Epigenetic Induction of Avian Immune Response by Sirtuin Modulators

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ABSTRACT

With the parallel increase in the global demand for poultry production and rise in the threat of antimicrobial resistance, the development of novel antibiotic alternatives is integral to the security of the food-animal industry and public health. Recently, the induction of host defense peptides (HDPs) of the innate immune system has emerged as a host-directed therapeutic approach to simultaneously mitigate poultry infections and disease resistance. This study is focused on comparing the capacity for several epigenetic compounds, specifically four sirtuin modulators and a known histone methyltransferase inhibitor (BIX01294), to induce the avian innate immune response. To evaluate the *in vitro* response, chicken macrophage cells were treated with each compound (resveratrol, SRT2104, sirtinol, and EX527) with or without BIX01294 for 24 h. Following treatment, RNA isolation, reverse transcription, and qPCR analysis of select innate immune genes *(AvBD3, AvBD9, AvBD10, CLDN1, IL-1β*, and *MUC2*) were performed to profile the avian immune response. Despite the hypothesized upregulation of the innate response strictly from sirtuin inhibitors, both the sirtuin inhibitors and activators synergized with BIX01294 to induce a significant avian HDP response with minimized inflammation. Although no further expression of mucosal or tight junction barrier protective genes was induced by any of the four modulators, this study furthers the need to understand sirtuin modulator epigenetic activity with future studies of histone profiling. Despite the unknown mechanisms of sirtuin modulators, the significant synergy response to these treatments validates the promising potential for these compounds to be actively explored as antibiotic alternatives.

1. Introduction

Annually, approximately 700,000 global deaths are attributed to antibiotic-resistant infections with a projected increase to 10 million annual deaths by 2050 (WHO, 2019). Despite U.S. bans on growth promotion antibiotics in 2017, the continued threat of antimicrobial resistance in animal agriculture furthers the demand for novel antibiotic alternatives (Nhung *et al.,* 2017). In response, host-directed therapeutics, which modulate the host immune response rather than target the pathogen, have emerged as promising antibiotic alternatives.

Host defense peptides (HDPs) are evolutionarily conserved components of the innate immune system. These short-chain, cationic peptides exhibit immunomodulatory, barrier protective, and antimicrobial properties. Although this broad-spectrum activity is often attributed to the amphipathic interaction of HDPs with bacterial membranes, the specific mechanism of action remains undefined (Robinson *et al*., 2018). However, epigenetic modulation of these HDPs has emerged as a promising therapeutic alternative with minimized potential to induce antibiotic resistance (van Dijk *et al*., 2018).

In recent high throughput screenings conducted by the Zhang laboratory, 14 epigenetic compounds were identified for their potential to independently upregulate HDP synthesis in avian (HTC) and porcine (3D4/31) macrophage cell lines (Lyu *et al*., 2018; Deng *et al*., 2018). Among these compounds, histone methyltransferase inhibitors (HMTis) and histone deacetylase inhibitors (HDACs), specifically sirtuin modulators, were noted for their ability to induce HDPs in both species cell lines. HMTis and HDACis reversibly regulate DNA accessibility, and therefore gene expression, by decreasing histone methylation and increasing histone acetylation, respectively (Deng *et al*., 2018). Sirtuins, or conserved cell maintenance proteins, have garnered recent interest in the scientific community due to their anti-aging properties attributed to broad inflammatory, stress resistance, and apoptotic functions (Dai *et al*., 2019).

The objective of this project is to further investigate and compare the ability for several of these identified sirtuin modulators to induce HDPs independently and in combination with a known HMTi, known as BIX01294. Specifically, two sirtuin activators, resveratrol and SRT2104, and two sirtuin inhibitors, sirtinol and EX527, were analyzed with BIX01294 to evaluate their ability to synergistically affect poultry innate immune response. Based on previous studies, it is hypothesized that only the sirtuin inhibitors will synergistically induce avian HDPs and barrier function genes due to their HDACi activity. The results of this study will direct further investigation into understanding sirtuin activator and inhibitor molecular mechanisms in relation to innate immune gene expression. Collectively, these results will determine the potential for these compounds to be utilized as host-directed therapeutics in infected poultry.

2. Experimental Details

Cell Culture & Treatment

HTC cell stocks were maintained at 37ºC and 5% CO_2 in complete cell media containing RPMI1640, 5% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin. These cells stocks were subcultured every 2-3 days and replaced after every two months. For experiment use, HTC cells were split into 12-well plates at 3×10^5 cells/well 14-18 hours prior to 24 h compound stimulation.

The four sirtuin modulators used for cell stimulation were reconstituted in DMSO, and each compound aliquot was limited to 1-2 freeze-thaw cycles for experiment use. Initially, independent dosage trials were performed to determine the optimal concentration of the two sirtuin activators and two inhibitors for maximized avian HDP gene response and RNA concentrations. Optimal conditions were identified by treating HTC cells with varying concentrations of each individual compound (0, 10, 20, 40 μM) in triplicate for 24 h. These initial concentrations were selected based on previous graduate research performed on resveratrol and sirtinol in the Zhang laboratory and published research on structurally related sirtuin modulators (Chauhan *et al*., 2011).

From these results, 10 μM concentrations of each sirtuin modulator were selected for use in synergy trials based upon HDP gene fold-change and RNA concentrations. Therefore, cells were independently treated with 10 μM resveratrol, SRT2104, sirtinol, or EX527 in combination with 2.5 or 5 μM BIX01294 for 24 h.

Isolation of Total RNA

Following treatment, cells were lysed with RNAzol®RT for total RNA isolation. RNA concentrations were quantified using the NanoDrop^{TM} One/One^{C} Microvolume UV-Vis Spectrophotometer and were stored at -80ºC until further analysis.

Gene Expression & Data Analysis

Isolated RNA was subjected to reverse transcription using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad) and quantitative real-time polymerase chain reaction (RT-qPCR) was performed using 2x iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad) and innate immune gene primers. During the independent dosage trials, HDP expression was characterized by the response of *AvBD9*, a representative poultry HDP gene expressed consistently throughout the proximal and distal gastrointestinal tracts (Cuperus *et al*., 2013), normalized to *GAPDH*. During synergy trials, the innate immune response was further profiled by the response of barrier protective (*MUC2, CLDN1*), inflammatory (*IL-1β*), and additional avian HDP genes (*AvBD3, AvBD10*). Between qPCR runs, all cDNA was stored at -20ºC.

qPCR data was analyzed to quantify innate immune gene response in independent dosage and synergy trials. The fold-change was calculated using the ΔΔCt method, and statistical significance was analyzed through GraphPad Prism 8 using one-way ANOVA ($P \leq 0.05$) and post hoc Tukey's Test.

3. Results

Based upon the ubiquitous expression of *AvBD9* throughout the poultry gastrointestinal tract and its identification as the most readily inducible HDP gene in response to previous compound studies (Cuperus *et al*., 2013; Sunkara *et al*., 2011), this gene was used as a

preliminary indicator for synergy. Despite their opposing epigenetic functions, both the sirtuin activators (*Figure 1A*) and inhibitors (*Figure 1B*) synergistically induced *AvBD9* response when paired with BIX01294. Specifically, 2.5 and 5 µM BIX01294 independently enhanced *AvBD9* expression by approximately 30- and 370-fold, respectively. Comparatively, the four sirtuin modulators induced little to no fold-change, with resveratrol inducing the greatest response of an approximately 6-fold increase. However, when each modulator was combined with BIX01294, maximum fold changes of approximately 10,900-, 1,200-, 2,800-, and 350-fold for resveratrol, SRT2104, sirtinol, and EX527 were observed, respectively.

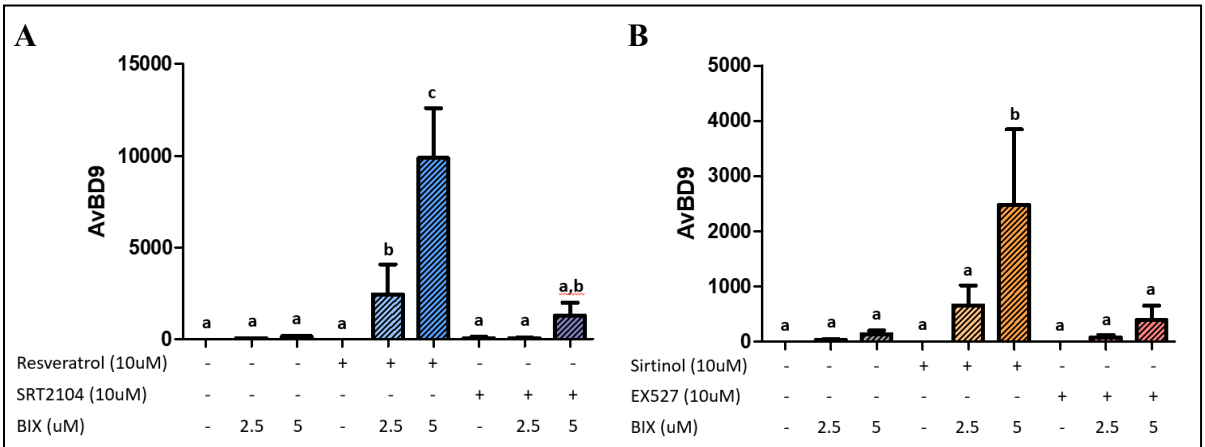

***Figure 1* | Synergistic effect of sirtuin activators and inhibitors with BIX01294 on *AvBD9*.**

HTC cells were treated with 2.5 or 5 µM of BIX01294 with or without 10 µM resveratrol or SRT2104 **(A)**, or sirtinol or EX527 **(B)** for 24 h. The efficacy of these compounds was determined by isolating RNA from treated cells, performing reverse transcription, and quantifying RT-qPCR analysis of *AvBD9* gene expression. The results are expressed as means ± standard errors of the means (SEM) of 3 independent experiments. The treatments without common superscripts are considered statistically different ($P \leq 0.05$) by one-way ANOVA and post hoc Tukey's Test.

In *Figure 2*, two other avian HDP genes were tested to determine the scope of the sirtuin modulator and BIX01294 interactions. *AvBD3* and *AvBD10* are predominantly expressed in the respiratory tract; however, *AvBD10* is additionally expressed in the distal gastrointestinal tract and male and female reproductive tracts (Cuperus *et al*., 2013) The sirtuin activators resulted in an additive response from *AvBD3*; however, statistically significant synergy between resveratrol and BIX01294 was observed in *AvBD10* (approximately 10-fold). Comparatively, sirtinol and BIX01294 resulted in statistically significant synergy of *AvBD3* induction (approximately 20-fold); however, no significant *AvBD10* response was induced.

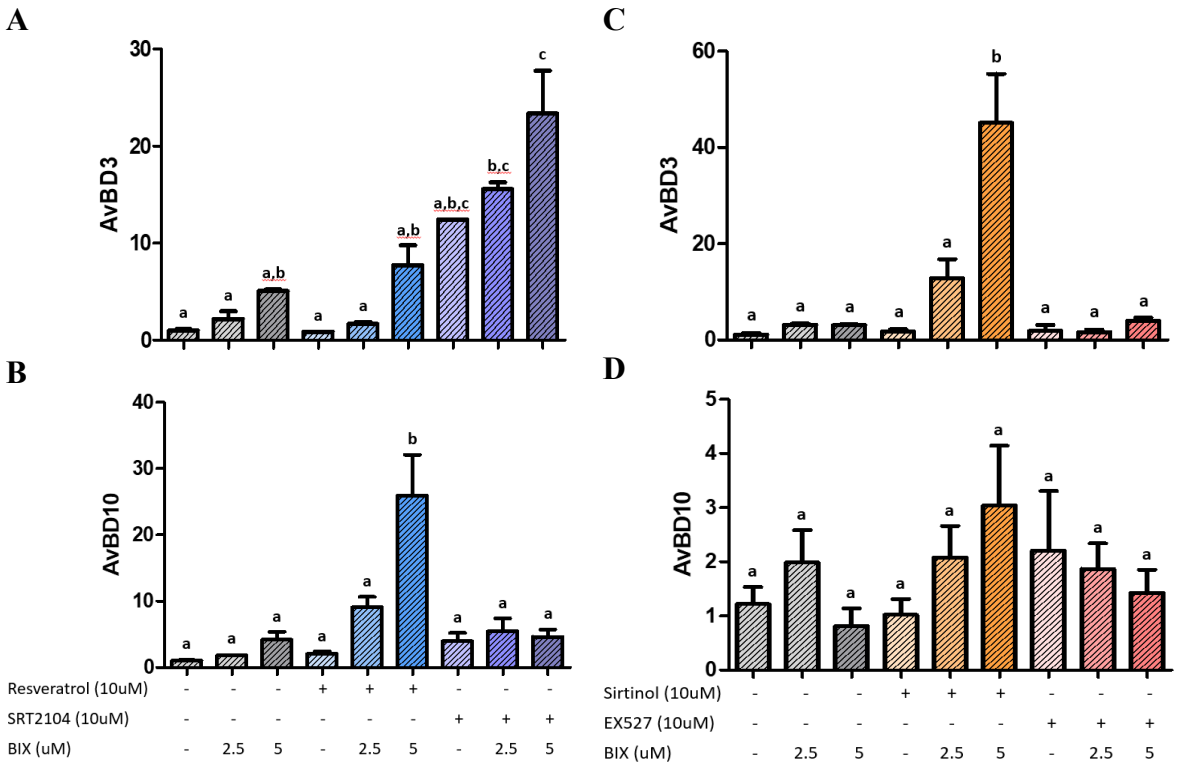

***Figure 2* | Effect of sirtuin modulators and BIX01294 on *AvBD3* and *AvBD10* expression.**

HTC cells were treated with 2.5 or 5 µM BIX01294 with or without 10 µM resveratrol, SRT2104, sirtinol, and EX527 for 24 h. qPCR analysis of *AvBD3* and *AvBD10* induction was used to further profile avian HDP response. The results are presented as means ± SEM of the same 3 independent experiments analyzed in *Figure 1*. Based upon one-way ANOVA and post hoc Tukey's Test, treatments without common superscripts are considered statistically different ($P \leq 0.05$).

In addition to HDP genes, the barrier protective response induced by the compounds in HTC cells was analyzed by *MUC2* and *CLDN1* gene expression (data not shown). *MUC2* encodes the mucin 2 protein secreted by epithelial tissue, which is essential to the composition of the mucosal membranes (Liu *et al*., 2020); similarly, *CLDN1* encodes the claudin 1 protein, which is necessary for epithelial tight junction formation (Milatz *et al*., 2015). Additionally, *IL-1β*, which encodes interleukin-1 β, was analyzed due to its role as a key mediator of inflammatory response. However, all sirtuin compounds did not statistically induce these barrier protective or inflammatory immune responses independently or in combination with BIX01294.

4. Discussion and Conclusions

Epigenetic modulation of the innate immune response has emerged as a promising in-feed prophylactic and therapeutic alternative to treat and mitigate the rise of antimicrobial resistant poultry infections. Based upon previous compound screenings, decreased histone methylation and increased histone acetylation both significantly contribute to enhanced HDP response. However, this study demonstrates that inhibitors and activators of sirtuin-led histone deacetylation both synergize with histone demethylation by BIX01294 to induce avian HDPs with minimized inflammation. Additionally, this study provides further evidence that beyond avian HDPs, no significant enhancement of barrier function is induced by this therapeutic combination.

Globally, approximately 70% of antimicrobial agents are applied to food-animal production. With the increasing demand for livestock and poultry production driving an estimated 67% increase in antimicrobial use by 2030 (Hedman *et al*., 2020), the therapeutic application of sirtuin modulators and BIX01294 provides a necessary alternative for the poultry industry. Due to the coevolution of the nonspecific functions of HDPs alongside antibiotic-resistant bacteria, bacterial resistance is less likely to manifest from controlled induction of HDPs (van Dijk *et al*., 2018; Lazzaro *et al*., 2020). Additionally, recent studies have identified that the translocation of commensal gut bacteria induced by antibiotics has the potential to further the proinflammatory disease state, enhancing tissue damage (Knoop *et al*., 2015). Within this study, all epigenetic compounds studied had little to no induction of inflammatory response, making these compounds ideal candidates for antibiotic alternatives.

Despite the unexpected induction of HDPs by sirtuin activators, this dual induction of HDPs by sirtuin inhibitors and activators with a known HMTi either suggests that the acetylation status of HDP genes does not directly dictate the expression or that the nonspecific nature of sirtuins and HMTis result in an unidentified epigenetic interaction affecting HDP expression. In support of this latter reasoning, although resveratrol is repeatedly characterized as a sirtuin activator (Dai *et al*., 2018), recent studies characterizing sirtuin modulator activity suggest that this activator status is sirtuin-protein specific. Within mammals and poultry, there are seven sirtuin proteins (cSIRT1-7). Recently, mammalian sirtuin studies (SIRT1-7) indicate that resveratrol has inhibitory effects on SIRT1, SIRT3, and SIRT5 depending on the substrate present. (Dai *et al*., 2018; Gertz *et al*., 2012). Comparatively, the other selected sirtuin activator, SRT2104 is a highly specific SIRT1 activator (Hoffman *et al*., 2012) with no characterized inhibitor effects. This difference in specificity may be indicative of the differences observed between the two activators effects on HDP genes. Similar to SRT2104, the two sirtuin inhibitors are both highly specific to SIRT1 inhibition (Napper *et al*., 2005). Despite this shared specificity, the two sirtuin inhibitors parallel the differences in efficacy observed between the two sirtinol activators, which suggests that sirtuin protein specificity does not independently justify the observed synergy.

Prior to expanding this study to live chicken trials, repeated *in vitro* trials using structurally related sirtuin modulators must be studied to determine the possible mechanism for this

shared induction by both classes of sirtuin modulators. Additionally, chromatin immunoprecipitation (ChIP) assay will be applied to analyze the HDP gene promoter acetylation status in response to the four sirtuin modulators (Eckmann *et al*., 2005), and an antimicrobial assay will be performed to establish the broad or narrow-spectrum effects of these compounds in synergy.

The threat of antibiotic resistance continues to dampen the treatability of common poultry and livestock infections. Therefore, the application of these HDP-inducing therapeutic alternatives provides a novel approach to treating poultry infections with minimized inflammation and resistance development.

5. Summary

Based upon previous compound screenings and known sirtuin activity (Lyu *et al*., 2018; Deng *et al*., 2018; Dai *et al*., 2018), it was hypothesized that only the sirtuin inhibitors would synergize with the epigenetic activity of BIX01294. However, the results of this study reveal that despite sirtuin activators and inhibitors having opposing effects on histone acetylation status, both modulators significantly synergize with the HMTi activity of BIX01294 to induce avian HDPs. Additionally, the selected sirtuin activators (resveratrol and SRT2104) induced approximately 10,900- and 1,200-fold increases in *AvBD9* gene expression, comparative to the 2,800- and 350-fold increases by the sirtuin inhibitors (sirtinol and EX527).

The dual induction by activators and inhibitors of sirtuin HDAC activity suggests that histone acetylation may not be the primary determinant of HDP activity in these studies. This reasoning is further confirmed by the minimized induction of *AvBD3* and *AvBD10* by BIX synergy trials with resveratrol and SRT2104, respectively. Additionally, this difference is supported by the minimized induction of *AvBD3* by EX527 and *AvBD10* by both sirtuin inhibitors. The observed synergy by both modulators and the differences between the two activators and two inhibitors on HDP induction suggests the need for testing structurally related sirtuin modulators and quantifying the effects on histone epigenetics.

Despite these differences in HDP induction, the four compounds had no statistically significant effects on the mucosal and tight junction barrier protective genes tested; this suggest that these induced epigenetic changes are not universally beneficial to the innate immune response. Additionally, inflammatory response quantified by *IL-1β* expression was minimally induced by all four compounds. Although further understanding of the synergy mechanism of sirtuin modulators and BIX01294 is necessary, the combined induction of avian HDPs and minimized inflammatory response makes these compounds effective and attractive candidates for therapeutic alternatives.

6. Appendices

6a. Acknowledgements

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