Inhibition of Bacterial Mutagenesis through Polyubiquitination

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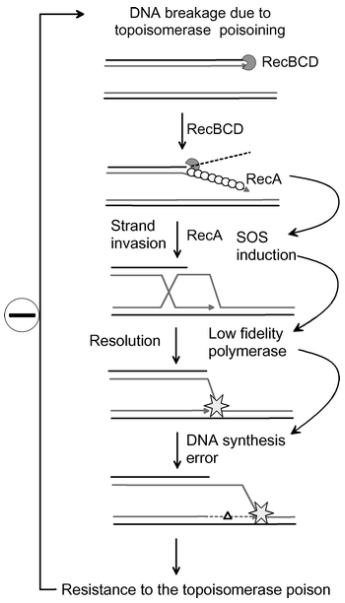
Bacterial cells can have DNA damage due to transcriptional error, or through the effect of an antibiotic. The SOS response is a bacterial cell program for coping with DNA damage, in which the cell cycle is arrested, and DNA repair is induced. The repairs have high probability in leading to mutagenesis in the bacteria, which can lead to antibiotic resistance. The RecA protein in bacteria is responsible for the activation of the SOS response; therefore, making it a target for inhibition. I elected to use the ubiquitination system, natively used for apoptosis, as a means of targeted degradation of the RecA protein in bacteria prone to mutations. Polyubiquitination of misfolded proteins leads to the breaking down of the protein with the aid of proteasomes, which break down unnecessary proteins through a chemical reaction known as proteolysis. Using random forest-predictors, I determined a statistically high likelihood of ubiquitination of the RecA protein in MRSA, Tuberculosis, and other high risk bacterial infections. I hypothesized that I could foster ubiquitin-tagging on RecA by forcing the protein to misfold. Chaperones are proteins which interact with each other to prevent specific sets of proteins from misfolding. CHIP (C terminus of HSC70-Interacting Protein) is a biomolecule that inhibits interactions between the chaperones of RecA. Adding CHIP, ubiquitin, and 26s proteasomes into the bacterial system, should theoretically lead to the degradation of the RecA protein inside the bacteria. I tested my hypothesis by conducting an assay for monitoring CHIP-mediated ubiquitination, and conducted analysis on the assay using SDS- Page gel electrophoresis, and Western-blotting. The resulting data showed signs of polyubiquitination on the RecA protein, with chains of five or more ubiquitin, showing high drug potential. Adding an antibody drug conjugate, containing all the necessary components of a CHIP-mediated ubiquitination reaction, to common antibiotics can lead to the inhibition of bacterial mutagenesis, and higher antibiotic drug potency.

Keywords: Ubiquitin-Proteasome System; Drug Discovery; Antibiotic Drug Resistance; Bacterial Mutagnesesis; SOS-Response

1. INTRODUCTION

During the cell cycle, if a cell's DNA ever undergoes any damage, it activates the SOS response, a cell program designed to repair DNA [5]. Unfortunately, the SOS response is extremely error prone, and most of the time ends up leading to mutagenesis [6]. Due to this, bacterial cells can sometimes mutate in a manner that gives them resistance to many antibiotics [7]. There are two main proteins involved in the SOS response, LexA, and RecA [8]. LexA makes sure that SOS response remains off when the cell is healthy [8], and RecA makes sure that SOS response is activated when the cell's DNA is damaged [8]. Degradation of the RecA protein can prevent the activation of the SOS response, greatly reducing the risk of mutation [9]. One such way that proteins such as RecA can be antagonized, is through the human ubiquitination system. The ubiquitination system consists of the proteins ubiquitin and proteasomes. Ubiquitin tags any protein which is misfolded, signaling that something is wrong with the protein [10]. If any protein is polyubiquitinated, then proteasomes will come in and destroy that protein through a process called proteolysis [4]. In order to destroy the RecA protein in bacteria using the ubiquitination system, RecA must be forced to misfold [10]. Proteins have specialized molecules with them, called chaperones, which prevent them from misfolding [11]. Without the presence of chaperones, proteins will not be able to maintain their structure, and misfold [11]. CHIP (C terminus of HSC70-Interacting Protein) is a special biomolecule, that inhibits the interactions between the chaperones specific to the RecA protein [12]. So the presence of CHIP in the bacterial system, would cause the misfolding of RecA, leading to the forced ubiquitination of the protein.

FIGURE 1. Diagram explaining SOS response mechanics [5]



2. BACKGROUND

During the cell cycle, if a cell's DNA ever undergoes any damage, it activates the SOS response, a cell program designed to repair DNA [5]. Unfortunately, the SOS response is extremely error prone, and most of the time ends up leading to mutagenesis [6]. In fact the SOS response is one of the major causes of bacterial mutagenesis [6]. Due to this, bacterial cells can sometimes mutate in a manner that gives them resistance to many antibiotics [7]. There are two main proteins involved in the SOS response, LexA, and RecA [8]. LexA makes sure that SOS response remains off when the cell is healthy [8], and RecA makes sure that SOS response is activated when the cell's DNA is damaged [8]. Degradation of the RecA protein can prevent the activation of the SOS response, greatly reducing the risk of mutation [9]. One such way that proteins such as RecA can be antagonized, is through the human ubiquitination system. The ubiquitination system consists of the proteins ubiquitin and proteasomes. Ubiquitin tags any protein which is misfolded, signaling that something is wrong with the protein [10]. If any protein is polyubiquitinated, then proteasomes will come in and destroy that protein though a process called proteolysis [4]. In order to destroy the RecA protein in bacteria using the ubiquitination system, RecA must be forced to misfold [10]. Proteins have specialized molecules with them, called chaperones, which prevent them from misfolding [11]. Without the presence of chaperones, proteins will not be able to maintain their structure, and misfold [11]. CHIP (C terminus of HSC70-Interacting Protein) is a special biomolecule, that inhibits the interactions between the chaperones specific to the RecA protein [12]. So the presence of CHIP in the bacterial system, would cause the misfolding of RecA, leading to the forced ubiquitination of the protein.

FIGURE 2. Diagram explaining reaction theory [17]

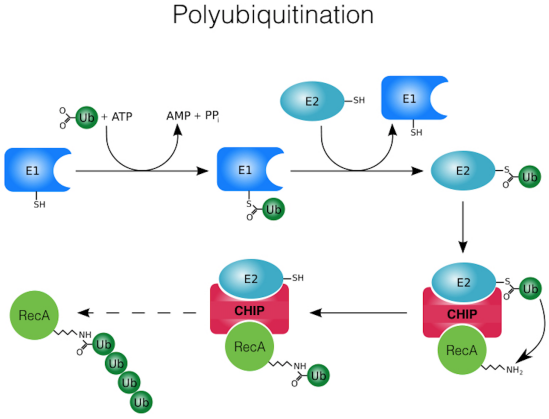
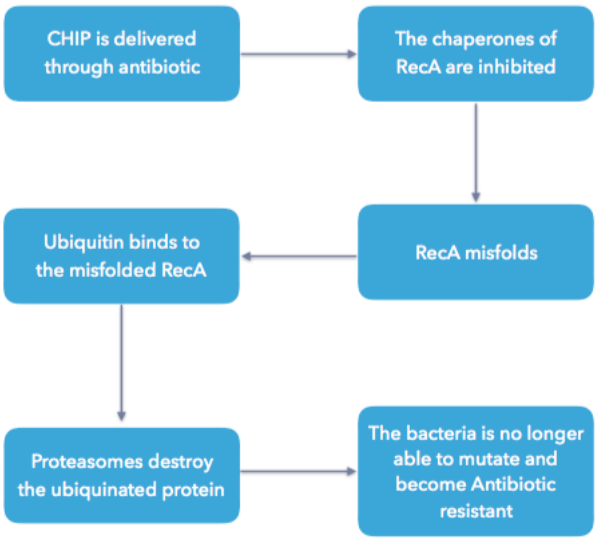


FIGURE 3. Diagram explaining project theory



3. MATERIALS AND METHODS

In order to test for CHIP-mediated ubiquitination on the RecA protein, I had to conduct an assay simulating the bacterial system in vitro, and containing all the necessary components of a Polyubiquitination reaction. I used an ubiquitination assay kit from UBPbio [2], in which CHIP was supplied as the Ubiquitin-E3-Ligase. The kit contained 10x Human Ubiquitin (500 uM), 20x Ubiquitin-activating enzyme (E1, 2 uM), 10x 6xHis-UbE2D3 (E2, 20 uM), 20x 6XHis-CHIP (E3, 40 uM), 10x Hsp70 (20 uM), 5x Ubiquitination Buffer, 20x ATP (40 mM), 5x SDS Sample Buffer. Both the E1 and the E2 act as catalysts in the reaction, and are not required

for an in vivo reaction [4, Image 3]. I conducted several assays with this kit, supplying my own substrate protein (RecA for E. Coli) [18]. I analyzed each assay with Western Blotting and SDS-Page gel electrophoresis lab techniques. By comparing the observed size of RecA with that of the resulting molecule from my assay, using gel electrophoresis, I was able to support my hypothesis that the CHIP-mediated reaction did in fact lead to polyubiquitination. To further confirm this theory, I conducted Western Blots with both anti-RecA [3], and anti-Ubiquitin [19], to confirm the presence of both proteins in the assay synthesized molecule.

Figure 1

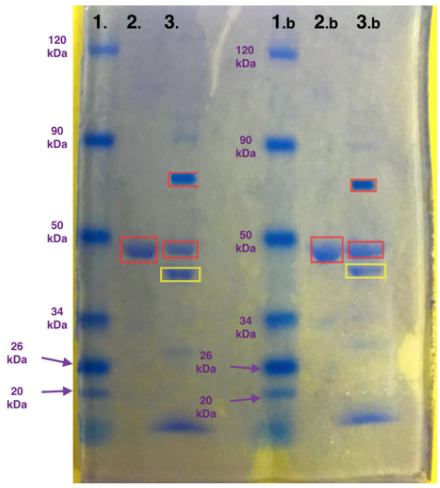


Figure 2

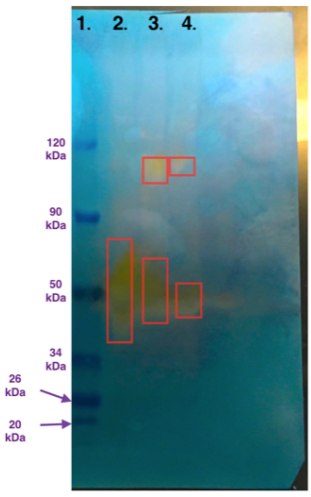
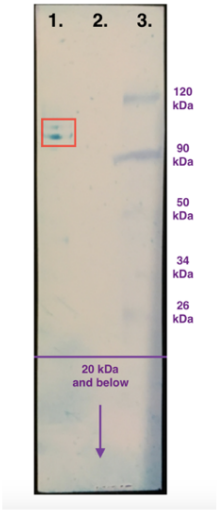


Figure 3



4. RESULTS

4.1. Results Breakdown

I conducted several CHIP-mediated ubiquitination reactions, and tested their success through gel electrophoresis, and Western Blotting. When running gel electrophoresis, I loaded a sample of unreacted RecA, as a control, and a sample of RecA that had gone through the entire ubiquitination reaction via assay. The gel showed that most of the sample in the lane containing unreacted protein had landed around 50 kDa, while most of the sample in the lane containing the reacted RecA had landed around 90 kDa.This showed that the reacted protein had been successfully tagged by several ubiquitin, increasing its size to almost 90 kDa. However, the reacted lane was also showing several other places where very small amounts of the sample had landed. Since it was a gel, it could be showing other components of the ubiquitination reaction, so to verify that the smaller components were not actually RecA, I conducted a Western Blot. The anti-RecA antibody I used in my Western Blot confirmed that all the RecA in the sample was deposited either at the 90 kDa site or the 50 kDa site, and that the other lines showing up in the gel were not RecA. To further prove that the line occurring at the 90 kDa site was a polyubiquitinated version of RecA and not a dimer, and that the line occurring around 50 kDa in both lanes was pure RecA, I conducted another Western Blot using an anti-Ubiquitin antibody. The results from this Western Blot, showed that the sample which went through the entire ubiquitination reaction was in fact ubiquitinated, and there was no presence of ubiquitin anywhere on the membrane, except at the 90 kDa site in the reacted sample lane.

4.2. Data Explanation

Diagram 1

Lanes 1 and 1.b contain the molecular weight ladder. Lanes 2 and 2.b contain the unreacted RecA protein. Lanes 3 and 3.b contain the RecA protein that went through the entire reaction, and was being verified for ubiquitination.

This is an image of an SDS-Page gel, so the markings on this gel are not as accurate or specific as that of a western blot. The red boxes show predicted sites of the RecA protein. The yellow boxes show unidentified protein samples; theses marks will no longer be visible post- Western Blot. The presence of a mark at around the 80 kDa area in both lanes 3 and 3.b show that the sample most likely did ubiquitinate. A Western Blot will confirm this data.

Diagram 2

Lane 1 contains the molecular weight ladder. Lane 2 contains the unreacted RecA protein. Lanes 3 and 4

contain the RecA protein that went through the entire reaction, and was being verified for ubiquitination. This was the first Western Blot I conducted, so there was some error in diluting the primary antibody. The large amount of anti-RecA antibody caused for negative staining, but you can still clearly see where the Western marked points of RecA concentration. The presence of a mark at around the 100 kDa area in both lanes 3 and 4, show that the sample did most likely ubiquitinate. This anti-RecA Western Blot confirms that both the proteins at the top do contain RecA. Ubiquitination will be confirmed by an anti-Ubiquitin Western Blot.

Diagram 3

Lane 1 contains the RecA protein that went through the entire reaction, and was being verified for ubiquitination. Lane 2 contains the unreacted RecA protein. Lane 3 contains the molecular weight ladder.

This anti-Ubiquitin western blot shows a mark in the same region as the anti-RecA Western Blot, showing the presence of both RecA and Ubiquitin in the same sample. The presence of a mark around the 100 kDa area only in the lane containing the reacted sample confirms that the reaction did in fact ubiquitinate the protein.

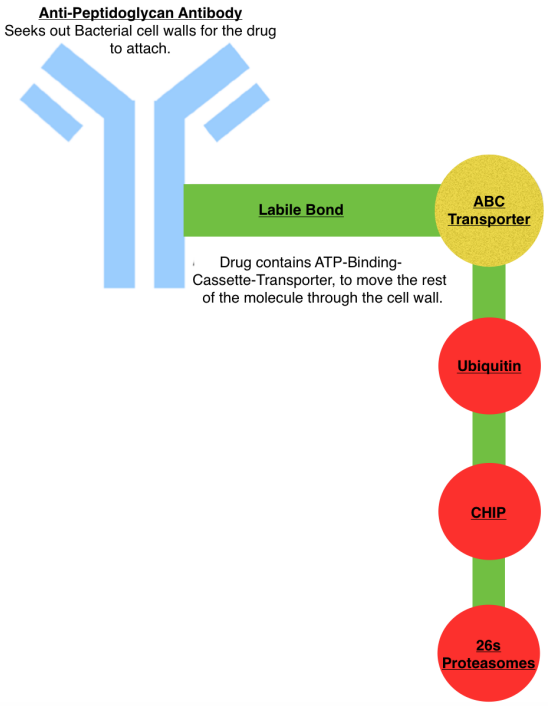
5. CONCLUSIONS

Based on the readings from both the multiple SDS-Page gels, and the Western Blots, we can conclude that the RecA protein can be ubiquitinated through a CHIP-mediated ubiquitination reaction. A polyubiquitinated RecA protein can be degraded via proteasomes, successfully thwarting the SOS response. This research can be used as a first step towards total inhibition of bacterial mutagenesis, to fight antibacterial drug resistance.

6. DRUG DELIVERY

Promoting a CHIP-mediated ubiquitination reaction inside the bacterial system may be a challenge, due to the difficulty of transporting all the necessary components across the cell wall of the bacterium. One possible drug design and delivery mechanism is the Antibody-Drug-Conjugate, or ADC. An ADC is an intravenous drug which consists of an antibody to locate the drug target, and the drug, which has to be delivered at the target [20]. An ADC containing, Anti-Peptidoglycan, ubiquitin, CHIP, 26s proteasomes, and a ATP-Binding-Cassette-Transporter all attached with labile bonds, has promise for inhibition of bacterial mutagenesis.

FIGURE 4. Proposed ADC mechanics for promotion of CHIP-mediated ubiquitination reactions in the bacterial system



ACKNOWLEDGEMENTS

Thank you to the entire staff at Dx-Sys Inc. who welcomed me to their lab with open arms, and allowed me to use their facility for my experimentation. This project would not have been possible without them.