ABSTRACT

Transition metal inorganic compounds, also known as polyoxometalates (POM), have many biological applications such as antiviral, antitumor, and antibacterial therapies. The objective of this study was to determine if the POM α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ could overcome the resistance of methicillin resistant *Staphylococcus aureus* (MRSA). To determine the minimal inhibitory concentration (MIC) and possible synergistic effects, multiple dilutions of *oxacillin* and POM were combined with inoculums of MRSA. Susceptibility (MIC ≤ 0.25 µg/mL *oxacillin*) was achieved at a concentration of 5 µM of α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$. The POM enhanced the efficacy of *oxacillin*, and additionally, the POM showed low toxicity to mammalian cell cultures *in vitro*. The effect of the POM on *mecA* gene transcription was assessed using reverse transcriptase quantitative PCR (RT-qPCR) and showed a reduction in mRNA transcription at effective POM doses, but increased transcription was observed at higher POM *oxacillin* doses. This pilot study illustrates that α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ could be used in conjunction with *oxacillin*. However, further testing would need to be completed.

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Abbreviations:

methicillin susceptible *Staphylococcus aureus*	MSSA
methicillin resistant *Staphylococcus aureus*	MRSA
Reverse Transcriptase Quantitative Polymerase Chain Reaction	RT-qPCR
Polymerase Chain Reaction	PCR
Tungsten	W
Infrared Spectroscopy	IR
Lethal Concentration 50	LC50
Nuclear Magnetic Resonance Spectroscopy	NMR
Sheep Blood Agar	SBA
Clinical Laboratory Standard Institute	CLSI
Dulbecco's Modified Eagle Medium	DMEM
Sodium Dodecyl Sulfate	SDS

Index Terms: methicillin resistant *Staphylococcus aureus*, Antimicrobial Agents, Polyoxometalates

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INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) resistance is due to a variety of mechanisms. MRSA is capable of evading beta-lactam antibiotics by genetic alterations, including the *mecC* and *mecA* genes[1]. This study focused on the *mecA* gene, which codes for an alternative penicillin binding protein (PBP2A). Beta-lactam antibiotics, such as *oxacillin*, are unable to efficiently bind with this alternate form of PBP2A, and thus are unable to act as cell wall synthesis inhibitors[2]. MRSA's resistance to antibiotics leaves healthcare professionals with limited treatment options. Patients infected with MRSA have an average hospital stay of 2.6 days longer than patients with a methicillin susceptible *Staphylococcus aureus* (MSSA) infection, and an average cost of $13,901 more than MSSA patients[3]. Due to the increase of asymptomatic carries, MRSA is one of the leading causes for hospital acquired infections. Because of this increase, hospitals are enforcing pre-surgical screenings for MRSA to try to decrease the chance of transmission to other patients. Two methods of pre-surgical screenings are used, and once a patient is positive on screening he or she is put on immediate isolation precautions[4].

Polyoxometalates (POM) are inorganic compounds that contain a transition metal and have been studied for a variety of uses. In high concentrations they induce apoptosis in some types of cancer cells. They also have an inhibitory effect on some viral infections by disrupting a step between attachment and penetration[5,6]. While transition metals are notorious for being toxic in high doses, tungsten (W) has been shown to be relatively non-toxic compared to other transition metals such as mercury. There are many hypotheses as to why tungsten containing compounds are not as toxic. The most probable theory is that when tungsten is in an oxoanionic state, it is water soluble[7].

This study analyzed the effect of the POM α-$K_6P_2W_{18}O_{62}$ and *oxacillin* against MRSA. The POM was also tested against mammalian cells to determine the lethal concentration that was required to kill 50% of the cell population (LC50). It was discovered that the POM was effective at overcoming the methicillin resistance in MRSA and showed a low toxicity at bio-reactive concentrations. To determine if the POM had an effect on the presence of *mecA*, mRNA levels of *mecA* were measured using RT-qPCR. In previous research, RT-PCR was used, and it was suggested that POMs were overcoming resistance by decreasing the expression of *mecA*[2]. In this study, a more sensitive and accurate method was used, RT-qPCR. α-$K_6P_2W_{18}O_{62}$ was found to decrease mRNA expression at low concentrations, yet it increased mRNA expression at higher concentrations. This is counter to previous research and may suggest an alternative effect on the *mecA* gene, perhaps a post-translational modification.

MATERIALS AND METHODS

α-$K_6P_2W_{18}O_{62}$ Synthesis

α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ was synthesized according to procedures in a previously published work[8]. While stirring vigorously and insulating the system with warm water, 250 mL of 4M HCl was slowly added to 301.7 g of Na_2WO_4· $2H_2O$ (formula weight 329.87) until the solution became limpid. 250 mL of 4M H_3PO_4 (aq) was added slowly with an addition funnel, still stirring vigorously to the previous solution. The product was then refluxed under positive pressure of argon gas for twenty-four hours. The solution went from colorless to a light green. 150 g of KCl powder was added to the refluxed solution and a white precipitate was formed. This precipitate was then filtered and air dried by aspiration. Once the solution was completely

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dry, 650 mL of distilled H_2O was added. A limpid solution was obtained after five days of stirring continuously at room temperature. After the solution was dissolved and colorless, it was then allowed to reflux at 80 degrees Celsius for three days and then cooled to room temperature. Once at room temperature, the solution was placed on ice for seventy-two hours and green crystals were produced. Proper structure of the compound was confirmed by comparing infrared spectroscopy (IR) and phosphorus (^{31}P) nuclear magnetic resonance spectroscopy (NMR) to previously published work[8]. Confirmation was determined by the correct Phosphorus-Oxygen stretching and vibration peak at 1086 cm^{-1} and 957 cm^{-1}, and by its ^{31}P chemical shift at -13.577 ppm, which is in the delta range when compared to referenced literature[8]. While the structural confirmation was performed, the stability of the POM over time was not tested.

POM Susceptibility Testing

To determine if the POM alone could inhibit bacterial growth, a minimum bactericidal concentration (MBC) and a minimum inhibitory concentration MIC of the POM was established for MRSA (ATCC 43300). Using a concentrated stock solution of the POM at 20,000 µM, 1:2 dilutions were made with 1 mL of Columbia Broth growth media down to 39 µM concentration. Then, 4 µL of a MRSA suspension was added to obtain a 0.5 McFarland standard concentration in each dilution tube. After twenty-four hours of incubation at 37 degrees Celsius, the tubes were recorded for growth or no growth based on turbidity. Those that demonstrated no visible growth had a sample taken from each tube that was then plated onto sheep's blood agar (SBA) media. These plates were then incubated at 37 degrees Celsius for an additional twenty-four hours then evaluated for colony growth.

MIC Panel Synergy Testing

Dilutions of 5,000 µM, 500 µM, 50 µM and 5 µM of α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ were tested in combination with preset concentrations of *oxacillin* on the minimal inhibitory concentrations (MIC) panels. These concentrations ranged from 0.25 to 4 µg/mL. Two sets of experiments were performed. The first was termed co-incubation. MicroScan™ MIC panels were inoculated with MRSA and various concentration of POM. This was done according to Clinical and Laboratory Standard Institute (CLSI) protocols and the manufacturers guidelines[9]. The MIC plates were then incubated for twenty-four hours at 37 degrees Celsius in an air incubator. The susceptibility results were read on a Beckman Coulter Microscan Walkaway™ instrument. In a second set of experiments, termed pre-incubation, the POM was allowed to incubate with MRSA before inoculation of the MIC panel. This was achieved by inoculating 3 mL of Columbia broth with MRSA and various concentrations of POM and letting them incubate overnight in an air incubator at 37 degrees Celsius while being agitated on a rocker. The dilutions were then prepared to a 0.5 McFarland standard, inoculated in the MIC panel and incubated for an additional twenty-four hours before being read on the Beckman Coulter Walkway™. Each combination of POM was run in triplicate. When the POM was added to the inoculums of saline and *oxacillin*, a blue color was observed. To ensure the POM did not interfere with the reading performance of the instrument, each MIC was confirmed by hand reading the panels.

Toxicity Study

HCT116 human colorectal carcinoma cells were obtained from ATCC as a model for mammalian cell cytotoxicity. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) in the presence of α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ at 0.5 µM, 5 µM, 50 µM and 500 µM for

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seventy-two hours. The cells were then fixed with ice-cold methanol and stained with crystal violet (0.5% in 20% MeOH/H_2O). To quantitate the stain, a 2% sodium dodecyl sulfate (SDS) solution was used to extract the crystal violet stain from the fixed cells and then analyzed at 595 nm on a Tecan UV-VIS spectrometer™. Dilutions were run in duplicate and an average of the absorbance reading was reported.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Six separate samples of MRSA (ATCC 43300) were cultured in tryptic soy broth for 2.5 hours at 37°C. Sample 1: MRSA, 2: MRSA with 4 µg/mL *oxacillin*, 3: MRSA with 5 µM α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$, 4: MRSA with 5 µM α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ and 4 µg/mL *oxacillin*, 5: MRSA with 50 µM α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$, 6: MRSA with 4 µg/mL *oxacillin* and 50 µM α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$. Following incubation, total RNA extraction was performed via TRIzol Max Bacterial RNA Isolation Kit by Ambion per manufacturer's guidelines. Each sample was then DNased to remove any residual DNA. cDNA libraries were generated from RNA extracts using M-MLV Reverse Transcriptase by Invitrogen per manufacturer's protocol using gene specific reverse primers for *16s rRNA* and *mecA*. Nucleic acid quantitation and sample purity was performed using standard absorbance at 260/280 nm using an EPOCH-2 microplate reader from Biotek™. RT-qPCR was performed on a QuantStudio 3™ Real-Time PCR instrument from Applied Biosystems. The reaction mixture consisted of PowerUP SYBR Green Master Mix, 300 nM of each primer, and 1uL cDNA. The *mecA* primers (F: 5'-ATCCACCCTCAAACAGGTGAAT-3', R: 5'GGAACTTGTTGAGCAGAGGTTC-3') were designed using the Benchling software with a product size of 139 base pairs (bp)[10]. The *16s* rRNA primers (F: 5'-CGTGCCTAATACATGCAAGTC-3', R: 5'-CCGTCTTTCACTTTTGAACCA-3') were designed per a previous publication[2]. A standard curve was created using cDNA from a MRSA isolate in a five point ten-fold dilution for both *16s rRNA* and *mecA*. qPCR was performed with the following conditions: 50°C for 2 min, 95°C for 2 min, and then 40 cycles of 95°C for 15 sec denaturation, 61°C (*mecA*) or 59°C (*16s*) annealing for 15 sec, and 72°C extension for 1 min. A melt curve was also performed to assess specificity. Relative quantification fold changes were calculated in the QuantStudio Design and Analysis software using the standard curve method that utilizes *16s rRNA* as the endogenous control and Sample 2 as the reference sample.

Statistical Analysis

The mean fold changes in *mecA* mRNA from various treated samples were compared using a web based statistical program for RT-qPCR data called BootstRatio[11]. Graphs and additional analysis were performed using the R software package[12].

RESULTS

Susceptibility Testing

To determine if the POM alone could inhibit the growth of MRSA, an MBC and MIC was established. When observing the tubes for growth or no growth, turbidity was noticed from 39 µM to 5,000 µM. A sample from all tubes presenting with no turbidity was then placed onto SBA media. Colony growth was not observed on any of the SBA plates. This indicated that the POM alone can inhibit the growth of MRSA at high concentrations. This resulted in the MIC and MBC of the POM at 10,000 µM. Two experiments were completed to establish any synergistic effects of POM and *oxacillin*; these were termed co-incubation and pre-incubation. The co-incubation study revealed that the POM and *oxacillin* resulted in MRSA being susceptible to

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oxacillin at 5 µM (Figure 1). However, the pre-incubation study of POM and *oxacillin* did not reach susceptibility until 500 µM. These results showed that the POM was most effective when co-incubated with *oxacillin* and MRSA in inducing susceptibility.

α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ Toxicity

To assess the viability of using α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ as a treatment option, toxicity studies against HCT-116 cancer cells revealed that α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ is not effective in killing mammalian cells, as the LC_{50} was not reached at concentrations as high as 500 µM (Figure 2). POM concentrations that were proven effective, 80%-90% of the cells survived. This indicates that the POM appears to have a low toxicity.

mecA mRNA Expression

Previous work has shown an effect of other POMs on the *mecA* gene expression in MRSA utilizing RT-PCR products visualized on an electrophoresis gel[2]. In this study, a more sensitive and accurate approach of RT-qPCR was utilized. The results of the RT-qPCR are shown in Figure 3. There was an average of 86.7% reduction (0.133 average fold decrease) in *mecA* mRNA expression when MRSA was incubated with α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ at a 5 µM concentration and a 20% reduction (0.807 average fold decrease) when incubated with 5 µM of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ and 4 µg/mL of *oxacillin*. The 20% reduction correlated with a susceptible phenotype in the MIC testing. Yet surprisingly, there was an observed increase in *mecA* mRNA production in both samples containing 50 µM of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ alone or in combination with 4 µg/mL of *oxacillin* (1.099 and 1.91 average fold increase respectively) even though this higher concentration of POM was also associated with a susceptible phenotype. These results taken together would suggest that α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ does not act directly against mRNA production as previously studies suggest.

DISCUSSION

With MRSA having an altered PBP2A, the ability to be resistant to beta-lactam antibiotics such as *oxacillin* has left healthcare providers with limited treatment options. POM's have been used against viruses and tumors at high concentrations. This study analyzed the effect of the POM α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ in combination with *oxacillin* against MRSA. It was proven that a combination of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ with *oxacillin* is effective at inhibiting MRSA in ATCC 43300. Co-incubation of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ with *oxacillin* made MRSA susceptible with as little as 5 µM of POM, while pre-incubation was not able to obtain susceptibility until 500 µM. The toxicity study revealed that at effective doses, the POM did not reach the LC_{50}. Previous research has used different POMs to try and produce a synergistic effect, they were able to do so at high concentrations. $K_6P_2W_{18}O_{62}$ · $14H_2O$ was able to do it at 5 µM. α-$K_6P_2W_{18}O_{62}$ was found to decrease mRNA expression at low concentrations yet increase the expression at higher concentration, which is counter to previous research and may suggest an alternative effect on the *mecA* gene. The use of the RT-qPCR method using endogenous controls and the standard curve method is more accurate and sensitive of a methodology compared to conventional RT-PCR and comparing electrophoresis band intensity. This research is an important pilot study since it illustrates that $K_6P_2W_{18}O_{62}$ · $14H_2O$ and *oxacillin* are capable of antimicrobial activity in the laboratory strain ATCC 43300.

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While this study was carefully designed, some limitations were obtained. This was a pilot study; therefore, the laboratory strain ATCC 43300 of MRSA was the only strain used for analysis. Other laboratory strains of MRSA, and clinical strains of MRSA would need to be obtained and tested in the same manner prior to any animal models being studied. If other strains of MRSA had the same conclusion as ATCC 4300, animal models with active MRSA infections would then be studied for routes of administering α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$. These studies may include: effectiveness of taking the POM orally, intravenously, and topically. While the MIC panels contain an abundance of antibiotics, *oxacillin* was the only one analyzed in this study. Other penicillins would need testing in the same manner as *oxacillin.* The POM structure was confirmed, however, the stability over long periods of time was not determined. To determine stability, a forced-stability, accelerated-stability, and real-time study may be performed. This would be accomplished by subjecting the POM to different temperatures, humidity, light, and time periods. Analysis of the product would then be conducted by liquid chromatography and mass spectrometry. This would help determine proper storage conditions, as well as shelf life of α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$. This pilot study provided evidence that α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ and oxacillin can overcome resistance in ATCC 43300.

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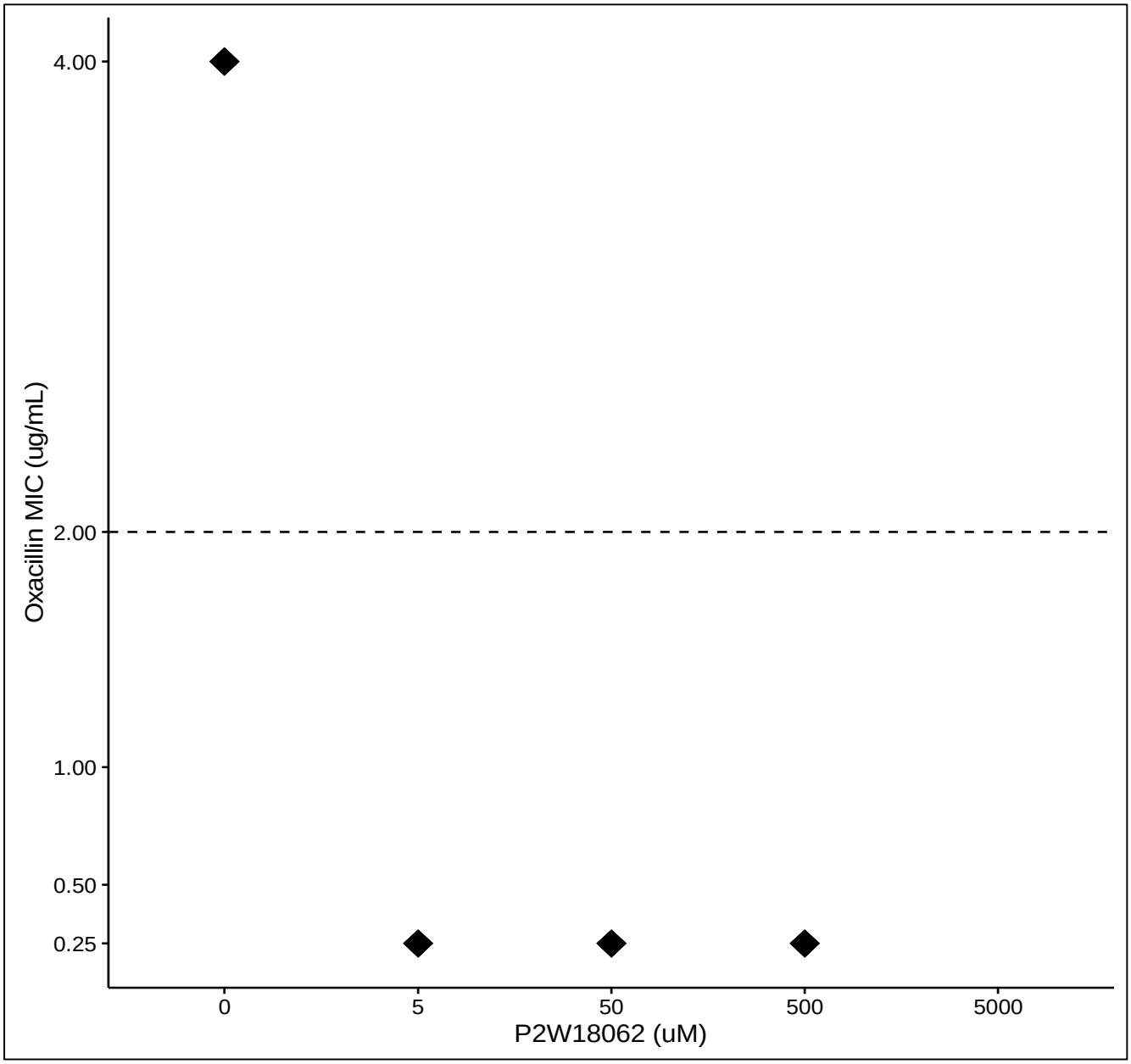

Figure 1. Co-incubation Effect of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ on Methicillin Resistant *Staphylococcus aureus* MIC of MRSA in combination with oxacillin (ug/mL) and three different concentrations of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ (uM) prepared by co-incubation techniques. Each concentration was ran in triplicate and obtained the same MIC value which are represented by the diamonds. The dashed line represents the oxacillin breakpoint at which *Stayphylococcus aureus* is considered resistant or susceptible.

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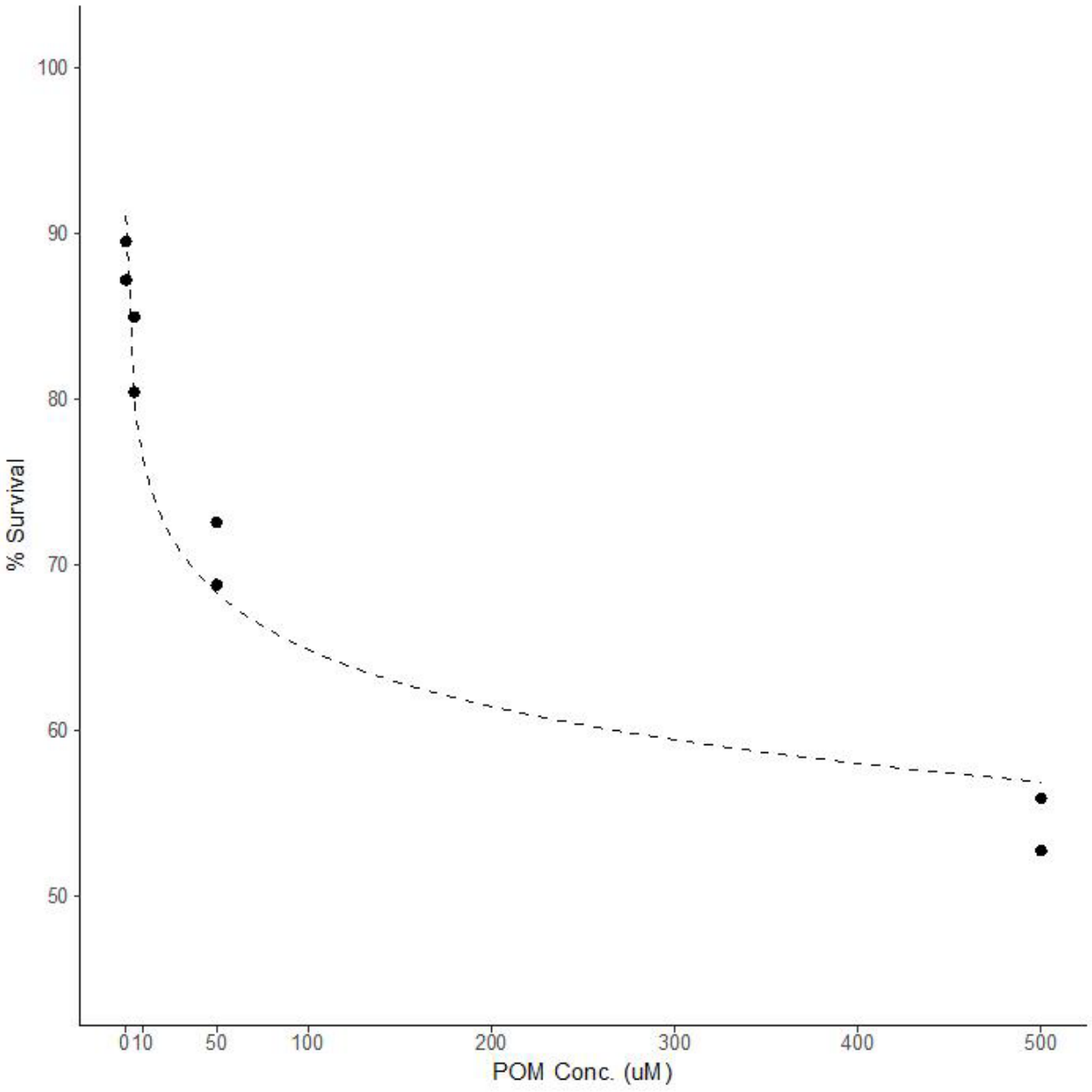

Figure 2. Toxicity of α-$K_6P_2W_{18}O_{62} \cdot 14H_2O$ on HCT116 cells

Points represent % cell survival for five concentrations of POM [0.5,5, 50 and 500uM]. Each concentration was run in duplicate and compared to control samples to calculate % survival. Dashed line represents a fitted logarithmic curve.

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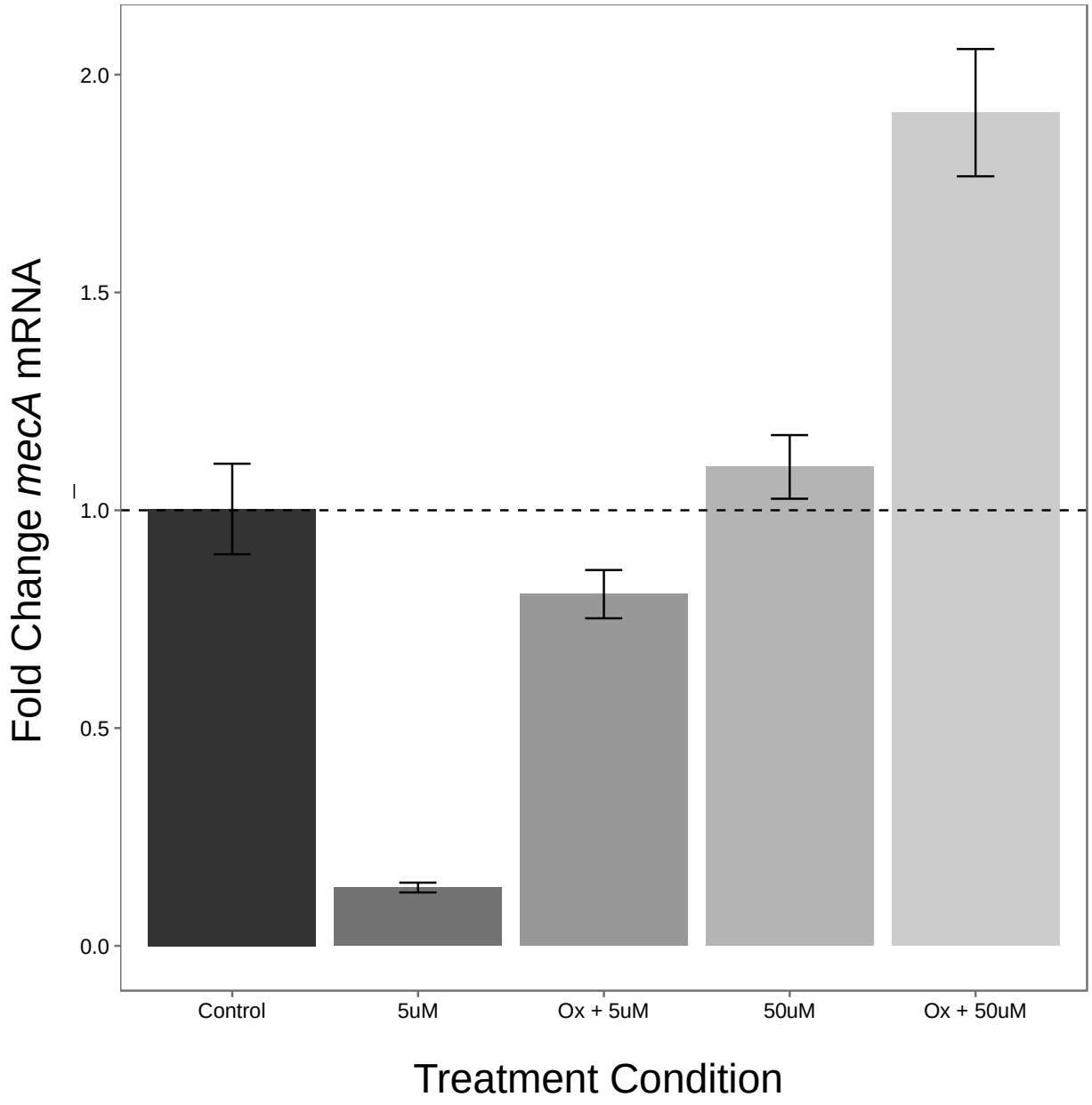

Figure 3. The effects of α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ on mecA expression MRSA. Overnight cultures of MRSA were incubated for three hours at 37 degrees Celsius without α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ in the presence of 4 μg/mL oxacillin to induce *mecA* expression [Control], α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ alone [5uM and 50uM], and combinations of 4 ug/mL oxacillin (Ox) and α-$K_6P_2W_{18}O_{62}$ · $14H_2O$ [Ox + 5uM, Ox + 50uM]. Data represent mean fold changes (error bars = +/- 2 SEM) in mecA expression determined by RT-qPCR. All fold changes values were calculated using a standard curve method using the 16s rRNA gene as the endogenous control and the Control sample as the reference.

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