Characterization of Host Defense Peptide Inducing Compounds in Human HT-29 Cells:

Investigating the Innate Human Immune System as an Alternative to Antibiotics

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Abstract

Inducing the innate human immune system's host defense peptide activity presents a novel alternative for antimicrobial therapy in the fight against antibiotic-resistant bacterial strains. The human body has only one cathelicidin host defense peptide, known as LL-37. The compound butyrate has already been identified as a positive control for inducing LL-37 gene expression, however a high-throughput screening assay has identified several additional potential compounds as possible host defense peptide inducing compounds in animal cell lines. This study investigates the efficacy of LL-37 induction for three structurally similar histone deacetylase inhibiting compounds, Mocetinostat, Chidamide, and Entinostat in an in vitro study utilizing the human HT-29 cell line. Total RNA isolation and REAL Time PCR amplification techniques were used to identify relative fold-change in gene expression. All three compounds exhibited significant fold-increase in LL-37 gene expression, with Mocetinostat demonstrating the highest efficacy out of the three compounds. The results of this investigation confirm previous findings and provide a basis for future time course and in vivo investigations on the path to utilizing the innate human immune system as a possible novel alternative to antibiotics.

Keywords: LL-37, host defense peptides

Introduction

Antibiotic resistance is an alarming problem that has been puzzling the medical community for years. While scientists are always working to develop more effective antibiotics, pathogenic bacterial strands continually adapt more quickly than medical developments, resulting in several deaths each year. The solution to this problem may not lie in a stronger antibiotic, but in a novel approach to antimicrobial therapy. Host-defense peptides (HDP) are a component of the innate immune system in mammals whose primary role is a rapid and broad antimicrobial response when the body is exposed to pathogens.

The human body contains only one cathelicidin antimicrobial peptide (CAMP) or more commonly known by the encoding gene, LL-37. This peptide is extremely small, with a chain of only 37 amino acids in its active form[1]. The peptide is amphipathic and positively charged for rapid transport through the body. It utilizes its positive charge allows to quickly attract to and bind to pathogens, such as negatively-charged bacteria[2]. The peptide utilizes those electrostatic interactions with the bacterial membrane to disrupt it completely and eliminate its pathogenic potential[3]. LL-37 has shown to be effective against gram-negative bacteria, gram-positive bacteria, fungi, and even some parasitic pathogens[2]. LL-37 is transcriptionally regulated by histone deacetylase activity, and studies have already proven a direct correlation between decreased histone deacetylase activity and increased LL-37 expression[2]. By inducing activity of this peptide, it is possible that in the future induction of the body's innate immune response could be used to respond to bacterial infections typically treated with prescription antibiotics.

In this cell-line, dosage-response study, human HT-29 cells were induced with three compounds at varying concentrations to test their efficacy in inducing a fold-increase in LL-37 gene expression, and thus LL-37 peptide activity. These drugs had already been targeted in a

high-throughput screening process as potential host-defense peptide inducing compounds and have been experimentally proven to induce host-defense peptide expression in chicken cell lines[4]. Human HT-29 cells are colon epithelial cells and were selected because of their ability to mimic in vivo responses and because of their high replication rate[4]. Additionally, LL-37 naturally exhibits a majority of its antimicrobial activity in the intestinal lining of human body due to high concentrations of pathogens[4], making HT-29 cells the most ideal cell line for in vitro investigations. Butyrate and its analogs have already been identified and proven for inducing a wide variety of host-defense peptide expression[5], therefore it was used as the positive control for this investigation as a means of comparison for test compound efficacy. Similar or greater fold increase than butyrate could indicate a more effective compound at inducing LL-37 expression.

Mocetinostat, Chidamide, and Entinostat were the test compounds used in this investigation that had been previously identified through high-throughput screening. All of these compounds are histone deacetylase inhibitors[6, 7, 8], meaning they could potentially inhibit the activity of the LL-37 transcriptional regulator and allow increased fold-change expression of the gene. Mocetinostat and Chidamide have been identified as potential compounds in cancer research for decreasing activity of immune suppressive cells and allowing for increased immune response against cancer cell types[6, 7]. Entinostat has been identified in preliminary studies to up-regulate LL-37 expression through both histone-deacetylase inhibition, and more specifically by activation of transcription factor STAT3 as outlined in the Miraglia et. al. study. Additionally, Entinostat has been proven to exhibit antimicrobial activity against cholera bacteria[9], specifically linking increased LL-37 regulation and expression, with antimicrobial peptide activity making the compound an ideal compound not only for this investigation, but as a potential novel alternative to antibiotics.

Methodology

Identification of induced activity of host defense peptides is most easily noted by the measurement of fold increase in LL-37 gene expression. Methods for identifying gene expression involved treating cells with selected compounds, isolating and purifying RNA, and amplifying complementary cDNA strands through REAL Time PCR to obtain fold increase.

Cell Culture and Treatment

Following cell maintenance protocol outlined in Jiang et al.[5], human HT-29 cells were grown in 1640 RPMI medium with 10% FBS serum solution and 1% penicillin/streptomycin antibiotic concentration (2013). Cells were incubated at 37ºC and subcultured every four to five days to maintain viability. Subculturing was done using 1640 RMPI medium and Trypsin enzyme for cell dislodgement. Cells were seeded into 12-well plates at $2.5x10^5$ cells per well in order to optimize cell growth during treatment. After seeding, cells were returned to 37ºC incubation for adaption and growth for 24 hours before treatment. Cells were treated on a dosage-response regimen with the compounds and concentrations outlined in Figure 1. Control wells received no additional compounds at the time of stimulation. Cells were lysed 24 hours after treatment as this was identified as the ideal time course for LL-37 inducing efficacy for Entinostat in the Miraglia et al. investigation[8].

Control 1	Control 2	5uM Mocetinostat	5uM Mocetinostat
2uM Butyrate	2 uM Butyrate	10uM Mocetinostat	10uM Mocetinostat
2uM Mocetinostat	2uM Mocetinostat	20uM Mocetinostat	20uM Mocetinostat

5uM Chidamide	5uM Chidamide	5uM Entinostat	5uM Entinostat
10uM Chidamide	10uM Chidamide	10uM Entinostat	10uM Entinostat
20uM Chidamide	20uM Chidamide	20uM Entinostat	20uM Entinostat

Figure 1: Treatment compounds used to stimulate HT-29 cells in 12-well plates. The first two trials tested 40uM Mocetinostat in replacement of 2uM Mocetinostat after high concentration of Mocetinostat demonstrated low efficacy.

Total RNA Isolation

Cells were harvested with 250ul of RNAzol per well. DNA was removed and RNA was purified through multiple ethanol washes and centrifugations at 4ºC. Isolated RNA was solubilized in RNAse free H2O at 55ºC. The concentration of solubilized RNA was taken via NanoDrop and all samples were diluted to 300ng/ul. All solubilized RNA was stored at -80 ºC and kept on ice during procedures to maintain viability. Complimentary cDNA strands were generated with diluted RNA samples following the BioRad iScript Reverse Transcriptase cDNA synthesis kit protocol.

REAL Time PCR and Statistical Analysis

cDNA samples were amplified utilizing SYBR-based REAL Time PCR targeting LL-37 gene expression. ΔΔ Ct mean calculations revealed relative fold increase in gene expression of LL-37 in reference to human actin beta gene and a negative control. The experiment was repeated three times to confirm results and reduce the impact of human error. One-way ANOVA and *post hoc* Tukey's test were used to determine statistical significance. All results had a p value ≤ 0.05, which was the marker used for determining significance of results.

Results

Relative-fold increase expression of LL-37 in HT-29 is summarized below for Mocetinostat, Chidamide, and Entinostat in comparison to their relative control. The results are means ± standard errors of three independent trials with **P < 0.01, ***P <0.001, ****P < 0.0001, relative to the control by Tukey's test. Results for known positive control butyrate are not outlined in this report, however fold-increase for those samples were confirmed as expected in comparison from previous investigations[5].

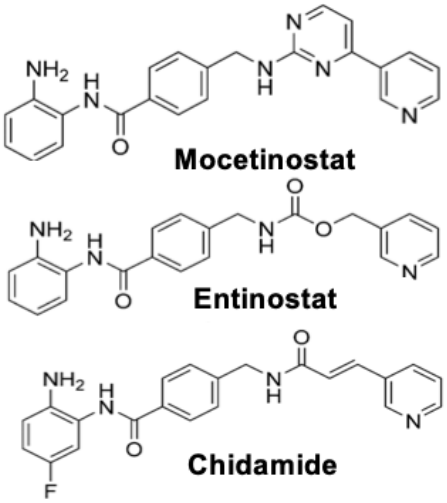

Figure 2: Chemical structures of the three test compound used in this investigation

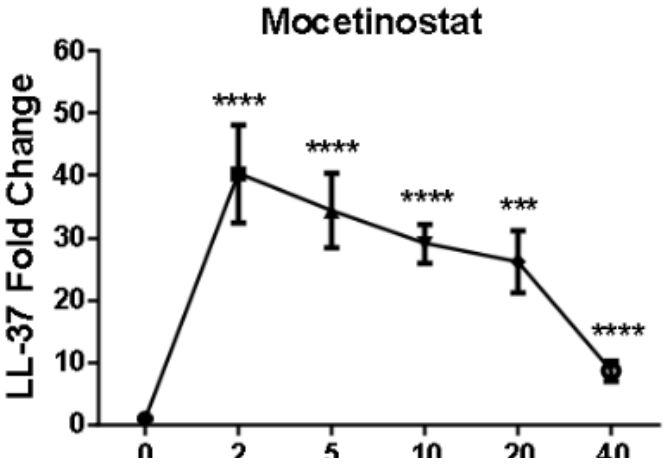

Figure 3: LL-37 fold change in HT-29 cells treated with 2uM to 40uM of Mocetinostat in a 24 hour period.

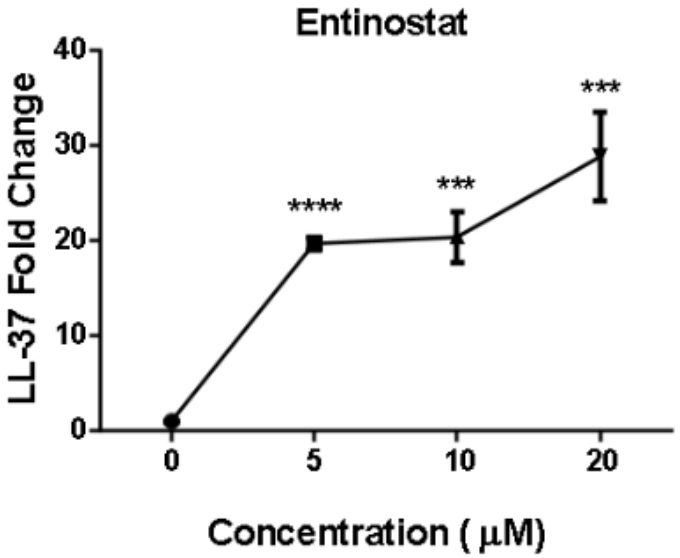

Figure 4: LL-37 fold change in HT-29 cells treated with 2uM to 20uM of Entinostat in a 24 hour period.

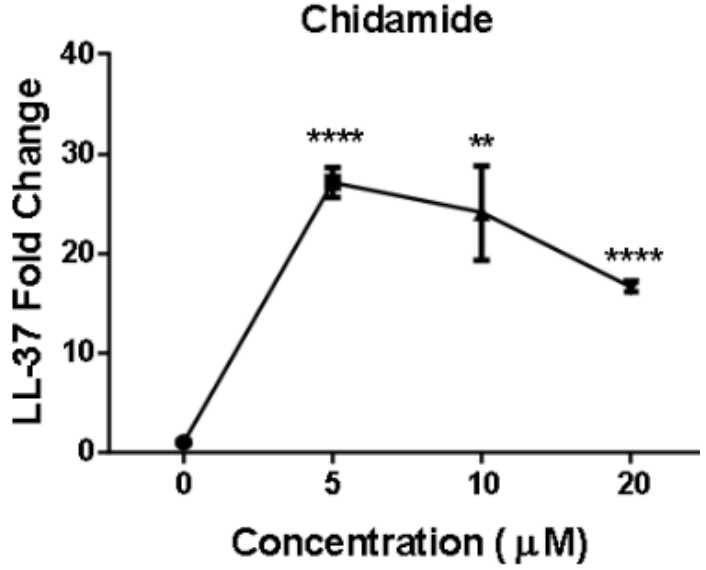

Figure 5: LL-37 fold change in HT-29 cells treated with 2uM to 20uM of Chidamide in a 24 hour period.

Conclusions

All test compounds demonstrated significant fold-increase expression of the LL-37 gene after normalization to a reference gene (hACTB) and relative comparison to negative-control groups. During the first two trials, Mocetinostat concentration was tested at 5uM, 10uM, 20uM, and 40uM however lack of efficacy at 40uM prompted the investigation of a lower concentration dosage, with the final two trials investigating 2uM, 5uM, 10uM, and 20uM. A negative correlation between HDP induction and Mocetinostat concentration clearly exists, with highest efficacy at 2uM Mocetinostat concentration.

Chidamide exhibits similar correlation between concentration and efficacy, with the highest efficacy at 5uM. Entinostat demonstrated a positive correlation between concentration and efficacy of the compound to induce fold change, with the highest fold change expression at 20uM. Entinostat treated cells, however, did show lowest data precision in comparison to the other compounds. Mocetinostat exhibited the highest fold-change out of all the compounds at 2uM concentration.

Discussion

The results of this investigation confirmed that Mocetinostat, Chidamide, and Entinostat all induce LL-37 gene expression. With different dosage-response trends, further investigations can identify both lower concentrations (Figure 3, 5) or higher concentrations (Figure 4) to determine if more effective dosage responses exist beyond the scope of this study. Additionally, further cell-line studies can take ideal dosage concentrations to determine ideal treatment time through time-course studies beyond that outlined in current literature.

As the most effective compound, Mocetinostat (Figure 3) should be further analyzed as novel alternative to antibiotics. In addition to dosage-response and time course treatments, this

compound should be tested for its induction of inflammation gene expression in addition to its expression of LL-37. Inflammation response is the biggest concern of inducing the innate immune system and overactive inflammation response can cause more harm than benefit. Future studies should focus on known immune system inflammation gene activity alongside LL-37 gene expression. Additionally, looking into the enzyme-substrate relationship between the test compounds and histone deacetylase could provide insight into how that relationship can be further manipulated via inhibition or allosteric regulation to allow increased expression of LL-37. Further investigations into the regulation of LL-37 beyond histone deacetylation could provide additional ways to manipulate regulatory mechanisms for inducing LL-37 expression.

Once ideal dosage, time, and coupled compounds for controlled inflammation response have been identified, this study should move into in vivo trials. Animal trials have already begun for investigation of host-defense peptide inducing compounds in both chicken and porcine subjects and have demonstrated positive results[4]. The demand for antibiotic-free livestock and a more effective antimicrobial therapy in human pharmaceuticals creates a unique opportunity for enhanced cooperation between animal and human medicine to maximize resources and funding for continuing the investigation of identification of a novel alternative to antibiotics. Animal trials in mice would serve as the precursor to human clinical trials and help determine the proper conditions for optimal in vivo induction.

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