Antimicrobial Chemotherapy 49.6 (2002): 1049.
3. Bonev, B., J. Hooper, and J. Parisot. "Principles of Assessing Bacterial Susceptibility to Antibiotics Using the Agar Diffusion Method." Journal of Antimicrobial Chemotherapy 61.6 (2008): 1295-301.
4. Emri, Tamás, László Majoros, Viktória Tóth, and István Pócsi. "Echinocandins: Production and Applications." Applied Microbiology and Biotechnology 97.8 (2013): 3267-284.
5. Kagan, Leonid, Pavel Gershkovich, Kishor M. Wasan, and Donald E. Mager. "Dual Physiologically Based Pharmacokinetic Model of Liposomal and Nonliposomal Amphotericin B Disposition." Pharmaceutical Research 31.1 (2014): 35-45.
6. Olson, J. A., J. Schwartz, D. Hahka, A. George, R. T. Proffitt, and J. P. Adler-Moore. "Differences in Efficacy and Cytokine Profiles following Echinocandin or Liposomal Amphotericin B Monotherapy or Combination Therapy for Murine Pulmonary or Systemic Aspergillus Flavus Infections." Antimicrobial Agents and Chemotherapy 56.1 (2011): 218-30.
7. Revankar, Sanjay G., and Jack D. Sobel. "Overview of Fungal Infections." Fungi: Merck Manual Professional. Merck Manuals, Jan. 2014. 04 Feb. 2015.