IDENTIFICATION OF HOST DEFENSE PEPTIDE-INDUCING COMPOUNDS AS ALTERNATIVES TO ANTIBIOTICS USING A CELL-BASED HIGH THROUGHPUT SCREENING ASSAY

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Abstract:
In-feed antibiotics has been banned from food animals for production purposes. Effective alternatives to antibiotics are needed to maintain animal health and production performance. Modulating of the synthesis of endogenous host defense peptides (HDPs) shows potential as a novel antibiotic alternative strategy to disease control and prevention. To identify small-molecule compounds with the ability to induce HDP synthesis, a high throughput screening (HTS) assay based on a stable HTC/*AvBD9-luc* cell line expressing a 2-kb avian β-defensin (*AvBD9*) gene promoter-driven luciferase reporter gene was developed. Libraries of natural and synthetic compounds totaling 5,586 were screened. Using a minimum Z-score of 2.0, 131 hits were identified. After further validation in HTC/*AvBD9-luc* cells and parental HTC macrophage cells, most compounds showed a strong capacity to induce *AvBD9* gene expression in a dose-dependent manner. For example, wortmannin, a phosphoinositide-3-kinase inhibitor, was confirmed to stimulate *AvBD9* expression in multiple chicken cell types and the duodenum of chickens. Wortmannin was also shown to synergize with butyrate in inducing *AvBD9* gene expression and enhancing the antibacterial activity of chicken monocytes. Additionally, mocetinostat, a histone deacetylase inhibitor, was highly potent in *AvBD9* induction in HTC/*AvBD9-luc* cells and parental HTC cells. Mocetinostat was more efficacious in *AvBD9* induction and enhancing the antibacterial activity of chicken cells than its structural analogs, Chidamide and MS-275, two compounds that are highly effective in HDP induction in pigs and humans, respectively. In addition to *AvBD9*, most other HDP genes were found to be simultaneously induced by wortmannin and mocetinostat in chicken cells. Collectively, these newly-identified HDP inducers have the potential to be developed as novel antibiotic alternatives for disease control and prevention in poultry and possibly other animal species including humans.

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CHAPTER I

INTRODUCTION

Routine in-feed use of antibiotics in the livestock industry has been suspected to be a main reason for antimicrobial resistance in humans and poses a serious threat to public health. Although various antibiotic alternatives such as prebiotics, probiotics, phytochemicals, organic acids, feed enzymes, vaccines, and bacteriophages are currently available on the market, very few are as effective as antibiotics. There is an urgent need to develop novel, more effective antibiotic alternatives.

Host defense peptides (HDPs), also known as antimicrobial peptides, exist in almost all species of life as a part of innate immunity active against a broad spectrum of bacteria. HDPs consist of two major families in vertebrates: defensins and cathelicidins. Human have six α-defensins (NP1-4 and HD5, HD6), more than 30 β-defensins (HBDs), and only one cathelicidin (LL-37), while chickens possess 14 avian β-defensins (AvBD1-14) and four cathelicidins (CATH1-3 and CATHB1).

As the first line of host defense, HDPs protect the host from infection by directly killing microbes and by acting as immunomodulators. HDPs kill bacteria mainly by interacting with and eventual lysis of bacterial membranes. As immunomodulators, HDPs facilitate chemotaxis of immune cells such as neutrophils and monocytes and promote differentiation and maturation of antigen presenting cells.

Modulation of endogenous HDP synthesis has become a promising approach to disease control and prevention. Recently, various classes of dietary compounds have been found to induce HDP synthesis. For example, butyrate and vitamin D_3 could potently induce HDP expression in humans and cyclic adenosine monophosphate synergizes with butyrate in promoting HDP expression in chickens.

In this dissertation, we summarized the latest progress on transcriptional regulation of HDPs (Chapter II). To identify additional compounds with a better ability to enhance the synthesis of endogenous HDPs, we established a cell-based high throughput screening (HTS) approach, identified a number of promising leads, and evaluated their potential as antibiotic alternatives (Chapter III and V). In the HTS assay, we first screened 584 natural products (Chapter III) and identified 21 compounds with Z-score of no less than 2.0, meaning two standard deviations above the average luciferase activity of all compounds. We further confirmed the HDP-inducing activity of these hits in chicken HTC macrophages and jejunal explants. A top candidate compound, wortmannin, was found to improve the *AvBD9* gene expression in the chicken duodenum and synergize with butyrate in enhancing the antibacterial activity of chicken monocytes.

We also conducted another larger-scale HTS of 5,002 small-molecule compounds and identified a total of 110 hits with a minimum Z-score of 2.0 from the primary screening (Chapter IV). We selected the top ten compounds for further conformational studies. Dose-response experiments were conducted in parental HTC cells and mocetinostat appeared to be the most potent in *AvBD9* gene induction. We further found that mocetinostat could enhance all other chicken HDP expression, in addition to *AvBD9.* Desirably, mocetinostat was also potent in promoting the expression of several representative barrier function genes. Furthermore, mocetinostat were more efficient in inducing *AvBD9* gene expression and augmenting the antibacterial activity of chicken macrophages than MS-275 and chidamide, two of its structural analogs that have been found to improve HDP expression in humans and chickens, respectively.

Collectively, we have successfully established a HTS assay that has allowed for large-scale screening of HDP inducers. Discovery of multiple HDP-inducing compounds is paving the way for their further development as alternatives to antibiotics for both livestock and human applications. Studies on the mechanism of action of these newly identified HDP inducers is needed. Furthermore, These HDP inducers will also needed to be evaluated for their influence on growth performance and their efficacy in disease control and prevention in liver animals.

CHAPTER II

REVIEW OF LITERATURE

TRANSCRIPTIONAL REGULATION OF ANTIMICROBIAL HOST DEFENSE PEPTIDES[†]

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ABSTRACT

Host defense peptides (HDPs) are of either myeloid or epithelial origin with antimicrobial and immunomodulatory functions. Due to HDP's ability to physically disrupt bacterial cell membranes and profoundly regulate host innate and adaptive immunity, microbial resistance to these peptides is rare. As an important first line of defense, HDPs are mostly present in epithelial cells of the digestive, respiratory or urogenital tracts as well as in the granules of neutrophils, macrophages or intestinal secretory Paneth cells. HDPs are either directly released or inducibly expressed upon exposure to microbes or microbial products, although certain pathogens such as Shigella have evolved an ability to down-regulate HDP synthesis as an immune invasion strategy. Even if a majority of HDPs are induced by infection and inflammation, it is undesirable to augment HDP synthesis and host immunity using pathogen-associated molecular patterns because of an excessive inflammation that is usually accompanied. Recently, several different classes of small-molecule compounds have been identified with the capacity to specifically induce HDP synthesis without triggering extensive inflammatory response. A few HDP-inducing compounds even synergize with each other in HDP induction. In this review, we summarized the recent progress on transcriptional regulation of HDPs by infection and inflammation, and by small-molecule compounds. We suggested the potential of dietary regulation of HDPs as a novel antibiotic-alternative strategy to antimicrobial therapy, as oral supplementation of HDP-inducing compounds has shown promise of preventing and controlling infections in humans and several animal species.

Keywords: antibiotic alternatives, antimicrobial resistance, cathelicidins, defensins, host defense peptides, nutritional regulation.

INTRODUCTION

Host defense peptides (HDPs), also known as antimicrobial peptides, constitute a diverse group of small peptides of mostly less than 120 amino acid residues [1-3]. These HDPs exist naturally in almost all forms of life, acting as an important mechanism of the first line of defense. Due to the presence of an excess number of arginine, lysine, and histidine, HDPs are generally positively charged with an amphipathic structure [1-3]. Two main families of HDPs, namely defensins and cathelicidins, exist in vertebrate animals. While defensins consist of six cysteines forming three disulfide bonds [4], cathelicidins are characterized by the presence of a highly conserved cathelin domain in the precursor [5]. Based on the folding pattern of the three disulfide bonds, defensins are categorized into three subgroups, i.e., α-, β-, and θ-defensins [4].

The β-defensins have been found in almost all vertebrates, whereas α-defensins exist in a small number of mammals and θ-defensins are specific to primates [6]. Only a pseudogene for θ-defensin is encoded in the human genome [6]. Phylogenetic analysis of vertebrate defensins revealed that β-defensins are the most ancient group of defensins, from which found in vertebrates, from which α- and θ-defensins are originated [7, 8]. This is consistent with the fact that all human α- and θ-defensin genes are flanked by the β-defensin genes on chromosome 8p21-23 [7, 8]. Importantly, the variation in the copy number of the α-defensin genes has a profound impact on the susceptibility of individuals to infections [9].

Cathelicidins exist in a variety of vertebrate animals including mammals, birds, fish, and reptiles [5]. Cathelicidins are so named because of presence of a highly conserved cathelin domain, which was discovered initially as a cysteine proteinase inhibitor in pig leukocytes [10]. It is unclear why certain mammalian species such as primates, rodents, and rabbits only harbor a single cathelicidin gene, whereas other species like pigs and ruminants encode over 10 genes [5]. Largely devoid of the antimicrobial activity, the cathelin domain is cleaved proteolytically from the precursor to

give rise to biologically active, mature peptides, which are highly diverse cross species [5]. While many mature cathelicidins are largely α-helical, some adopt a β-hairpin structure and others are unstructured due to the presence of an unusually high proportion of amino acids such as arginine, proline or tryptophan [5].

This review will briefly summarize the tissue expression patterns of defensins and cathelicidins in multiple animal species. We will also highlight transcriptional regulation of HDPs by infection and inflammation as well as different classes of dietary compounds (Fig. 1). The mechanisms of regulation will also be discussed. It is exciting to reveal a number of nutritional compounds with the ability to stimulate the synthesis of many HDPs in different animal species without provoking excessive inflammatory response, suggesting the potential of employing these HDP-inducing compounds for disease control and prevention.

TISSUE EXPRESSION PATTERNS OF HDPS

HDPs are derived from either myeloid or epithelial origin showing tissue-specific expression patterns. Myeloid cells, especially neutrophils and macrophages, are main sources of a majority of HDPs. In mammals, four human α-defensins (HNP-1, -2, -3, -4) are firstly synthesized in promyelocytes, and then stored in azurophilic granules of neutrophils, constituting approximately 5–10% of total proteins in these cells [11]. Two other human α-defensins (HD5 and HD6) are synthesized and stored in the granules of Paneth cells [4], a special type of secretory cells located at the base of the small intestinal crypts [12]. Defensins in the granules, like other antimicrobial granular proteins, are released upon exposure of phagocytes or Paneth cells to microbes [4]. In avian species, the mRNAs for several β-defensin are abundantly expressed in the bone marrow, whereas β-defensin proteins exist in the granules of heterophils, which are equivalent to mammalian neutrophils [3, 13]. Most vertebrate β-defensins are also expressed in epithelial cells lining the gastrointestinal, respiratory or urogenital tract [14].

Most cathelicidins are present in both neutrophils (or heterophils in birds) and mucosal epithelial cells [5]. For example, human cathelicidin LL-37 is expressed in a wide range of tissues including bone marrow, neutrophils, skin keratinocytes, and epithelial cells lining the gastrointestinal, respiratory, and reproductive tracts [5]. Eleven cathelicidins have been detected in bone marrow and neutrophils of pigs [15]. Among them, PR-39 could also be detected in the kidney, liver, thymus, spleen, and mesenteric lymph nodes [16]. Chicken cathelicidin-1 and -2 mRNAs are expressed predominantly in bone marrow and also in mucosal tissues [17, 18]. Interestingly, chicken cathelicidin-B1, a distant member of cathelicidin, is predominantly synthesized in the bursa of Fabricius, excreted from epithelial cells, and finally accumulates in M cells [19], a cell type critical for induction of adaptive immune response to mucosal antigens [20].

HDP REGULATION BY INFECTION AND INFLAMMATION

Defensins

While most myeloid HDPs are constitutively expressed and secreted upon microbial exposure, a majority of epithelial HDPs are inducible in response to infection and inflammation. Neutrophil α-defensins are mostly constitutive, but can be induced in few cases such as pulmonary tuberculosis, septicemia, bacterial meningitis [21-23]. Similarly, IL-18 and hepatitis C viral infection increase human α-defensin expression in intestinal cells and peripheral blood mononuclear cells, respectively [24, 25]. Mouse α-defensins (cryptidins) are also induced in response to *Toxoplasma gondii* via toll-like receptor 9 (TLR9)-dependent pathway [26]. Neuropathogenic *Escherichia coli* enhances the expression of α-defensins in the intestine of 9-, but not 2-day-old rats [27]. However, in human THP-1 monocytic cells, a panel of human α-defensins are suppressed by *Ureaplasma spp.,* bacterial species that commonly colonize the urogenital tract, suggestive of an immune evasive strategy employed by the bacteria [28].

Human β-defensin 1 (hBD-1) expression is primarily constitutive, but inducible by lipopolysaccharides (LPS), and interferon (IFN)-γ and suppressed by *Shigella dyserteriae*, *Vibrio*

cholera, and several bacterial exotoxins [29-31]. The expressions of HBD 2-4 are up-regulated by various bacteria, bacterial products, and cytokines such as interleukin (IL)-1α, IL-1β, IL-17A, IL-22, IL-32, tumor necrosis factor (TNF)-α, and IFN-γ in keratinocytes and/or monocytes [32, 33]. Human immunodeficiency virus (HIV)-1 and rhinovirus-16 enhance hBD-2 and hBD-3 expression in epithelial cells [6]. Protozoa such as *Cryptosporidium parvum* up-regulate hBD-2, but down-regulate hBD-1 expression, with no effect on hBD-3 in colon epithelial cells [34]. Certain mouse β-defensins are also regulated by mechanical injury or tumor growth factor (TGF)-α and TNF-α [35]. Mouse β-defensin 3 (mBD-3) expression is augmented in the esophagus and tongue by *E. coli*, while mBD-1 expression is inhibited in *C. parvum*-infected mice, suggesting the immune evasion by microbes [34, 36].

Intestinal ischemia/reperfusion stimulates rat β-Defensin-2 (rBD-2) expression [37], and methicillin-resistant *Staphylococcus aureus* enhanced the expression of rBD-3 in the rat lung [38] Similarly, *Actinobacillus actinomycetemcomitans* increases rBD-1 and rBD-2 expression in gingival epithelium [39]. Testicular and epididymal β-defensin expression is enhanced in rats treated with LPS through activation of NF-κB and inhibition of histone deacetylase 1 and DNA methyltransferase [40, 41]. Porcine β-defensins are also differentially regulated in response to infections. *Salmonella* infection enhances the gene expression of pBD-1 and pBD-2, but heat-killed or colistin-treated *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritis or *Arcobacter cryaerophilus* fails to increase pBD-2 mRNA levels [42, 43]. The pBD-1 expression is up-regulated in intestinal epithelial cells by *Fusarium* toxin, whereas the pBD-2 expression is suppressed [44]. Altered expression of the defensin genes in the skin of dogs is observed with atopic dermatitis and other inflammatory skin disorders [45, 46]. Treatment of primary tracheal epithelial cells with LPS, coronavirus or parainfluenza virus led to a decreased expression of several canine β-defensins [47].

In cattle, the expressions of tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP) and bovine β-defensin 5 (BNBD5) genes are up-regulated in response to infection particularly in mammary, lung, and uterine tissues [48]. Several bovine β-defensins also showed increased expression in response to inflammatory mediators, bovine viral diarrhea virus, *Mycobacterium paratuberculosis*, *Pasteurella haemolytica* and *Mannheimia haemolytica* [49-51]. Similarly, *S. aureus* or LPS treatment of umbilical endothelial cells potentiated the expression of LAP, BNBD1 and BNBD4 [52]. Activation of NF-κB appears to be responsible for *M. haemolytica*-induced β-defensins [53], whereas autocrine production of TNF-α is involved in β-defensin induction in umbilical endothelial cells stimulated with LPS or *S. aureus* [52].

Intrauterine infusion of *E. coli* in goats resulted in rapid activation of local innate immune response, characterized by up-regulation of β-defensin 2 (gBD2) expression [54]. Infection of intestinal epithelial cells with *Eimeria spp.* resulted in down-regulation of the gBD2 gene [55]. In sheep, the β-defensin 1 (sBD1) expression is increased with parainfluenza virus type 3 infection and decreased by *M. haemolytica*, with no difference seen with sBD2 [56]. Like their mammalian counterparts, many avian β-defensins (AvBD) are inducible in response to inflammatory mediators, bacteria, viruses and parasites in the intestinal, reproductive, and respiratory tracts [57-60].

Cathelicidins

Expression of cathelicidins is readily modulated by microbes, microbial products, inflammatory cytokines such as IL-1, IL-15, and IL-32 [33, 61, 62]. For examples, human cathelicidin LL-37 is induced by *S. enterica*, *E. coli*, *S. aureus, Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Mycobacterium spp.* in epithelial cells, keratinocytes, alveolar macrophages, monocytes, and neutrophils [63-68]. In addition, injury, endoplasmic reticulum stress, and inflammatory disorders increase LL-37 expression in keratinocytes [69-71]. On the other hand, *S. dysenteriae*, *V. cholera,* and *N. gonorrhoeae* as well as bacterial exotoxins have been shown to down-regulate LL-37

expression in intestinal epithelial cells, suggestive of an immune invasion mechanism employed by these bacteria [29, 31, 72]. Similarly, LL-37 is suppressed by *Ureaplasma spp.* in human THP-1 monocytic cells [28]. LL-37 is also reduced in certain autoimmune disorders like atopic dermatitis, which is consistent with the fact that the patients are more prone to infections [73].

Murine cathelicidin CRAMP is enhanced in the skin in response to injury and in mast cells in response to LPS stimulation [74]. Porcine cathelicidins such as protegrins and PR-39 show an increased expression in bone marrow cells treated with different *Salmonella* strains, LPS, and IL-6 [75, 76]. In addition, PR-39 is increased in mucosal and lymphatic tissues of the respiratory tract of pigs infected chronically with *A. pleuropneumoniae* but not in acute infections [77]. Similarly, in large ruminants, *E. coli* or LPS stimulation of neutrophils results in an increased production of bovine cathelicidin Bac-5 [78]. The expression of avian cathelicidins is also differentially regulated by infection and inflammation. For example, certain chicken cathelicidins are augmented in response to *S. enterica* serovar Typhimurium in cecal tonsils, but down-regulated by *Campylobacter jejuni* in peripheral blood leukocytes and by *Eimeria praecox* in the duodenum and jejunum [57, 79, 80].

NUTRITIONAL REGULATION OF HDP EXPRESSION

Besides infection and inflammation, several different classes of nutritional compounds including fatty acids (e.g., butyrate and propionate), vitamins (e.g., vitamin D_3 and A), sugars (e.g., lactose), amino acids (e.g., arginine and isoleucine), minerals (e.g., zinc), and phytochemicals (e.g., epigallocatechin gallate and resveratrol) are capable of inducing HDP expression in several animal species. Some of these nutritional compounds also show synergistic activities in HDP induction. Unlike infectious agents that non-discriminatorily trigger the synthesis of HDPs together with a large array of inflammatory mediators, nutritional compounds often induce HDP synthesis with a negligible effect on inflammation, making dietary modulation of HDP synthesis

a promising approach to infectious disease control and prevention without relying on antibiotics [81, 82].

Defensins

Among the most well-known HDP stimulators is butyrate, a four-carbon short-chain fatty acid [81, 82]. Butyrate and its analog, 4-phenylbutyrate, have potent hBD-1-inducing properties in human lung epithelial cells [83]. Short-chain fatty acids mediate HDP induction likely by acting as histone deacetylase (HDAC) inhibitors, allowing DNA to remain acetylated in the relaxed state enabling gene transcription [84]. Sebum-free fatty acids like lauric acid, palmitic acid or oleic acid induce hBD-2 expression in sebocytes, enhancing the antimicrobial activity against *Propionibacterium acnes* [85]. Oleic acid was also proven to induce mBD4 in the mouse skin [85]. Short-chain fatty acids ranging from 3-8 carbons in length are able to induce an array of β-defensins both *in vitro* and *in vivo* in pigs, chickens, and cattle [86-88]. Oral supplementation of butyrate has led to an enhanced bacterial clearance in the cecum of chickens infected with *S. enterica* serovar Enteritidis. Butyrate analogs such as glyceryl tributyrate, benzyl butyrate, and 4-phenylbutyrate are also strong inducers of β-defensins in swine [89], suggesting fatty acid regulation of HDPs is a conserved innate immunity mechanism. In addition, unsaturated fatty acids such as linoleic, linolenic, and conjugated linolenic acids also trigger β-defensin synthesis in chicken macrophage cell lines, albeit with a much reduced efficiency as compared to short-chain fatty acids [90].

Vitamin D_3 is another natural compound that potentiates hBD-2 expression through the vitamin D receptor [91]. Vitamin D_3 and its derivatives also increase LAP, bBD-1, and psoriasin expressions in bovine mammary epithelial cells, resulting in reduced internalization of *S. aureus* [92]. Sugars like glucose induce hBD-1 mRNA levels in both embryonic kidney and colonic epithelial cells [93, 94]. Hyaluronan, a sugar found in breast milk, induces hBD-2 in human keratinocytes, colonic and vaginal epithelial cells [95, 96]. Hylauronic acid is also known to increase mBD-2

expression in the epidermal layer of the murine skin, and when given orally, adult mice show mBD-2 induction in the intestinal mucosa, with enhanced resistance to pathogenic *Salmonella* infection. [96].

Minerals such as zinc and calcium have also been shown to regulate β-defensin expression. For example, zinc gluconate, which is used for treatment of inflammatory dermatoses, up-regulates hBD-2 expression in humans [97], and the same effect has been observed with pBD-1,-2, and -3 in porcine jejunal cells when treated with different concentrations of zinc [98]. It was also noted that in immunocompromised Paneth cells, hBD-5 is down-regulated, which is correlated with reduced levels of zinc, linking the role of zinc in host immunity [99]. Calcium at high concentrations up-regulates hBD-2 mRNA levels in human gingival cells [100], and when calcium is removed, there is a significant down-regulation of hBD-2 [78, 101]. In four human epidermal cell lines, increased hBD-1, -2 and -3 levels are echoed with elevated calcium levels, suggesting a close connection between calcium and HDP induction [102].

Amino acids like arginine and isoleucine up-regulate hBD-1 in human epithelial cells [103]. L-isoleucine and several of its derivatives are also able to induce β-defensins in bovine kidney epithelial cells [104]. A similar effect was seen when multidrug resistant *M. tuberculosis* H37Rv-positive mice were given L-isoluecine, resulting in a significant increase in mBD-3 and -4 and a reduction in the bacterial load and tissue damage [105]. A similar up-regulation of pBD-1, -2, and -3 is seen in porcine jejunal cells after incubation with L-isoluecine [98], showing a conservation of the HDP-inducing capacity of amino acids across species.

Phytochemicals are biologically active components of plants and have been used to treat diseases for centuries. A natural sugar in avocado, named AV119, was found to induce hBD-2 and hBD-3 expression in human keratinocytes [106]. Epigallocatechin gallate, an active component of green tea, up-regulates hBD-1 and hBD-2 in gingival epithelial cells [107], and was also able to restore HD-5 and HD-6 levels in human intestinal epithelial cells depleted by an anticancer drug [108].

Resveratrol, a phytochemical found in grapes, was also able to enhance expression of hBD-2 and hBD-3 in human keratinocytes, while suppressing pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α [109-111].

Cathelicidins

Similar to defensins, cathelicidins are also specifically modulated by short-chain fatty acids such as butyrate. For example, butyrate, isobutyrate, and 4-phenylbutyrate are all capable of up-regulating human LL-37 production in gastric, intestinal, hepatic, and lung epithelial cell lines as well as in monocytic cells [83, 112-118]. Other butyrate analogs such as benzyl butyrate, trans-cinnamyl butyrate, glyceryl tributyrate, and phenethyl butyrate showed a similar potency in induction of human LL-37 when compared to butyrate [119]. Butyrate also induces cathelicidin expression in epithelial and monocytic cells of chickens, pigs, and cattle [86, 89]. In addition to short-chain fatty acids, many known HDAC inhibitors enhance LL-37 expression in human colonic epithelial cells [120]. In rabbits, butyrate up-regulates the synthesis of CAP-18, a rabbit cathelicidin [121], reinforcing the notion that short-chain fatty acid-mediated HDP induction is a phylogenetically conserved host defense mechanism.

Vitamin D_3 is also a strong inducer of human cathelicidin LL-37 [122]. In human bronchial epithelial cells and primary lung cells, vitamin D_3 was able to enhance LL-37 production, inhibiting the growth of antibiotic-resistant *Mycobacterium tuberculosis* [123, 124]. Vitamin D_3 and its derivatives have shown cathelicidin-inducing activities when administered to psoriatic skin biopsies [125], acute skin injuries [126], and chronic ulcers [126]. Vitamin D_3 deficiency is linked to diminished synthesis of LL-37 in human patients [73, 127], and conversely, supplementation of vitamin D_3 re-establishes LL-37 expression [128]. Furthermore, increased vitamin D_3 synthesis through IL-15, IL-32, and sun baths can stimulate macrophages to synthesize LL-37, resulting in enhanced clearance of intracellular *M. tuberculosis* [33, 62, 129].

Several mono- and di-saccharide sugars have also shown the capabilities to induce cathelicidins. For example, lactose induces LL-37 in a dose- and time-dependent manner in several different human cell lines, aiding to the innate immunity of adults, but even more importantly in immune incompetent infants [130]. Trace minerals like zinc and its analogs have also shown cathelicidin-inducing capabilities. In humans, zinc supplementation results in an up-regulation of LL-37 in colon epithelial cells [131].

Phytochemicals such as resveratrol and pterostilbene are capable of stimulating human LL-37 synthesis in monocytes and keratinocytes [132]. In mice, resveratrol augments the expression of murine cathelicidin (CRAMP), leading to significant suppression of the growth of *S. aureus* [133]. Curcumin is another phytochemical that up-regulates LL-37 in human colonic epithelial cells and keratinocytes [134]. Forskolin (FSK), a phytochemical found in the *Indian Coleus* plant and a natural adenylate cyclase agonist, was shown to enhance the expression of LL-37 in human colonic epithelial cells [135]. In chickens, when FSK was orally supplemented, AvBD9 expression was increased in the intestinal tract [136].

Desirably, several of these compounds are synergistic with each other in HDP induction. For example, a combination of butyrate and vitamin D_3 or a combination of butyrate and lactose show a dramatic synergy in inducing LL-37 in several human epithelial cell lines [137, 138]. FSK also showed an obvious synergy with butyrate in chicken cells and also in live animals [136]. These results suggested the potential of using a combination of HDP-inducing compounds in augment immunity and disease resistance.

CONCLUSIONS

As an important mechanism of animal innate immunity, HDPs are preferentially expressed in the phagocytic cells and mucosal epithelial cells. Inflammatory cytokines and pathogen-associated molecular patterns produced during infection and inflammation often trigger release or induction of HDPs, although certain bacteria employ mechanisms to down-regulate HDP synthesis to evade

the host immune response. Because of unwanted proinflammatory response, it is undesirable to use microbial products for HDP induction. Several different classes of small-molecule compounds have been shown to be strong inducers of multiple HDPs in humans and other animal species without causing excessive inflammation. A combination of several compounds have synergistic activities in HDP induction. Because of no direct interactions with microbes, these HDP-inducing compounds are capable of enhancing immunity and bacterial clearance without triggering resistance. Therefore, dietary modulation of endogenous HDP expression has promise for further development as a novel antibiotic-alternative approach to disease control and prevention.

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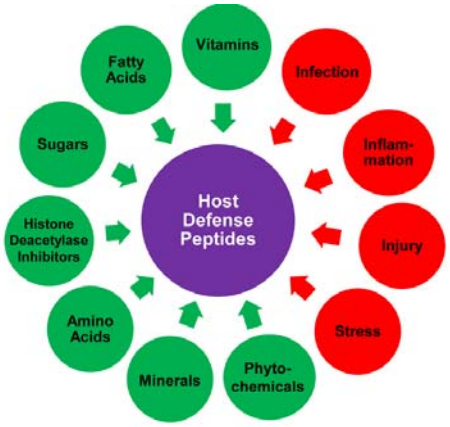

Fig. 2.1. Transcriptional regulation of host defense peptide expression. A large array of proinflammatory mediators and small-molecule compounds are capable of inducing host defense peptide gene expression in multiple animal species.

CHAPTER III

HIGH THROUGHPUT SCREENING FOR NATURAL HOST DEFENSE PEPTIDE-INDUCING COMPOUNDS AS NOVEL ALTERNATIVES TO ANTIBIOTICS[†]

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ABSTRACT

A rise in antimicrobial resistance demands novel alternatives to antimicrobials for disease control and prevention. As an important component of innate immunity, host defense peptides (HDPs) are capable of killing a broad spectrum of pathogens and modulating a range of host immune responses. Enhancing the synthesis of endogenous HDPs has emerged as a novel host-directed antimicrobial therapeutic strategy. To facilitate the identification of natural products with strong capacity to induce HDP synthesis, a stable macrophage cell line, known as HTC/AvBD9-luc, expressing a luciferase reporter gene driven by a 2-Kb avian β-defensin 9 (AvBD9) gene promoter was constructed through lentiviral transduction and puromycin selection. A high throughput screening assay was subsequently developed using the stable cell line to screen a library of 584 natural products. A total of 21 compounds with a minimum Z-score of 2.0 were identified. Secondary screening in chicken HTC macrophages and jejunal explants further validated most compounds with a potent HDP-inducing activity in a dose-dependent manner. A follow-up oral administration of a lead natural compound, wortmannin, confirmed its capacity to enhance the AvBD9 gene expression in the duodenum of chickens. Besides AvBD9, most other chicken HDP genes were also induced by wortmannin. Additionally, butyrate was also found to synergize with wortmannin and several other newly-identified compounds in AvBD9 induction in HTC cells. Therefore, these natural HDP-inducing products may have the potential to be developed individually or in combinations as novel antibiotic alternatives for disease control and prevention in poultry and possibly other animal species including humans.

Keywords: host defense peptides, antimicrobial peptides, defensins, high throughput screening, HDP inducers, wortmannin, host-directed antimicrobial therapy, antimicrobial resistance

INTRODUCTION

Antimicrobial resistance is posing a major threat to public health. While it is necessary to continue the development of antibiotics with direct antimicrobial activities, host-directed therapies have emerged as attractive alternative strategies to combating infectious and non-communicable diseases [1]. Host defense peptides (HDPs), also known as antimicrobial peptides, are represented by a large diverse group of small peptides that are synthesized primarily by phagocytic cells and epithelial cells lining the gastrointestinal, respiratory, and urogenital tracts [2]. With antimicrobial, immunomodulatory and barrier protective activities, HDPs constitute an important phylogenetically conserved first line of defense in virtually all species of life [3-5]. Two main HDP families, namely defensins and cathelicidins, exist in vertebrate animals [6, 7]. One cathelicidin known as LL-37, six α-defensins, and a minimum of 39 β-defensins exist in humans [8], whereas four cathelicidins (CATH1-3 and CATH-B1) and 14 avian β-defensins (AvBD1-14) are present in chickens [9, 10].

While HDPs are being directly explored as novel antimicrobials or vaccine adjuvants against drug-resistant infections [3, 4, 11], modulating the synthesis of endogenous HDPs has shown promise in the treatment of shigellosis, pulmonary tuberculosis, cholera, and enteropathogenic *E. coli*-induced diarrhea [12-15]. In fact, a number of small-molecule compounds such as butyrate, vitamin D_3, bile acids, and histone deacetylase inhibitors have been shown to induce HDP synthesis in humans without provoking inflammation [16-19]. A high throughput screening (HTS) luciferase reporter assay was recently developed to identify multiple compounds with the ability to induce human *LL-37* gene expression [20].

To facilitate the identification of HDP-inducing compounds for use in other animal species, particularly in poultry, here we report the establishment of a cell-based HTS assay. Our earlier studies revealed that, among multiple chicken HDPs, *AvBD9* is the most readily inducible gene in

response to butyrate and several other compounds in chickens [21, 22]. Here we constructed a stable chicken macrophage cell line integrated permanently with a lentiviral luciferase reporter vector under control of chicken *AvBD9* gene promoter. Such a stable cell line was further employed to screen a library of 584 natural products. Multiple *AvBD9*-inducing compounds were identified and further validated for their HDP-inducing activities *in vitro*, *ex vivo*, and *in vivo*. We confirmed several natural compounds such as wortmannin to have a strong ability to enhance HDP gene expression with good potential for further development as novel antibiotic alternatives for application in poultry and possibly other animal species.

MATERIALS AND METHODS

Chemicals

Cell culture media and supplements such as RPMI 1640, DMEM, PBS, and antibiotics (penicillin, streptomycin, and puromycin) were purchased from Lonza (Allendale, NJ), Fisher Scientific (Pittsburgh, PA) or Santa Cruz Biotechnology (Dallas, TX). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Flowery Branch, GA). Sodium butyrate and sanguinarine were procured from Sigma-Aldrich (St. Louis, MO). Trichostatin A (TSA), apicidin, HC toxin, LY294002, PX866, CAL-101, MK2206, Triciribine, GDC0068, Rapamycin, AZD8055, and BEZ235 were obtained from Cayman Chemical (Ann Arbor, MI). Tetrandrine was acquired from Santa Cruz Biotechnology, and (–)-depudecin was purchased from BioVision (Milpitas, CA) and MyBioSource (San Diego, CA). Datiscetin was ordered from BOC Sciences (Shirley, NY), while wortmannin and CUDC-907 were procured from Selleck Chemicals (Houston, TX).

Cell culture

Chicken HTC macrophage cells [23], kindly provided by Dr. Narayan C. Rath of USDA-ARS, were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 U/ml penicillin, and

100 μg/ml streptomycin. Stable HTC cell lines (HTC/*AvBD9-luc*) transduced with the *AvBD9*-driven luciferase gene were maintained in the same complete medium supplemented additionally with 0.5 μg/ml puromycin. Human 293T embryonic kidney epithelial cells (HEK 293T) were obtained from ATCC (Manassas, VA) and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C and 5% CO_2 and subcultured every 3-4 days.

Construction of the *AvBD9* luciferase reporter plasmids

Chicken genomic DNA was extracted from the liver of a Cobb broiler chicken using Quick-gDNA Microprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's recommendations. A series of *AvBD9* gene promoter constructs were cloned from chicken genomic DNA using CloneAmp HiFi PCR Premix (Takara Bio USA, Mountain View, CA) with different forward primers paired with a common reverse primer (Table 1). It is noted that the 5'-end of gene-specific reverse primer begins at the third nucleotide upstream of the start codon of the *AvBD9* mRNA (GenBank accession number NM_001001611). PCR products were then cloned into a *Kpn*I-linearized luciferase reporter vector, pGL4.21[*luc2P*/Puro] (Promega, Madison, WI), using a ligation-independent In-Fusion HD PCR Cloning Kit (Takara Bio USA). The presence of the insert in each recombinant plasmid was confirmed with direct Sanger sequencing. Recombinant plasmids were propagated in Stellar *E. coli* HST08 competent cells (Takara Bio USA) and purified with QIAprep Spin Plasmid Miniprep Kit (Qiagen, Germantown, MD) for transient transfections as described below.

Transient transfection and luciferase assay

HTC cells were seeded overnight in 24-well tissue culture plates before being transfected with 50 ng/well of different *AvBD9* promoter-driven luciferase reporter plasmids using FuGENE HD Transfection Reagent (Promega). After 24 h, cells were stimulated in duplicate with or without 8

mM sodium butyrate for another 24 h. Luciferase activity was measured by adding an equal volume of Steady-Glo Substrate to each well for 10 min using Steady-Glo Luciferase Assay System (Promega) according to the manufacturers' instructions. The luminescence was detected using GloMax 20/20 Single-Tube Luminometer (Promega).

Development of a stable HTC/*AvBD9-luc* luciferase reporter cell line

A 2.0-Kb *AvBD9* gene promoter fragment was cloned into a lentiviral luciferase reporter vector, pGreenFire1-mCMV-Puro (System Biosciences, Palo Alto, CA) using In-Fusion HD PCR Cloning Kit (Takara Bio USA) and gene-specific primers (Table 1). The PCR product in the recombinant plasmid was confirmed by Sanger sequencing. Pseudolentiviral particles were packaged by transfecting HEK 293T cells in a 10-cm tissue culture dish with 1 μg of recombinant *AvBD9* reporter lentivector and 5 μg of the pPACKH1 plasmid mix (System Biosciences) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The cell culture medium containing pseudolentiviral particles was collected 48 h after transfection and stored at -80°C. For viral transduction, HTC cells were seeded at 1×10^5 cells/well in a 6-well plate overnight and then incubated with 2 ml HEK 293T cell culture medium containing the pseudolentiviruses for 4 h before being replenished with 4 ml fresh cell culture medium. After 3 days of incubation, transduced HTC cells were expanded to 10-cm dishes in complete RPMI 1640 medium containing 0.5 μg/ml puromycin for a week of selection, with medium change every 2-3 days. Single cell clones were obtained by limiting dilution of stable cells in 96-well plates in complete RPMI 1640 medium in the presence of 0.5 μg/ml puromycin. After 10-14 days, individual cell clones were gradually expanded and assessed for their responsiveness to sodium butyrate. The most responsive cell clones, named HTC/*AvBD9-luc*, were chosen for the development of a high-throughput screening (HTS) assay.

Optimization of a cell-based HTS assay for *AvBD9*-inducing compounds

Stable HTC/*AvBD9-luc* cells were grown overnight at different densities in the presence or absence of FBS in a 96-well white tissue culture plate with clear bottom (Santa Cruz Biotechnology). Cells were stimulated with different concentrations of sodium acetate, propionate or butyrate for 24 h, followed by luminescence detection with Steady-Glo Luciferase Assay System (Promega) on L-Max II Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA). To assess the robustness of the HTS assay, Z'-factor [24] was used, which is expressed as $Z'=1-\frac{(3\sigma_p+3\sigma_n)}{|\mu_p-\mu_n|}$, where σ_p and σ_n are standard deviations of positive and negative controls, while μ_p and μ_n are the mean luciferase activity of positive and negative controls, respectively. To assess the Z'-factor, HTC/*AvBD9-luc* cells were grown at 4×10^4 cells/well overnight in 96-well white plates in 50 µl complete RPMI 1640 medium containing 0.5 µg/ml puromycin, followed by stimulated with or without 8 mM butyrate in 48 technical replicates for another 24 h. Luciferase activity was measured with Steady-Glo Luciferase Assay System on L-Max II Luminescence Microplate Reader.

Screening of natural product libraries

HTC/*AvBD9-luc* cells were seeded at 4×10^4 cells/well overnight in 96-well white tissue culture plates in complete RPMI 1640 medium containing 0.5 µg/ml puromycin. The natural products and rare natural products libraries consisting of 584 compounds were previously purchased from BIOMOL International (Plymouth Meeting, PA) [25], dissolved in DMSO at 10 mg/ml, and further diluted in RPMI 1640 to 0.2 mg/ml. Compounds were then added to individual wells to a final concentration of 20 µg/ml for 24 h, followed by luciferase assay with Steady-Glo Luciferase Assay System (Promega) on L-Max II Luminescence Microplate Reader (Molecular Devices). Cell viabilities were also assessed by adding alamarBlue Reagent (Thermo Fisher Scientific, Waltham, MA) to cell culture to a final concentration of 0.2% 4 h before luciferase assay.

Fluorescence was detected on FLx800 Multi-Detection Microplate Reader (BioTek, Winooski, VT) at the excitation/emission wavelengths of 570 nm and 590 nm, respectively. The relative luciferase activity was determined for each compound after normalization to the cell viability. For selection of positive compounds, Z-score [26] was calculated, which is defined as $Z=\frac{x-??}{??}$, where x is relative luciferase activity of an individual compound, μ is the mean luciferase activity of all test compounds, and σ is the standard deviation of all test compounds in a 96-well plate. A compound with a minimum Z-score of 2.0, meaning that its luciferase activity is two standard deviations above that of the mean, was considered a hit [26].

Secondary screening of the hit compounds

Dose-response experiments were conducted in 96-well plates seeded with HTC/*AvBD9-luc* cells and treated with three different concentrations (5, 20, and 80 μg/ml) of all hits in duplicate for 24 h. Cell viability and luciferase assays were conducted as described above. For those compounds showing a robust dose-dependent response, their HDP-inducing activities were further validated in parental HTC cells (6×10^5/well) at different concentrations in 12-well plates. After 24 h stimulation, cells were subjected to RNA isolation and real-time RT-qPCR as described below.

RNA extraction and RT-qPCR

After stimulation, cells were directly lysed in RNAzol RT (Molecular Research Center, Cincinnati, OH), followed by total RNA extraction. Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) or iSCRIPT RT Supermix (Bio-Rad) was used for cDNA synthesis and qPCR was performed using QuantiTect SYBR Green qPCR Master Mix (Qiagen, Valencia, CA) or iTaq Universal SYBR Green Supermix (Bio-Rad) as described [21, 22, 27]. The expression levels of various chicken HDP genes as well as a house-keeping gene, glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*), were evaluated using gene-specific primers, and relative fold changes in gene expression were calculated using the ΔΔCt method as described [21, 22, 27].

Intestinal explant culture

Chicken jejunal explants were prepared as described [22]. Briefly, an approximately 10-cm jejunal segment was collected from 1- to 2-week-old broiler chickens, washed thoroughly in cold PBS containing 100 μg/ml of gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin, and dissected into a series of small segments (approximately 5 mm × 5 mm). Jejunal segments were then placed individually in 6-well plates containing 4 ml RPMI 1640 medium supplemented with 10% FBS, 20 mM HEPES, 100 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The segments were treated in triplicate with different concentrations of a compound and then incubated in a Hypoxia Chamber (StemCell Technologies, Vancouver, BC, Canada) flushed with 95% O_2 and 5% CO_2 at 37°C for 24 h. Total RNA isolation and RT-qPCR analysis of chicken HDP gene expression were performed with jejunal explants after stimulation.

Oral gavage of HDP-inducing compounds to chickens

A total of 72 newly hatched male broiler chickens were obtained from Cobb-Vantress Hatchery (Siloam Springs, AR), housed on floor cages, divided randomly into groups of 6, and provided *ad libitum* access to a commercial antibiotic-free diet (DuMOR Chick Starter/Grower 20%) and tap water. After 3 days of acclimation, for each treatment group, 12 chickens in two cages were orally gavaged every 12 h for three times with 0.5 ml of PBS alone or PBS containing 5, 10, 20 or 40 μM wortmannin or 40 mM sodium butyrate. After 36 h of initial gavage, all birds were euthanized with carbon dioxide and cervically dislocated. A segment of the mid-duodenum was collected, snap frozen in liquid nitrogen, and stored at -80°C for future homogenization in RNAzol RT and RNA extraction. All animal procedures were approved by the Institutional Animal Care and Use Committee of Oklahoma State University under protocol number AG1610.

Minimum inhibitory concentration (MIC) assay

The MICs of wortmannin and butyrate were determined using a standard broth microdilution assay as recommended by the Clinical and Laboratory Standards Institute [28] as we previously described [29-31]. Briefly, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076) and *Escherichia coli* (ATCC 25922) were streaked onto trypticase soy agar (Fisher Scientific) plates, followed by subculture of 2–3 individual colonies in trypticase soy broth (Fisher Scientific) with shaking at 37◦C for 3 h to reach the mid-log phase. Bacteria were then diluted to 5×10^5 CFU/ml in Mueller Hinton Broth (Fisher Scientific). After dispensing 90 µl/well in 96-well tissue culture plates, 10 µl of wormannin were added in duplicate to final concentrations of 5, 10, 20, 40, 80, 160, 320 µM with or without 4 mM sodium butyrate. MIC was determined as the lowest concentration of the compound or compound combination that gave no visible bacterial growth after overnight incubation at 37°C.

Antimicrobial activity of chicken monocytes

The antibacterial activities of chicken monocytes treated with wortmannin, butyrate or their combination was assessed as described previously [21, 32] with slight modifications. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated venous blood of 1- to 4-week-old male Cobb broilers through gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). Monocytes were obtained by seeding PBMCs at 3×10^7 cells/well in complete RPMI 1640 medium containing 10% FBS, 20mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin in 6-well plates overnight and washing off non-adherent cells twice with calcium- and magnesium-free washed with Hank's Balanced Salt Solution (HyClone, Pittsburgh, PA). Monocytes were replenished with fresh complete RPMI 1640 medium and stimulated in duplicate with 40µM wortmannin in the presence or absence of 4mM butyrate. After 24 h, cells were scraped, washed twice with calcium- and magnesium-free Hank's balanced salt solution,

and resuspended in 100 µl water. Cells were then frozen at −80°C for 20 min, thawed, and sonicated for 30 s, followed by centrifugation at 12,000 × g for 10min at 4°C. Cell supernatants were collected and 20 µl of the supernatants were incubated with 80 µl of *S. enteritidis* (ATCC 13076) at 2.5×10^5 CFU/ml in 20% trypticase soy broth containing 1mM NaH_2PO_4 and 25mM $NaHCO_3$ in a 96-well plate at 37°C. Bacterial turbidity was measured at OD_{600} using SpectraMax M3 (Molecular Devices, Sunnyvale, CA) at 3, 6, 9, and 24 h.

Statistical analysis

Data are expressed as means ± SEM. Statistics was performed with GraphPad Prism (San Diego, CA) using unpaired Student's two-tailed *t*-test. $P < 0.05$ was considered significant.

RESULTS

Selection of appropriate *AvBD9* gene promoter constructs to establish a stable cell line

AvBD9 has been shown to be the most inducible HDP gene in response to butyrate and several other small-molecule compounds in chickens [21, 22]. Therefore, the *AvBD9* gene promoter was chosen to drive luciferase reporter gene expression. In order to select an appropriate promoter segment to provide maximum luciferase activation, eight *AvBD9* promoter constructs of varying lengths were cloned into a luciferase reporter vector, pGL4.21[*luc2P*/Puro] (Promega). The recombinant vectors were separately transfected into chicken HTC macrophage cells and stimulated with 8 mM sodium butyrate for 24 h. Luciferase assay revealed that *AvBD9* promoter activity was clearly length-dependent, with the 2-Kb promotor construct giving a maximum 13-fold increase in luciferase activity relative to a basic promoterless construct (Fig. 1A). Consequently, the 2-Kb *AvBD9* construct was used for subsequent stable cell line development. Besides butyrate, the 2-Kb *AvBD9* construct was also confirmed to respond to two other short-chain fatty acids, namely acetate and propionate, in a dose-dependent manner after transfection into HTC cells (Fig. 1B).

It is noted that omission of either of two promoter regions (120-300 bp or 611-950 bp) upstream of the *AvBD9* start codon resulted in greatly diminished luciferase activity in response to butyrate (Fig. 1A), implying the presence of consensus binding sites for critical transcription factors in these two regions. Conversely, inclusion of a 950-bp segment upstream of the 2998-bp region obviously suppressed luciferase activity (Fig. 1A), suggesting existence of the binding site for a negative regulator. A preliminary scanning for putative transcription factor binding sites in those regions revealed several candidate transcription factors (data not shown), which are currently being experimentally verified.

Establishment of a cell-based HTS assay to identify *AvBD9*-inducing compounds

To establish a stable luciferase reporter cell line driven by the *AvBD9* gene promoter, the 2-Kb *AvBD9* promoter construct that gave the highest fold increase in response to butyrate (Fig. 1A) was cloned into a lentiviral luciferase reporter vector, pGreenFire1-mCMV-Puro (System Biosciences). Pseudoviruses were generated in 293T cells and subsequently used to infect chicken HTC macrophages. After one week of selection in puromycin, a portion of surviving cells were subjected to limiting dilution in 96-well plates. Individual cell clones were gradually expanded, followed by evaluation of their responsiveness to butyrate. Among 27 cell clones analyzed, 1D4 and 1F10 showed the highest fold increase, and both were superior to the mixture of cells prior to limiting dilution (Fig. 2A). These two cell clones were further confirmed to contain the 2-Kb transgene by PCR (data not shown) and gave a similar 300-fold increase in luciferase activity following 24-h stimulation with 8 mM sodium butyrate (Fig. 2B). Therefore, these two stable reporter cell clones, named HTC/*AvBD9-luc,* were used interchangeably and 8 mM butyrate was used as positive control in subsequent HTS assays. To further evaluate the robustness of the HTS assay, we assessed the Z'-factor [24] by measuring luciferase activity of stable cells stimulated with or without 8 mM butyrate in a 96-well plate. Positive controls (8 mM

butyrate) were clearly separated from negative controls (no stimulation) (Fig. 2C), and the Z'-factor was calculated to be 0.80, indicating that the HTS assay is excellent [24].

Identification and validation of natural HDP-inducing compounds

To identify natural small-molecule compounds with the ability to induce *AvBD9*, natural product and rare natural product libraries of 584 compounds were screened at a final concentration of 20 μg/ml in 96-well plates. The Z-scores of all compounds tested were shown in Fig. 3. Using a Z-score of 2.0 as the threshold [26], 21 hits were identified and they represent a structurally diverse group of natural products, with a majority being flavonoids and alkaloids (Table 2). To our surprise, many compounds are involved in epigenetics by regulating histone modification and DNA repair (Table 2). It is noted that none of the compounds had a Z-score of less than -2.0, suggesting that none had a strong activity to suppress *AvBD9* gene expression.

To further validate the *AvBD9*-inducing activity of the 21 hits identified in the primary screening, dose-response experiments were conducted in stable HTC/*AvBD9-luc* cells. When applied at 5, 20, and 80 μg/ml for 24 h, all compounds showed an obvious dose-dependent change in luciferase activity, and at least one concentration of each compound resulted in an increased luciferase activity (Fig. 4A), indicative of the validity of our primary screening. Out of 21 compounds, eight including datiscetin, wortmannin, tetrandrine, trichostatin A, HC toxin, (–)-depudecin, apicidin, and sanguinarine had a higher than 10-fold increase in luciferase activity, and were consequently chosen for further confirmation of mRNA expression in parental HTC cells by RT-qPCR. As expected, all compounds, except for (–)-depudecin, induced *AvBD9* mRNA expression (Fig. 4B), signifying the reliability of the HTS assay in identifying *AvBD9*-inducing compounds. It is currently unknown why (–)-depudecin, a known HDP inducer in human cells [33], purchased from two different vendors including BioVision (Milpitas, CA) and MyBioSource (San Diego, CA) failed to work. It is likely because of a variation in structural

integrity among difference sources as (–)-depudecin is chemically instable due to the presence of two oxirane rings separated by a *trans* double bond [34].

Among the compounds that induced *AvBD9* mRNA expression, wortmannin, tetrandrine, datiscetin, and sanguinarine were the most potent (Fig. 4B). Wortmannin and tetrandrine, when used in the μM range, showed a comparable, if not superior, fold increase to 2 or 4 mM butyrate that gave a maximum 100- to 250-fold *AvBD9* mRNA induction. We further confirmed in a time-course experiment that wortmannin gave a peak induction of *AvBD9* mRNA expression at 24 h (Fig. 4C). It is worth mentioning that higher doses of most compounds showed diminished *AvBD9* induction, suggesting the existence of a negative feedback mechanism.

Ex vivo and *in vivo* confirmation of *AvBD9* induction

To verify the ability of individual compounds to induce *AvBD9* expression in the intestinal tract, chicken jejunal explants were prepared and stimulated with different concentrations of wortmannin, tetrandrine, datiscetin, and sanguinarine for 24 h. All four compounds showed an obvious dose-dependent induction of *AvBD9* in jejunal explants. The optimal dose for wortmannin, tetrandrine, and datiscetin was 20 μM with a 15- to 40-fold induction of *AvBD9* mRNA, while 2 μM sanguinarine gave a peak induction of approximately 2.5-fold (Fig. 5). To further confirm whether wortmannin is capable of inducing *AvBD9 in vivo*, 3-day-old broiler chickens were given 5, 10, 20 or 40 μM wortmannin or 40 mM butyrate by oral gavage every 12 h for 36 h. RT-qPCR analysis of *AvBD9* gene expression in the duodenum revealed that 5 and 10 μM wortmannin increased *AvBD9* mRNA expression by approximately 30- and 50-fold, respectively, which was superior to 40 mM butyrate showing a 10-fold increase (Fig 6).

Augmentation of the Antibacterial Activity of Chicken Monocytes by Wortmannin

HDP inducers such as butyrate and vitamin D3 are capable of enhancing the antibacterial activity of host cells [21, 32, 35-37] and alleviate disease symptoms [13, 38, 39]. To confirm whether

wortmannin or the combination of wortmannin and butyrate can augment the antibacterial activity of host cells, chicken monocytes were isolated and stimulated with 40 μM wortmannin, 4 mM sodium butyrate or their combination for 24 h, followed by incubation of the cell lysate with *S. enteritidis* (ATCC 13076) and measurement of the bacterial turbidity [21, 32]. Consistent with our earlier observation [21], butyrate-treated monocytes exhibited an obviously enhanced ability to suppress bacterial growth (Figure 9). Wortmannin also improved the ability of monocytes to kill bacteria. Importantly, a combination of wortmannin and butyrate resulted in nearly complete suppression of bacterial growth up to 24 h, suggestive of their synergistic activity.

To rule out the possibility that augmented bacterial killing of chicken monocytes is due to direct antibacterial activity of wortmannin or butyrate, a standard broth microdilution assay [28] was performed using two reference bacterial strains, *S. enteritidis* (ATCC 13076) and *E. coli* (ATCC 25922) exposed to wortmannin in serial 2-fold dilutions in the presence or absence of 4 mM butyrate. Wortmannin alone or in combination with 4 mM butyrate showed no obvious antibacterial activity, with the MIC beyond 320 μM, the highest concentration that we tested (data not shown), implying that wortmannin, particularly the wortmannin/butyrate combination, could enhance HDP synthesis and bacterial clearance without exerting selective pressure on bacteria, thus reducing the likelihood of triggering bacterial resistance against HDP inducers.

Induction of multiple chicken HDP genes by natural compounds and their synergy with butyrate

Besides *AvBD9*, 13 other β-defensins and 4 cathelicidins exist in chickens [9, 10] and butyrate can induce more than a half number of them [21]. To examine how other chicken HDP genes are regulated by wortmannin, HTC cells were stimulated with or without three different doses (10, 20, and 40 μM) of wortmannin for 24 h, followed by RT-qPCR of individual β-defensins and cathelicidins. Among those HDP genes that can be detected in HTC cells, all but three were

obviously induced by wortmannin, albeit with a reduced magnitude of induction relative to *AvBD9* (Fig. 7). Clearly, different HDP gene showed different patterns of induction. Wortmannin dose-dependently increased *AvBD2, AvBD13,* and *CATHB1* gene expression, while *AvBD3, 5, 7, 8, 9, 10,* and *12* had a peak induction at 10 or 20 μM (Fig. 7). On the other hand, *AvBD1, 6*, and *14* was dose-dependently suppressed by wortmannin.

Intrigued by the synergy between butyrate with other small-molecule compounds such as vitamin D_3 [40], lactose [41], and forskolin [22], we sought to evaluate a possible synergistic action between butyrate and several newly-identified HDP-inducing compounds. A dramatic synergy was observed between butyrate and any of wortmannin, tetrandrine and datiscetin in HTC cells, but not between butyrate and sanguinarine (Fig. 8A-D). For example, 4 mM butyrate and 40 μM wortmannin individually enhanced *AvBD9* expression by approximately 200- and 250-fold, respectively, while a combination of 4 mM butyrate and 40 μM wortmannin induced *AvBD9* expression by approximately 15,500-fold, which reflected an additional 60-fold increase over either compound alone (Fig. 8A). Similarly, the butyrate/tetrandrine (Fig. 8B) and butyrate/datiscetin combinations (Fig. 8C) also displayed a strong synergy separately. However, no synergy was observed between butyrate and sanguinarine (Fig. 8C), suggesting the mechanisms of action among different compounds are likely to be different.

Involvement of the PI3K/AKT/mTOR pathway in *AvBD9* gene induction

Phosphoinositide 3-kinases (PI3K) are a family of structurally related enzymes that are involved in a variety of cellular functions that often signal through protein kinase B (also known as AKT) and mechanistic target of rapamycin (mTOR) [42, 43]. Wortmannin is a well-known inhibitor of PI3K [44] and brassinin is a newly identified AvBD9-inducing compound (Table 2) also with a reported PI3K inhibitory activity [45]. To examine whether the PI3K/AKT/mTOR pathway is involved in *AvBD9* gene induction, specific inhibitors to PI3K (PX-866, LY294002, and CAL-

101), AKT (MK2206, GDC0068, triciribine) or mTOR (Rapamycin, AZD8055) or dual inhibitors to PI3K/mTOR (BEZ235) or PI3K/HDAC (CUDC-907) were applied to HTC cells separately for 24 h, followed by RT-qPCR analysis of *AvBD9* gene expression. Among all four PI3K inhibitors, only pan-inhibitors, wortmannin and its structural analog PX-866, gave a robust *AvBD9* induction, while another pan-inhibitor (LY294002) and an isoform-specific inhibitor (CAL-101) showed a minimum or no activity (Fig. 9), suggesting that specific inhibition of PI3K may have a limited effect on *AvBD9* induction. The reason that wortmannin and PX-866 work well is likely due to their non-specific activities. Wortmannin is highly efficient in suppressing PI3K in the low nanomolar range, but can non-specifically inhibit several other PI3K-related kinases such as DNA-dependent protein kinase (DNA-PK) at higher concentrations [46]. In this study, the micromolar concentrations of wortmannin are needed to induce chicken HDP genes, and no appreciable HDP gene induction was observed when wortmannin was used below 1 μM (data not shown). Therefore, it is likely that PI3K inhibition alone is insufficient for robust HDP gene induction. In agreement, none of the three AKT inhibitors or mTOR inhibitors had an obvious ability to induce *AvBD9* expression (Fig. 9), suggesting a minimum involvement of the PKA/AKT/mTOR signaling pathway in chicken *AvBD9* induction. Interestingly, dual inhibition of PI3K/mTOR or PI3K/HDACs gave obvious *AvBD9* expression in chicken HTC cells, albeit with a much reduced fold increase relative to wortmannin or PX-866 (Fig. 9).

DISCUSSION

Increased resistance to conventional antibiotics necessitates the development of novel antimicrobial strategies. With no or reduced likelihood of triggering resistance, host-directed antimicrobial therapies are gaining increased attention, with a number of products being approved for human use or evaluated at different stages of clinical trials [1]. Moderating the synthesis of endogenous HDPs has shown promise in reducing infections and alleviating clinical infections and is being actively explored as an alternative approach to antimicrobial therapy [16-18]. A

number of small-molecule compounds such as butyrate and vitamin D_3 have been identified to induce HDP synthesis in humans and other animal species [16-18]. A cell-based HTS assay was recently developed by employing human HT-29 intestinal epithelial cells transfected with a fusion of a 4-Kb human cathelicidin *LL-37* gene promoter and its entire open reading frame with a luciferase reporter gene [47]. Such an approach has led to discovery of a group of compounds with the ability to induce *LL-37* gene expression; however, a majority of the compounds are weak relative to many known LL-37 inducers such as butyrate [47]. To facilitate the identification of additional, perhaps more potent HDP inducers particularly for poultry applications, we developed a HTS assay by fusing a luciferase gene with a 2-Kb *AvBD9* gene promoter, followed by lentiviral transduction into a chicken macrophage cell line. After optimization, we obtained a Z'-factor of 0.80 for our HTS assay, which suggested that it is a robust system [24] and equivalent to the previously reported human HTS assay that had a Z'-factor of approximately 0.70 [47]. By employing such a HTS assay, we identified 21 natural compounds with a strong ability to boost *AvBD9* gene expression after a screening of 584 natural products.

Out of the 21 HDP-inducing compounds identified in this study, only forskolin, trichostatin A (TSA), apicidin, and (–)-depudecin have been reported earlier with the ability to induce HDP gene expression in humans and chickens [22, 33, 48]. The 17 remaining compounds are linked with a role in HDP induction for the first time. To our surprise, 14 out of 21 HDP-inducing compounds are involved in epigenetic modifications of DNA, histones or non-histone proteins (Table 2). TSA, HC toxin, parthenolide, apicidin, and (–)-depudecin are known histone deacetylase (HDAC) inhibitors [49], which contribute to histone hyper-acetylation, chromosomal relaxation, and often enhanced gene expression [50]. Consistently, human HDPs such as LL-37, β-defensin-1, and β-defensin-2 have been found to be upregulated by HDAC inhibitors such as butyrate, TSA, apicidin, sulforaphane, curcumin, MS-275, and resveratrol and its analogs [19]. Although it has not been definitively confirmed, datiscetin, tamarixetin, and robinetin are all

structurally similar to quercetin, a natural flavonol with the ability to regulate the activities of histone acetyltransferases, sirtuins, and classical HDACs [51-53]. Therefore, it is very likely these quercetin-like flavonoids have histone modifying functions as well. Besides histone acetylation, four compounds including sanguinarine, wortmannin, hypocrellin B, and radicicol are known to induce DNA damage, inhibit DNA repair or DNA topoisomerase II activity [54-57], which has been recently revealed to exert a positive role in initiation of gene transcription [58, 59]. Additionally, radicicol, sclerotiorin, and sanguinarine are capable of inhibiting heat shock protein 90 (HSP90) [25, 60, 61], which is known to interact with DNA topoisomerase II [55] and whose activity is regulated by reversible acetylation [62]. Thus, inhibition of DNA repair or HSP90 could positively impact the transcription of a subset of genes perhaps including many HDPs.

Wortmannin, tetrandrine, datiscetin, and sanguinarine are among the four most potent *AvBD9*-inducing compounds identified in this study. Wortmannin is a fungal metabolite and a well-known inhibitor of phosphoinositide 3-kinases (PI3K) [44], which are critically involved in a variety of cellular metabolism and immune functions [43]. However, the PI3K/AKT/mTOR pathway alone appears to play a minimum role in *AvBD9* gene induction because most specific inhibitors to PI3K, AKT and mTOR are largely ineffective in inducing *AvBD9*. The reason that wortmannin is highly efficient is likely due to its dual inhibitory role to both PI3K and DNA-PK [56], with the latter being required to repair double-strand DNA breaks via the non-homologous end joining pathway [63, 64]. Inhibition of both PI3K and DNA repair perhaps creates a synergistic effect on HDP gene induction.

Among the other three potent *AvBD9* inducers, tetrandrine is a *bis*-benzylisoquinoline alkaloid extracted from the root of a Chinese herb, *Stephania tetrandra* S. Moore [65, 66]. Datiscetin is a plant-derived flavonoid and structurally related to quercetin that are known to have epigenetic functions [67] and a strong ability to induce chicken HDP genes (data not shown). Therefore, it is of little surprise that datiscetin is capable of inducing *AvBD9*. Sanguinarine is a

benzophenanthridine alkaloid extracted from the bloodroot plant *Sanguinaria canadensis* [68] that can cause DNA damage [57], which could subsequently lead to an increase in *AvBD9* gene transcription. However, the mechanism by which tetrandrine induces AvBD9 expression remains unknown. Tetrandrine is a well-known calcium channel blocker and has been used as a Chinese traditional medicine for decades to treat hypertensive and arrhythmic conditions, inflammation, fibrosis, and silicosis [65, 66]. Whether tetrandrine augments *AvBD9* expression by acting as a calcium channel blocker warrants further investigations. We observed a strong synergy in *AvBD9* gene induction between butyrate, a well-studied pan-HDAC inhibitor, and any of wortmannin, datiscetin and tetrandrine, but not between butyrate and sanguinarine. The mechanism behind their synergy needs to be further investigated.

Although structurally divergent from each other, a common feature among wortmannin, tetrandrine, and sanguinarine is that they are all anti-inflammatory, antioxidative, anti-proliferative, and pro-apoptotic [44, 65, 66, 68]. So is likely the case with datiscetin because of the anti-inflammatory and antioxidative properties associated with structurally-related quercetin [69]. The ability to enhance HDP gene expression and antioxidative response without triggering inflammation makes these compounds desirable for further development as host-directed therapeutics for disease control and prevention. The fact that some of these compounds have demonstrated synergistic actions among each other suggests the potential of employing these compounds or their combinations as alternatives to antibiotics for poultry applications. Because HDAC inhibitors have been found to promote HDP synthesis across animal species [19], it is likely that a few, if not all, of the newly-identified HDP-inducing compounds are capable of enhancing HDP gene expression and disease resistance beyond chickens.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

GZ and WL conceived and designed the experiments. WL, ZD, LTS, SB, and KR performed the experiments. WL and GZ analyzed and interpreted the data. RM provided the reagents. WL and GZ drafted and revised the manuscript.

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Table 3.1. Primers used in this study[a,b]

Name	Sequence	Size (bp)
***AvBD9* forward primers**		
AvBD9-120-F	TGGCCTAACTGGCCGGTACCGTCCAGACCCACAGCCTTTA	118
AvBD9-300-F	TGGCCTAACTGGCCGGTACCTCTCTGGGTGCAGCCCA	298
AvBD9-399-F	TGGCCTAACTGGCCGGTACCCAACACCATGTCCAAGAGCCAC	397
AvBD9-611-F	TGGCCTAACTGGCCGGTACCAGATATCAAGGACAGGGATGGG	609
AvBD9-950-F	TGGCCTAACTGGCCGGTACCCCTCAAGAGTGGCATTTCTCAG	948
AvBD9-1999-F	TGGCCTAACTGGCCGGTACCGTGGATGCTGTTATTGCCTGGA	1997
AvBD9-2998-F	TGGCCTAACTGGCCGGTACCGAGATCTGCAGGAAAGCAGCT	2996
AvBD9-3948-F	TGGCCTAACTGGCCGGTACCAAACAGGAATTTCCACATGGCAG	3946
AvBD9-1999-Lenti-F	TTTTATCGATGAATTCGTGGATGCTGTTATTGCCTGGA	1997
***AvBD9* reverse primers**		
AvBD9-3-R	CCGGATTGCCAAGCTTTTGTCCTCTGCTGTGGAATAG	
AvBD9-3-Lenti-R	TACACGCCTAACTAGTTTGTCCTCTGCTGTGGAATAG	

Note: [a] Each forward primer consists of a common linker sequence at the 5'-end and a *Kpn*I site in the middle (as underlined) and a gene-specific sequence at the 3'-end, whereas the reverse primer included a different linker sequence at the 5'-end and a *Hind*III site in the middle (as underlined) and a gene-specific sequence at the 3'-end. The exceptions are AvBD9-1999-Lenti-F and AvBD9-3-Lenti-R that are composed of different linker sequences and restriction sites.

[b] The number associated with each primer indicates the upstream position relative to the start codon of the *AvBD9* mRNA reference sequence (GenBank accession no. NM_001001611).

Table 3.2. The Z-scores and major functions of 21 hits at 20 µg/ml from primary screening of the Natural Products Library

Compound Name	CAS Number	Mass (g/mol)	Z score	Structural Family	Major Function[a,b]
Sanguinarine	5578-73-4	332.1	7.92	Benzophenanthridine alkaloid	Inducer of DNA damage
Datiscetin	480-15-9	286.2	7.66	Hydroxylated flavonoid	?
Wortmannin	19545-26-7	428.4	5.86	Steroid	Inhibitor of PI3K/DNA-PK and DNA repair
HC toxin	83209-65-8	436.5	5.55	Cyclic tetrapeptide	HDAC inhibitor
Hypocrellin B	123940-54-5	528.5	5.18	Perylenequinone	Inducer of DNA strand breakage
Parthenolide	20554-84-1	248.3	4.99	Sesquiterpene lactone	HDAC inhibitor
Tetrandrine	518-34-3	622.8	4.88	Bisbenzylisoquinoline alkaloid	Calcium channel blocker
Apicidin	183506-66-3	623.8	4.42	Cyclic tetrapeptide	HDAC inhibitor
(–)-Depudecin	139508-73-9	212.2	3.95	Polyketide	HDAC inhibitor
Isotetrandrine	477-57-6	622.8	3.91	Bisbenzylisoquinoline alkaloid	Calcium channel blocker
Silibinin	22888-70-6	482.4	3.11	Flavonolignan	STAT3, cyclo- and lipoxygenase inhibitor
Sclerotiorin	549-23-5	390.9	3.1	Azaphilone	HSP90 and lipoxygenase inhibitor
Cytochalasin D	22144-77-0	507.6	3.04	Alkaloid	Actin polymerization inhibitor
Trichostatin A	58880-19-6	302.4	2.7	Hydroxamic acid	HDAC inhibitor
Radicicol	12772-57-5	364.8	2.31	Polyketide	HSP90 and DNA topoisomerase II inhibitor
Tamarixetin	603-61-2	316.3	2.29	O-methylated flavonoid	?
Carminic acid	1260-17-9	492.4	2.07	Glucosidal hydroxyanthrapurin	?
Forskolin	66575-29-9	410.5	2.06	Labdane diterpene	Adenylyl cyclase agonist
Dihydroergocristine mesylate	24730-10-7	707.8	2.06	Ergot alkaloid	Serotonin receptor antagonist
Brassinin	105748-59-2	236.4	2.04	Indole phytoalexin	STAT3 and PI3K/Akt/mTOR inhibitor
Robinetin	490-31-3	302.2	2.01	Hydroxylated flavonoid	?

Note: [a] Although there is little information on their biological activities, datiscetin, tamarixetin, and robinetin are expected to have similar epigenetic functions in histone acetylation to structurally related quercetin.

[b] Abbreviations: PI3K, phosphatidylinositide 3-kinases; DNA-PK, DNA-dependent protein kinase; HDAC, histone deacetylase; STAT3, signal transducer and activator of transcription 3; HSP90, heat shock protein 90; AKT, protein kinase B; mTOR, mechanistic target of rapamycin.

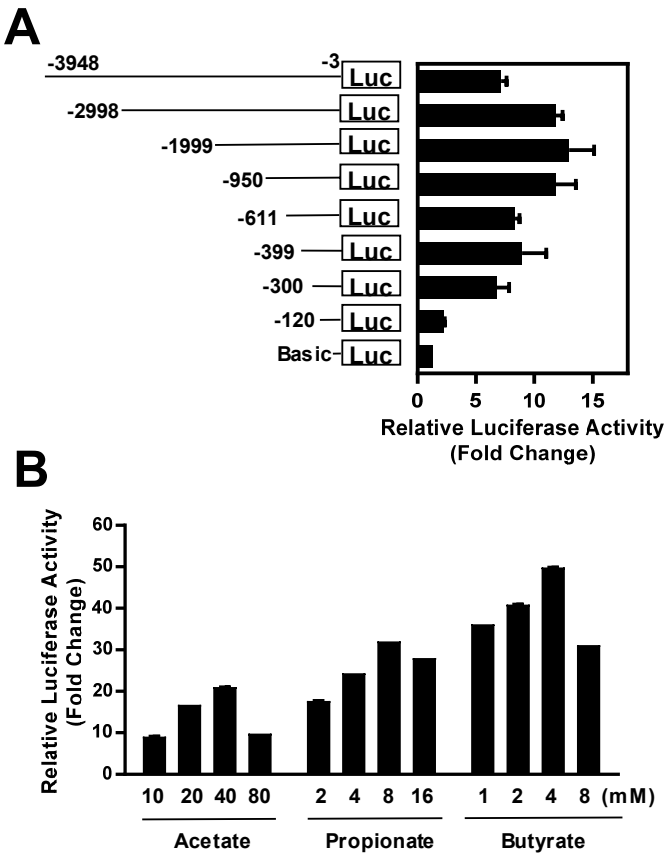

Figure 3.1. Promoter analysis of the *AvBD9* gene. (A) *AvBD9* gene promoter constructs of different lengths were cloned into pGL4.21[*luc2P*/Puro] luciferase reporter vector and transfected into chicken HTC macrophage cells, followed by stimulation with 8 mM sodium butyrate for 24 h. The fold change in luciferase activity of the cells transfected with each *AvBD9* promoter construct in response to butyrate relative to that of the cells transfected with the promoterless basic vector is shown. (B) Chicken HTC cells were transfected with the pGL4.21[*luc2P*/Puro] luciferase reporter vector driven by a 2-Kb *AvBD9* gene promoter construct, followed by stimulation with or without different concentrations of short-chain fatty acids for 24 h. For each short-chain fatty acid, fold change in luciferase activity of stimulated cells was calculated relative to that of the non-stimulation control. The results are means ± SEM of 2-3 independent experiments.

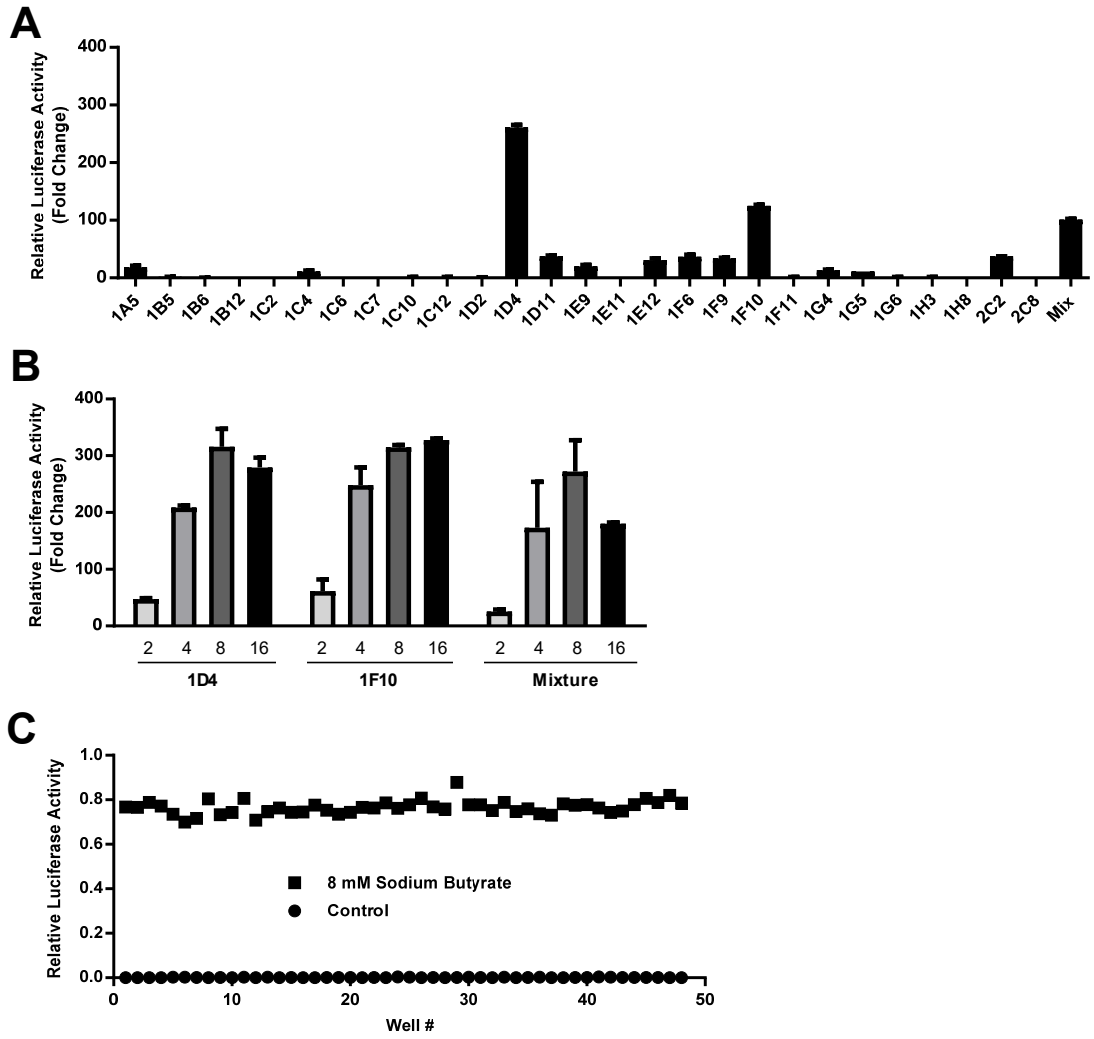

Figure 3.2. Characterization and optimization of stable HTC/*AvBD9-luc* luciferase reporter cells for high-throughput screening. (A) The fold change in luciferase activity of each cell clone in response to 8 mM butyrate relative to that of non-stimulation control. (B) Dose-dependent response of two selected stable cell clones and stable cell mixture to sodium butyrate (mM). The results in Panels A and B are means ± SEM of two independent experiments. (C) Relative luciferase activities of stable reporter cells in the presence or absence of 8 mM sodium butyrate for calculation of the Z'-factor.

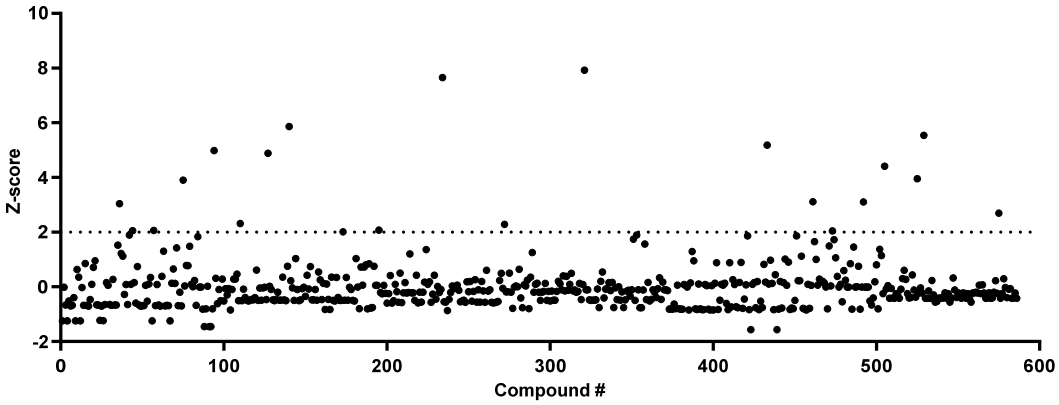

Figure 3.3. Z-scores of 584 natural products following a primary screening. Stable HTC/*AvBD9-luc* luciferase reporter cells were stimulated with 20 μg/ml of natural products for 24 h in 96-well plates, followed by the luciferase assay. The alamarBlue Dye was added 4 h before the luciferase assay to measure cell viability. The luciferase activity of each compound was normalized to cell viability before the Z-score was calculated.

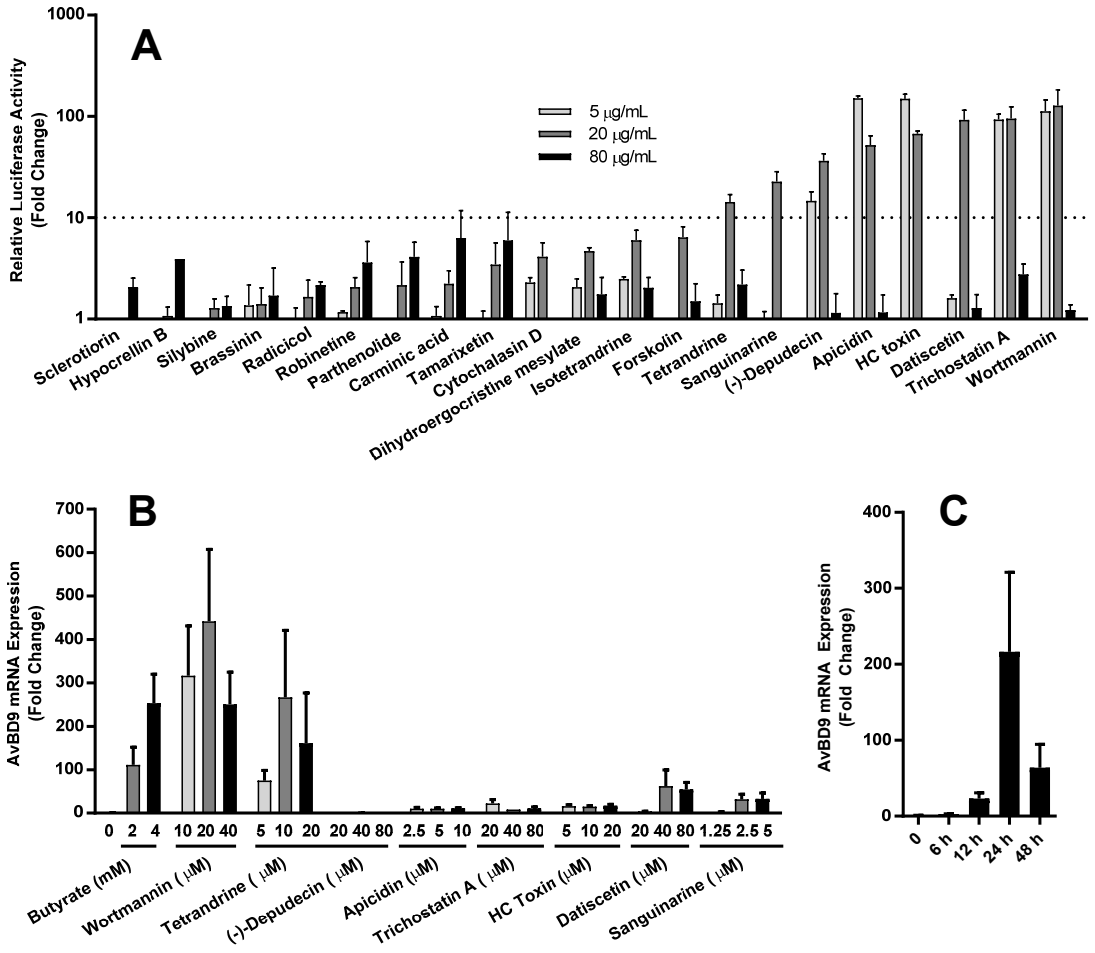

Figure 3.4. Secondary screening of newly identified natural HDP-inducing compounds. (A) Dose-dependent changes in luciferase activity in stable HTC/*AvBD9-luc* luciferase reporter cells in response to 21 hits identified in the primary screening. (B) Dose-dependent induction of *AvBD9* mRNA expression in parental HTC cells stimulated with selected compounds for 24 h by RT-qPCR. (C) Time-dependent changes in *AvBD9* mRNA expression levels in HTC cells treated with 10 μM wortmannin by RT-qPCR. The results are means ± SEM of three independent experiments.

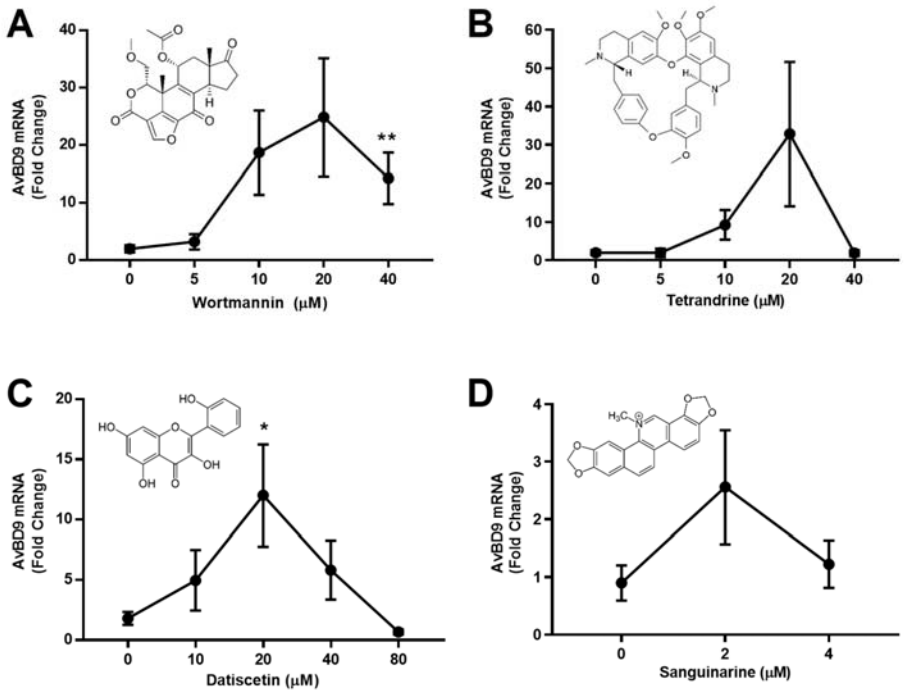

Figure 3.5. Dose-dependent induction of *AvBD9* mRNA expression in response to wortmannin, tetrandrine, datiscetin, and sanguinarine in chicken jejunal explants. Jejunal explants were prepared and treated with different concentrations of each compound for 24 h, followed by RT-qPCR analysis of *AvBD9* mRNA expression. The results are means ± SEM of 2-3 independent experiments. **P* < 0.05 and ***P* < 0.01 (by unpaired Student's *t*-test).

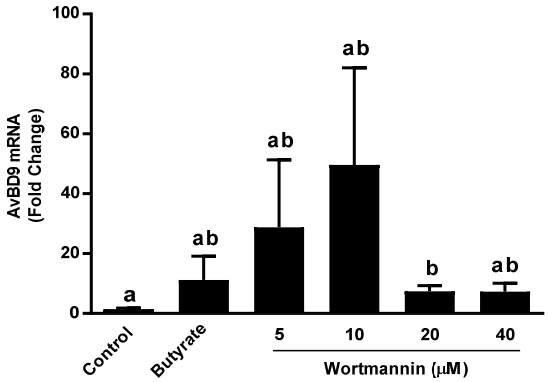

Figure 3.6. *In vivo* induction of *AvBD9* mRNA expression in the duodenum of chickens by wortmannin. Different concentrations of wortmannin or 40 mM sodium butyrate were administered to 3-day-old chickens (n = 12) by oral gavage every 12 h for 36 h. A segment of the mid-duodenum was collected and subjected to total RNA isolation and RT-qPCR analysis of *AvBD9* mRNA expression. Fold changes were calculated relative to the control chickens receiving an equal volume of saline three times. The bars without common superscript letters denote statistical significance (by unpaired Student's *t*-test).

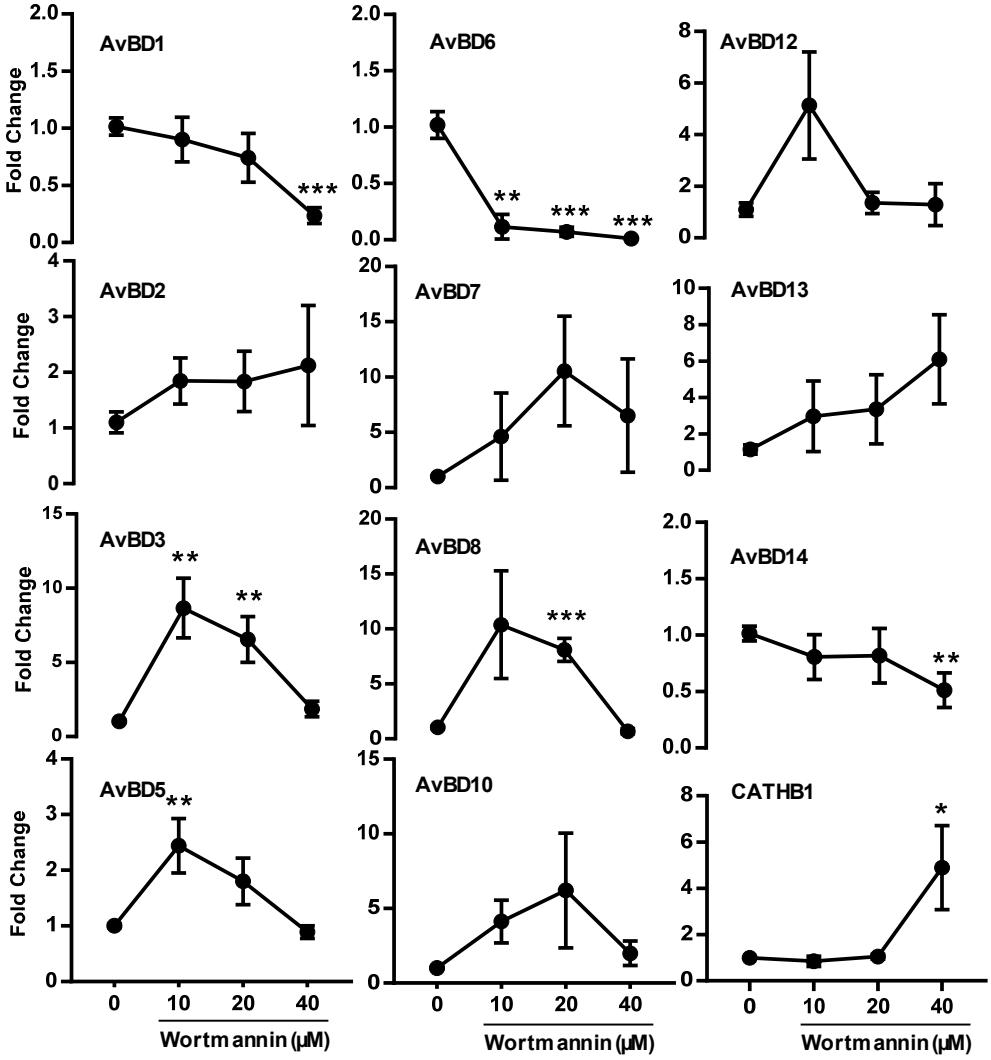

Figure 3.7. Dose-dependent changes in multiple chicken HDP mRNA expression levels in HTC cells by wortmannin. Chicken HTC cells were treated with or without three different concentrations of each compound for 24 h, followed by RT-qPCR analysis of mRNA expression of all chicken HDP genes that can be detected in HTC cells. The results are means ± SEM of three independent experiments. *$P < 0.05$ and **$P < 0.01$ (by unpaired Student's *t*-test).

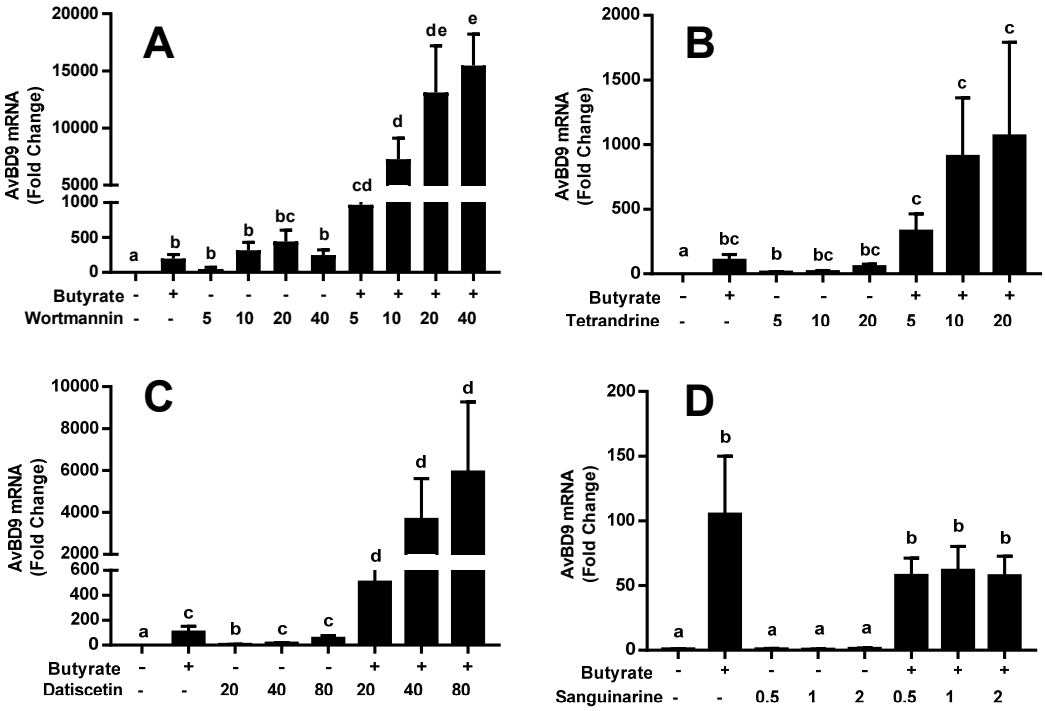

Figure 3.8. Synergistic induction of *AvBD9* mRNA expression in HTC cells between butyrate and three newly identified natural products. Chicken HTC cells were treated with 4 mM butyrate in the presence or absence of different concentrations of wortmannin (A), tetrandrine (B), datiscetin (C) or sanguinarine (D) for 24 h, followed by RT-qPCR analysis of *AvBD9* mRNA expression. The results are means ± SEM of 2-3 independent experiments. The bars without common superscript letters denote statistical significance (by unpaired Student's *t*-test). It is noted that an obvious synergy in AvBD9 gene expression was observed between butyrate and any of wortmannin, tetrandrine, datiscetin, but not between butyrate and sanguinarine.

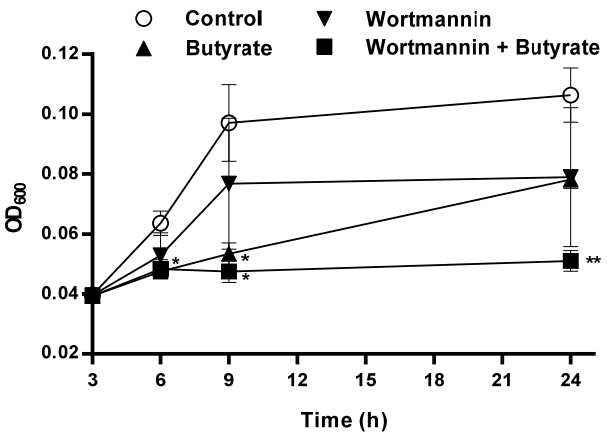

Figure 3.9. Augmentation of the antibacterial activity of chicken monocytes by wortmannin and butyrate. Chicken monocytes were stimulated with 40 μM wortmannin, 4 mM butyrate or their combination for 24 h. Cell lysates were then incubated with *Salmonella* enteritidis (ATCC 13076) at 37◦C. The bacterial turbidity was measured at OD600 at 3, 6, 9, and 24 h. The results are means ± SEM of two independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (relative to the unstimulated control).

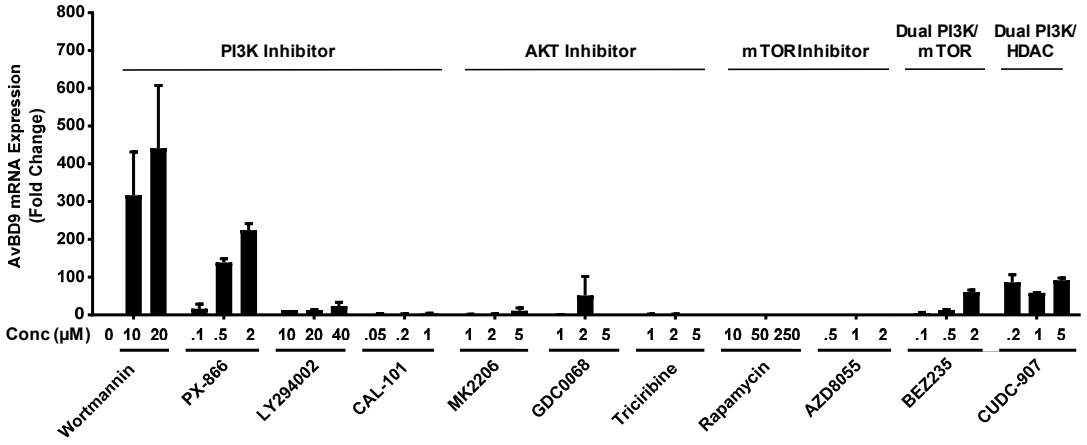

Figure 3.10. Involvement of the PI3K/AKT/mTOR signaling pathway in *AvBD9* mRNA induction. Chicken HTC cells were treated with or without different concentrations of specific inhibitors to PI3K (Wortmannin, PX-866, LY294002, and CAL-101), AKT (MK2206, GDC0068, triciribine) or mTOR (Rapamycin, AZD8055), dual PI3K/mTOR inhibitor (BEZ235) or dual PI3K/HDAC inhibitor (CUDC-907) for 24 h, followed by RT-qPCR analysis of *AvBD9* gene expression. The results are means ± SEM of 2-3 independent experiments.

CHAPTER IV

LARGE-SCALE IDENTIFICATION OF MULTIPLE CLASSES OF HOST DEFENSE PEPTIDE-INDUCING COMPOUNDS FOR ANTIMICROBIAL THERAPY

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ABSTRACT

Rapid emergence of antibiotic resistance demands new antimicrobial strategies that are less likely to develop resistance. Augmenting the synthesis of endogenous host defense peptides (HDPs) has proved to be an effective host-directed therapeutic approach. This study is aimed at identifying small-molecule compounds with a strong ability to induce endogenous HDP synthesis for further development as novel antimicrobial agents. By employing a stable chicken HDP promoter-driven luciferase reporter cell line known as HTC/*AvBD9-luc*, we performed a high throughput screening of 5,002 natural and synthetic compounds and identified 110 hits with a minimum Z-score of 2.0. Although they are structurally and functionally diverse, a half number of these hits are inhibitors of class I histone deacetylases, the PI3K/AKT/mTOR pathway, ion channels, and dopamine and serotonin receptors. Follow-up validation studies led to the identification of mocetinostat, a benzamide histone deacetyalse inhibitor, to be highly potent in enhancing HDP mRNA expression in chicken macrophage cell lines and jejunal explants. In addition to multiple HDP genes, mocetinostat was also capable of promoting the expression of several representative barrier function genes such as tight junction protein-1, claudin-1 and mucin-2 in chicken cells. Importantly, mocetinostat was more efficient than two of its structural analogs, MS-275 and chidamide, in inducing HDP gene expression and augmenting the antibacterial activity of chicken macrophage cells, albeit with no direct antibacterial activity up to 320 μM. Taken together, mocetinostat, with the ability to enhance HDP synthesis, barrier function, and the antibacterial activity of host cells, could be potentially developed as a novel antimicrobial in disease control and prevention for poultry and possibly other species as well.

Keywords: host defense peptides, high throughput screening, host defense peptide inducers, mocetinostat, antimicrobial resistance

INTRODUCTION

Antimicrobial resistance has become a major healthcare concerns worldwide [1]. Rapid emergence of antibiotic-resistant pathogens, coupled with a dwindling antibiotic pipeline, demands innovative antimicrobial strategies that are less likely to trigger resistance [2]. Host-directed immunotherapies have emerged as promising alternative approaches to disease control and prevention with a minimum risk of developing microbial resistance [3]. Host defense peptides (HDPs), also known as antimicrobial peptides, constitute a large diverse group of small molecules that act as an important component of innate immunity [4-6]. In vertebrate animals, HDPs are classified into two major families, namely cathelicidins and defensins, that are expressed mainly by phagocytic cells and mucosal epithelial cells [7]. A large array of HDPs are produced in each animal species to provide the first line of host defense in response to infection and inflammation [4-6]. For example, humans have one cathelicidin known as LL-37, six α-defensins, and more than 30 β-defensins [8, 9], while four cathelicidins (CATH1-3 and CATH-B1) and 14 β-defensins known as AvBD1-14 are encoded in the chicken genome [10, 11]. HDPs are capable of killing a broad spectrum of pathogens through membrane-lytic mechanisms [12, 13] and, at the same time, exert a profound influence on the regulation of both innate and adaptive immunity by recruiting and promoting differentiation and activation of different types of immune cells [10].

Augmenting the synthesis of endogenous HDPs has become an active host-directed approach to antimicrobial therapy, showing promise in conferring the host an enhanced ability to cope with infections and alleviate pathologies [10, 14]. Besides infectious and inflammatory agents, scores of small-molecule compounds such as butyrate and vitamin D_3 have been identified with the ability to induce HDP synthesis in humans and other animals [15]. Butyrate, a short-chain fatty acid, works primarily by acting as a histone deacetylase (HDAC) inhibitor to cause

hyperacetylation and relaxation of chromatin to allow gene transcription [16], while vitamin D_3 works by binding to vitamin D receptor [17].

A high throughput screening (HTS) assay based on a stable *LL-37* promoter-driven luciferase reporter cell line (MN8CampLuc), has been developed and led to the identification of multiple LL-37 inducers [18, 19]. We have also established a similar luciferase reporter cell line through stable integration of a 2-kb *AvBD9* promoter-driven luciferase reporter gene in chicken HTC macrophages [20]. Such a reporter cell line, termed HTC/*AvBD9-luc*, was subsequently employed to screen 584 natural products and identified scores of HDP-inducing compounds [20]. Here we report the screening of additional 5,002 natural and synthetic small-molecule compounds with much greater structural and functional diversities using the newly-established cell-based HTS assay. We revealed that nine out of the 10 most efficacious HDP inducers are HDAC inhibitors. Further characterization of these compounds may pave the way for their development as novel host-directed immune boosting antimicrobials.

MATERIALS AND METHODS

Chemicals and reagents

Cell culture reagents including RPMI 1640 and antibiotics (penicillin, streptomycin, puromycin, and gentamycin) were purchased from Lonza (Allendale, NJ), Santa Cruz Biotechnology (Dallas, TX) or Fisher Scientific (Pittsburgh, PA). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Flowery Branch, GA). Sodium butyrate were procured from Sigma-Aldrich (St. Louis, MO), while mocetinostat, MS-275, and chidamide were obtained from Cayman Chemical (Ann Arbor, MI).

Cell culture

Chicken HTC [21] and HD11 [22] macrophage cell lines were kind gifts from Dr. Narayan C. Rath and Dr. Hyun S. Lillehoj at USDA-Agricultural Research Service (ARS), respectively. Both cells were cultured in complete RPMI 1640 containing 10% heat-activated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. A stable luciferase reporter cell line, HTC/*AvBD9-luc*, was established and reported earlier through permanent lentiviral integration of the HTC cells with a firefly luciferase gene driven by a 2-kb promoter of a chicken HDP gene known as avian β-defensin 9 (*AvBD9*) [20]. HTC/*AvBD9-luc* cells were maintained in complete RPMI 1640 supplemented with 0.5 μg/ml puromycin. All cells were incubated at 37°C and 5% CO_2 and subcultured every 3-4 days.

High throughput screening for HDP-inducing compounds

HTS was conducted in the High Throughput Screening Facility at Vanderbilt University (Nashville, TN). A total of 5,002 small-molecule compounds from MicroSource Spectrum Collection of biologically and structurally diverse compounds (2,399), NIH Clinical Collections I and II of approved and experimental drugs used in human clinical trials (618), NCI Focused Natural Product Collection (819), Cayman Bioactive Lipid I Screening Library (823), Marnett Collection of NSAID derivatives (212), Enzo Screen-Well™ Kinase Inhibitor Library (80), and Selleck Chemicals Epigenetics Compound Library (51) were included in the screening (Fig. 1A). All compounds were dissolved in DMSO to 10 mM and used in HTS at the final concentration of 20 μM. For primary screening, HTC/*AvBD9-luc* cells were plated at 2×10^4 cells/well in 384-well white tissue culture plates in 20 μl of complete RPMI 1640 overnight, followed by stimulation with 20 μM of each test compound for 24 h as we previously described [20]. Cells that were treated with 4 mM sodium butyrate or left untreated were used as positive and negative controls, respectively. Cell viability was measured with alamarBlue™ Reagent (Thermo Fisher Scientific, Waltham, MA) 4 h before luciferase assay, which was performed using the Steady-Glo Luciferase Assay System (Promega). The relative luciferase activity of each compound was

determined after normalization to the cell viability. To evaluate the robustness of the HTS, Z'-factor [23] was calculated for each plate based on the relative luciferase activities of 12 positive and 12 negative controls. For hit selection, Z-score [24] was calculated for each test compound and a minimum Z-score of 2.0 was considered as a hit.

Validation of hit compounds

The hits were first confirmed for their relative HDP-inducing potency in HTC/*AvBD9-luc* cells. HTC/*AvBD9-luc* cells were seeded in duplicate in 96-well plates at 4×10^4 cells/well overnight and then treated with each hit compound at three different concentrations (5, 10, and 20 μM) for 24 h, followed by cell viability and luciferase assays. The relative luciferase activity of each compound was determined after normalization to the cell viability. For those with a minimum 20-fold increase in the relative luciferase activity over the unstimulated controls, the compounds were further validated for their ability to induce HDP mRNA expressions in parental HTC cells. HTC cells were stimulated in duplicate at 5, 10 and 20 μM in 12-well plates for 24 h and then subjected to RNA isolation. HDP mRNA expression levels were evaluated using quantitative RT-PCR (RT-qPCR) as described below.

RNA isolation and RT-qPCR

RNAzol RT (Molecular Research Center, Cincinnati, OH) was used for cell lysis and total RNA isolation. Reverse transcription of total RNA and qPCR were conducted using iSCRIPT RT Supermix (Bio-Rad, Hercules, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad), respectively, as previously described [16, 25, 26]. The mRNA expression levels of different chicken HDPs were evaluated using gene-specific primers with glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*) as the reference gene as described previously [16, 25, 26]. The relative fold changes in mRNA gene expression were calculated by using the ΔΔCt method.

Chicken intestinal explant culture

A 10-cm segment of the jejunum was collected from 1- to 2-week-old broiler chickens, and jejunal explants were prepared as previously described [25]. In brief, after thorough washes of a jejunal segment in cold PBS supplemented with 100 μg/ml of gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin, smaller segments (approximately 5 mm × 5 mm) were prepared and placed individually in 12-well plates containing 2 ml RPMI 1640 medium containing 10% FBS, 20 mM HEPES, 100 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin, followed by an addition of each compound in triplicate at different concentrations. The explants were incubated at 37°C for 24 h in a Hypoxia Chamber (StemCell Technologies, Vancouver, BC, Canada) flushed with 95% O_2 and 5% CO_2. Total RNA isolation and RT-qPCR analysis of chicken HDP gene expressions were performed with jejunal explants after stimulation.

Antibacterial assay of chicken HTC macrophages

The influence of HDP-inducing compounds on the antibacterial activity of chicken HTC cells was evaluate as previously described [16, 31]. In brief, HTC cells were seeded at 1×10^6 cells/well in 6-well plates in complete RPMI1640 medium. After overnight incubation at 37°C and 5% CO_2, cells were stimulated with 10 or 20 μM selected compounds for 24 h, followed by cell lysis and incubation of cell lysate with *Escherichia coli* (ATCC 25922) and *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076) at 2.5×10^5 CFU/ml in 20% trypticase soy broth containing 1 mM NaH_2PO_4 and 25 mM $NaHCO_3$ in a 96-well plate at 37°C. Bacterial growth was monitored at OD_{600} on SpectraMax M3 (Molecular Devices, Sunnyvale, CA) at 37ºC for 3, 6, 9, and 24 h.

Minimum inhibitory concentration (MIC) assay

A standard broth microdilution assay was used to evaluate the MICs of selected compounds in accordance with the recommendation of the Clinical and Laboratory Standards Institute [27] as preciously described [20, 28-30]. Briefly, *E. coli* (ATCC 26922) and *Salmonella* Enteritidis

(ATCC 13076) were streaked onto trypticase soy agar plates. After overnight incubation at 37°C, 2-3 individual colonies were picked and grown in trypticase soy broth (Fisher Scientific) at 37°C for 3 h, bacteria were diluted in Mueller Hinton Broth (Fisher Scientific) to 5×10^5 CFU/ml, followed by an addition of 90 μl/well in a 96-well tissue culture plate. Serially diluted compounds (10 μl) were added to each well in duplicate to final concentrations of 5, 10, 20, 40, 80, 160, and 320 μM. The MIC was defined as the lowest concentration of a compound that gave no visible bacterial growth at 37°C for 24 h.

Statistical analysis

Data are expressed as means ± SEM. GraphPad Prism (San Diego, CA) was used to conduct unpaired Student's two-tailed *t*-test between two groups or one-way ANOVA for more than two groups. Dunnett's multiple comparisons test was performed after ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

HTS for small-molecule compounds that induce HDP synthesis

We have developed a cell-based HTS assay using a stable *AvBD9* promoter-driven luciferase reporter cell line known as HTC/*AvBD9-luc*, which has led to the identification of scores of natural products with the ability to enhance HDP synthesis [20]. To discover additional HDP-inducing compounds, we employed the HTC/*AvBD9-luc* reporter cell line and conducted a larger scale screening of 5,002 natural and synthetic small-molecule compounds in the HTS Facility at Vanderbilt University (Fig. 1A). The average Z' factor [23] across 384-well plates was 0.52 ± 0.03, indicating that the HTS assay was robust and reliable. Based on our preliminary screening and an earlier experience [20], the final concentration of 20 μM was used for each test compound in HTS. Using the Z-score of 2.0 as the threshold [24], we identified 110 compounds, resulting in a hit rate of 2.20% (Fig. 1B). These 110 hits were largely scattered across libraries, with a larger

percentage being found in the epigenetic compound library (Fig. 1B), consistent with our earlier results that many epigenetic compounds and HDAC inhibitors in particular are HDP inducers [16, 17, 25].

All hits were further compared for their relative *AvDB9*-inducing activity in HTC/*AvBD9-luc* cells at 5, 10, and 20 μM using luciferase assay. Obviously, most showed increased luciferase activities in at least one concentration used (Supplemental Table S1). Among 18 compounds with a minimum 5-fold increase in luciferase activity at 20 μM (Table 1), each induced luciferase activity in an apparent dose-dependent manner. A half number of these hits were known HDAC inhibitors, while three others were involved in the PI3K/AKT/mTOR pathway, which was observed in an earlier study as exemplified by wortmannin [20]. The remaining included maprotiline hydrochloride, desloratadine, doxorubicin, tetrandrine, quinacrine, and promazine (Table 1), which are norepinephrine reuptake inhibitor, H_1-antihistamine, topoisomerase II inhibitor, calcium channel blocker, NF-κB inhibitor/p53 activator/ histamine N-methyltransferase inhibitor, and D_2 dopamine receptor inhibitor, respectively [32-38].

Top 10 compounds with a minimum 20-fold increase in luciferase activity at 20 μM were further selected to compare their relative potency in inducing *AvBD9* mRNA expression in HTC cells by RT-qPCR. As expected, all 10 compounds obviously induced *AvBD9* expression in a dose-dependent manner, and all but triciribine achieved a similar efficacy to butyrate (Fig. 2). Among them, mocetinostat and CUDC-907 were the most potent, triggering nearly a 1,000-fold *AvBD9* induction even at 5 μM, while sodium butyrate gave only 125-fold increase at the optimal concentration of 4 mM. On the other hand, mocetinostat at 20 μM enhanced *AvBD9* mRNA expression by more than 5000-fold (Fig. 2).

Confirmation of the *AvBD9*-inducing capacity of mocetinostat in chicken macrophages

Mocetinostat, also known as MGCD0103, is a benzamide HDAC inhibitor (Fig. 3A) undergoing clinical trials for various forms of cancers [39, 40]. Mocetinostat was the most potent HDP-inducing compound in our assays up to now, and therefore, became the focus of subsequent characterizations. Mocetinostat was first evaluated for its ability to promote *AvBD9* gene expression in a different cell line. It showed an obvious dose-dependent *AvBD9* induction in another chicken macrophage cell line, HD11 [22]. In fact, HD11 cells appeared to be more sensitive to mocetinostat than HTC cells. At each concentration used, a higher magnitude of *AvBD9* gene induction was seen in HD11 cells than in HTC cells (Fig. 3B). To investigate the kinetics of gene induction, a time-course experiment was conducted in HTC cells in response to 2 μM mocetinostat. An apparent 100-fold increase in *AvBD9* expression was observed as early as 6 h with the peak induction occurring around and beyond 24 h (Fig. 3B).

Induction of multiple HDP and barrier function genes by mocetinostat

To verify how other HDPs are regulated by mocetinostat, RT-qPCR was used to analyze the expression levels of multiple chicken HDP mRNAs in HTC cells in response to mocetinostat. Mocetinostat apparently enhanced the expression of all HDP genes that are expressed in HTC cells in a dose-dependent manner, although varying in the magnitude of induction (Fig. 4). For example, similar to AvBD9, *AvBD4* and *AvBD10* were upregulated by 20 μM mocetinostat by more than 1,000-fold, while *AvBD1, AvBD3*, *AvBD7*, *AvBD8*, and *AvBD14* were increased by at least 100-fold. However, the remaining HDP genes showed a peak induction of no than 50-fold in response to mocetinostat.

Several HDP inducers such as butyrate and vitamin D_3 have barrier protective properties [41]. To examine whether mocetinostat is capable of improving barrier function, three representative tight junction protein and mucin genes including tight junction protein-1 (*TJP1*), claudin-1 (*CLDN1*), and mucin-2 (*MUC2*) were analyzed following a 24-h stimulation of HTC cells with 5, 10, and 20

μM mocetinostat. Desirably, mocetinostat dose-dependently induced all three barrier function genes, showing a peak increase of *TJP1*, *CLDN1* and *MUC2* between 10- and 20-fold (Fig. 4).

Comparison of the HDP-inducing efficacy among mocetinostat, MS-275, and chidamide

MS-275, also known as SNDX-275 or entinostat, was recently identified among the most potent compounds to stimulate human *LL-37* synthesis [14, 19, 42]. MS-275 is also a benzamide HDAC inhibitor and a structural analog of mocetinostat (Fig. 3A). Chidamide is another structural analog of mocetinostat (Fig. 3A) and was found to be more potent than entinostat in porcine HDP induction in our recent HTS [43]. Mocetinostat, MS-275, and chidamide were compared directly for their relative HDP-inducing efficacy in chicken HTC cells and jejunal explants. Apparently, mocetinostat was much more potent in upregulating *AvBD9* mRNA expression in both HTC cells and jejunal explants than MS-275 and chidamide (Fig. 5). For example, 20 μM mocetinostat triggered approximately 20,000- and 200-fold induction of *AvBD9* mRNA in HTC cells and jejunal explants, respectively, whereas chidamide caused approximately 800-fold *AvBD9* increase in HTC cells and 25-fold increase in jejunal explants. On the other hand, 20 μM MS-275 only gave less than 20-fold induction in both cell types (Fig. 5).

Mocetinostat augments the antibacterial activity of chicken macrophages without directly killing bacteria

To evaluate whether mocetinostat is capable of boosting the antibacterial activity of host cells through upregulation of HDP synthesis, HTC cells were treated with 4 mM sodium butyrate or 10 μM of mocetinostat, chidamide, and MS-275 for 24 h, followed by cell lysis and incubation of cell lysate with *E. coli* (ATCC 26922) and *Salmonella* Enteritidis (ATCC 13076) for various lengths of time. Consistent with our previous studies [16, 43], butyrate enhanced the ability of HTC cells to kill both bacteria. Mocetinostat at 10 μM also significantly augmented bacterial

killing at 6, 9, and 24 h, while MS-275 failed to show a significant antibacterial activity at any time point and chidamide showed an intermediate activity (Fig. 6).

To ensure augmentation of the antibacterial activity of host cells is not due to direct antibacterial activity of mocetinostat, MS-275, and chidamide, we examined their MICs using a standard broth microdilution assay [27] with *E. coli* (ATCC 26922) and *Salmonella* Enteritidis (ATCC 13076). MICs of all three compounds were beyond 320 μM, the highest concentration tested, suggesting that they have no obvious antibacterial activity. It is, therefore, likely that HDP inducers such as mocetinostat boost the antibacterial capacity of host cells through modulation of endogenous HDP synthesis.

DISCUSSION

A number of small molecules have been found to be capable of inducing HDP synthesis without provoking inflammation [15, 44-46]. Moreover, HDP inducers are not expected to trigger resistance because they regulate host immunity with no direct interactions with bacteria. Modulating the synthesis of endogenous HDPs is, therefore, being actively explored as an alternative host-directed antimicrobial approach [15, 19]. We have developed a cell-based HTS assay to screen a natural products library of 584 compounds and identified a number of compounds with potential for further development as novel antimicrobials [20]. In this study, we broadened our screening effort to a list of 5,002 small-molecule compounds with much greater structural and functional diversities. From primary screening, we identified 110 hits with a minimum Z-score of 2.0. Approximately, a half number of the compounds function to inhibit HDACs, the PI3K/AKT/mTOR pathway, dopamine and serotonin receptors or calcium/sodium channels, with the HDAC and PI3K/AKT/mTOR blockers being among the most efficacious HDP inducers. The other half number of compounds are involved in a variety of other functions. Top 10 hits that gave a minimum 20-fold increase in luciferase activity in HTC/*AvBD9-luc* cells

at 20 μM were further confirmed to induce *AvBD9* mRNA expression in a dose-dependent manner in chicken HTC cells, suggestive of the validity and effectiveness of our HTS assay.

Histone acetylation is regulated by the opposing effects of HDACs and histone acetyltransferases (HATs), with the former functioning to remove the acetyl groups from the lysine residues of histones and the latter to add the acetyl group to histones [48, 49]. The balancing act of HDACs and HATs are to fine-tune chromatin structure, the accessibility of transcriptional factors to their binding sites, and subsequent gene transcription [50]. HDAC inhibitors works by tipping the HDAC/HAT balance, leading to relaxation of chromatin structure and enhanced gene transcription [51]. Modifying the acetylation status of the promoter has been shown to have a profound impact on the transcription of HDP genes in humans, rats, rabbits, cattle, pigs, and chickens [15]. Therefore, it is perhaps not surprising to see that nine out of 10 most efficacious HDP inducers are HDAC inhibitors, although two (CUDC-907 and CUDC-101) are known to be involved in other functions as well. These results are in line with our early screening, where five out of eight top HDP inducers are HDAC inhibitors in a library of 584 natural products [20]. A number of HDAC inhibitors have indeed been shown to be potent HDP inducers [46, 47].

The PI3K/AKT/mTOR pathway is critically involved in cell growth and metabolism [52]. Several inhibitors of this pathway such as wortmannin and CUDC-907 were shown to be potent in HDP induction in this study, which is consistent with our earlier HTS effort, in which wortmannin was identified as a top hit [20]. However, several other specific inhibitors to PI3K, AKT or mTOR were assessed and only gave a marginal effect on HDP induction in chicken HTC cells [20]. Additionally, butyrate, a well-known HDAC inhibitor and HDP inducer, was shown recently to enhance mTOR phosphorylation, and knockdown of mTOR significantly reduced butyrate-mediated β-defensin gene expression in mouse intestinal epithelial cells [53]. These lines of apparently conflicting evidence suggested that more studies are needed to implicate the PI3K/AKT/mTOR pathway in HDP synthesis.

Several calcium channel blockers and serotonin receptor antagonists were identified as HDP inducers in this study. Consistently, tetrandrine and isotetrandrine, two calcium channel blockers, and dihydroergocristine mesylate, a serotonin receptor antagonist, were also identified in our previous screening as top hits [20]. However, the mechanism by which these two classes of compounds induce HDP gene expression is currently unknown. So is the case with the role of dopamine receptor antagonists in HDP induction.

Although a majority of the hits obtained in the primary screening gave a dose-dependent increase in the follow-up luciferase assay, luciferase activities of approximately a half number of the compounds in the secondary screening were not necessarily correlated well with their Z-score in the primary HTS assay. More than a dozen hits failed to show an obvious increase in luciferase activity in the follow-up experiments. The reason for such a discrepancy is unclear, but could be due to the fact that many of the compounds used in the secondary screening were obtained from different batches of the libraries or procured from different vendors.

Mocetinostat has been identified as the most potent HDP inducer among all hits identified in this study. It is also known as MGCD0103 and inhibits preferentially HDAC1-3 and HDAC11 with a negligible effect on other HDACs [54]. Mocetinostat causes hyperacetylation of histones and induces apoptosis in cancerous cells and is currently in clinical trials for several different types of cancer [39, 40]. We have shown that mocetinostat potently induces the expression of multiple HDP genes in different cell types and is more potent than two of its structural and functional analogs, MS-275 and chidamide [55-57], in enhancing HDP synthesis and the antibacterial activity of host cells. MS-275 was identified earlier as a highly potent HDP inducer in humans [14, 18, 43], and chidamide was recently discovered to be more potent in HDP induction than MS-275 in porcine cells [43]. In this study, we revealed that mocetinostat is much more potent than both MS-275 and chidamide in chickens, which is consistent with their relative efficacy in inhibiting class I HDACs [58]. However, it will be important to evaluate them side-by-side in

human and porcine cells to see whether the results in chickens can be extended to other animal species or whether it is only a species-specific observation. It is perhaps the presence of an additional aromatic ring that confers mocetinostat an improved efficacy in HDP induction.

Collectively, we have identified multiple classes of HDP inducers and further characterizations of these compounds have led to the identification of mocetinostat being the most potent. Coupled with its ability to induce barrier function genes without killing bacteria directly, mocetinostat shows promise to be developed as a novel antimicrobial agent for disease control and prevention with a minimum risk of triggering resistance.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS

GZ, WL, and PV conceived and designed the experiments; WL and DM performed the experiments; WL and GZ analyzed, interpreted the data, drafted, and revised the manuscript.

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Table 4.1. Functional properties, Z-scores, and fold increases in relative luciferase activity of top 18 hits

Compound Name	Major Function[1]	Z-Score	Fold Increase		
			5 µM	10 µM	20 µM
CUDC-907	HDACi/PI3Ki	9.97	137.04	482.67	264.88
Triciribine	AKTi	3.25	37.36	77.73	156.08
Mocetinostat	HDACi	3.45	21.36	41.72	59.69
Scriptaid	HDACi	4.33	6.17	25.71	50.42
ITF2357 (Givinostat)	HDACi	5.68	52.38	113.64	46.13
PCI-24781	HDACi	2.85	77.51	82.80	37.43
CUDC-101	HDACi/EGFRi/HER2i	2.54	4.69	12.52	34.17
Belinostat (PXD101)	HDACi	6.58	23.05	41.44	33.96
Trichostatin A	HDACi	17.00	68.08	47.64	27.62
AR-42	HDACi	2.27	107.71	80.82	25.88
Vorinostat (SAHA)	HDACi	3.35	1.59	3.52	14.53
Wortmannin	PI3Ki	2.38	29.61	33.69	12.09
Maprotiline hydrochloride	NRi	5.61	1.53	3.85	7.01
Desloratadine	HH1Ri	3.00	1.73	2.47	6.83
Doxorubicin	TOPIIi	5.64	7.89	101.89	6.56
Tetrandrine	CaChi	2.17	1.22	1.97	6.42
Quinacrine	NF-κBi/p53a/HMTi	3.02	1.78	2.10	6.03
Promazine	D2DRi	2.82	0.64	3.24	5.39

[1] Abbreviations: HDACi; histone deacetylase inhibitor; PI3Ki, phosphatidylinositide 3-kinase inhibitor; AKTi, protein kinase B inhibitor; EGFRi, epidermal growth factor receptor antagonist; HER2, human epidermal growth factor receptor 2 antagonist; NRi, noradrenaline reuptake inhibitor; HH1Ri, histamine H1 receptor inhibitor; TOPIIi, topoisomerase II inhibitor; CaChi, calcium channel inhibitor; NF-κBi, inhibitor to nuclear factor kappa-light-chain-enhancer of activated B cells; p53a, p53 activator; HMTi, histone methyltransferase inhibitor; D2DRi, dopamine receptor D_2 inhibitor.

Supplemental Table 4.S1. Functional properties, Z-scores, and fold increases in relative luciferase activity of 110 hits

Compound Name	CAS Number	Z Score	Major Function	Fold Increase		
				5 µM	10 µM	20 µM
HDACi						
Mocetinostat	726169-73-9	3.45	HDACi	21.36	41.72	59.69
Scriptaid	287383-59-9	4.33	HDACi	6.17	25.71	50.42
ITF2357	732302-99-7	5.68	HDACi	52.38	113.64	46.13
PCI-24781	783355-60-2	2.85	HDACi	77.51	82.8	37.43
PXD101	414864-00-9	6.58	HDACi	23.05	41.44	33.96
Trichostatin A	58880-19-6	16.99	HDACi	68.08	47.64	27.62
AR-42	935881-37-1	2.27	HDACi	107.71	80.82	25.88
Vorinostat (SAHA)	149647-78-9	3.35	HDACi	1.59	3.52	14.53
Parthenolide	20554-84-1	4.14	HDACi	1.41	0.75	1.13
CUDC-907	1339928-25-4	9.97	PI3Ki/HDACi	137.04	482.67	264.88
Channel inhibitors						
Tetrandrine	518-34-3	2.17	CaChi	1.22	1.97	6.42
Amlodipine	88150-42-9	2.95	CaChi	0.94	1.57	3.02
Amiodarone HCl	19774-82-4	2.78	CaChi	1.05	1.05	2.12
Loperamide hydrochloride	34552-83-5	2.05	CaChi	0.65	0.66	1.34
Thioridazine HCl	130-61-0	2.17	CaChi/D2DRi/D4DRi	0.69	1.35	1.06
Propafenone	34183-22-7	3.53	NaChi	0.94	1.87	1.51
Gitoxin	4562-36-1	7.21	NaChi/Kchi	1.93	3.81	1.64
Dopamine modulators						
Promazine	53-60-1	2.82	D2DRi	0.64	3.24	5.39
Fluphenazine	69-23-8	2.56	D2DRi	0.69	1.41	3.42
Perphenazine	58-39-9	9.94	D2DRi	0.98	1.65	3.22
Prochlorperazine Dimaleate	2/6/1984	2.92	D2DRi	0.63	1.63	1.66
Chlorprothixene HCl	113-59-7	2.64	D2DRi	1.43	2.28	1.41
Thioridazine HCl	130-61-0	2.17	CaChi/D2DRi/D4DRi	0.69	1.35	1.06
Trifluoperazine	117-89-5	2.35	D2DRi/CaMi/Ari/5-HTl	0.66	0.71	1.71
Rimcazole	75859-04-0	3.17	DATi/SMAi	0.63	1.52	3.84
PI3K/AKT/mTOR inhibitors						
Wortmannin	19545-26-7	2.38	PI3Ki	29.61	33.69	12.09
CUDC-907	1339928-25-4	9.97	PI3Ki/HDACi	137.04	482.67	264.88
Triciribine	35943-35-2	3.25	Akti	37.36	77.73	156.08

Plumbagin	481-42-5	8.95	Akti/mTORi	1.01	3.93	3.27
CUDC-101	1012054-59-9	2.54	mTORi	4.69	12.52	34.17
Serotonin (5-HT) modulators						
Metergoline	17692-51-2	16.42	Ligand for various 5-HT and dopamine receptors	1.31	2.3	4.86
Periactin	969-33-5	2.43	Inhibitor of histamine H1 receptor and 5-HT receptor	1.08	2.13	3.91
Paroxetine	61869-08-7	2.2	Specific selective 5-HT reuptake inhibitor	1.58	1.6	2.25
Methiothepin	20229-30-5	3.02	Non-selective antagonist of 5-HT, dopamine, and adrenergic receptors	0.66	1.67	2.12
Pizotifen	11/7/5189	3.22	5-HT antagonist	1.24	1.33	2.06
Protriptyline hydrochloride	1225-55-4	6.62	Decreases the reuptake of norepinephrine and (5-HT) in the brain.	1	1.31	1.99
Trifluoperazine	117-89-5	2.35	Antagonist of adrenergic receptors, calmodulin, D2DR, and DNA repair; ligand of 5-HT receptors	0.66	0.71	1.71
Amitriptyline HCl	549-18-8	11.98	Inhibitor of 5-HT and norepinephrine transporters	0.63	1.52	1.25
Others						
Maprotiline hydrochloride	10347-81-6	5.61	Norepinephrine reuptake inhibitor	1.53	3.85	7.01
Desloratadine	100643-71-8	3	Histamine H1 receptor inhibitor	1.73	2.47	6.83
Doxorubicin	23214-92-8	5.64	Inhibitor of topoisomerase II	7.89	101.89	6.56
Quinacrine	6151-30-0	3.02	Inhibitor of NF-kB and histamine methyltransferase, and activator of p53	1.78	2.1	6.03
9-Aminoacridine	90-45-9	9.8	Dye	0.97	0.99	3.19
Tetrachloroisophthalonitrile	1897-45-6	4.94	Fungicide	1.04	1.43	2.91
ZM-447439	331771-20-1	2.69	Aurora inhibitor	0.68	1.03	2.16
Imatinib	152459-95-5	2.17	Inhibitor of tyrosine kinase, c-Abl, c-kit, and PDGFR	1.67	1.67	2.04
Protriptyline hydrochloride	1225-55-4	6.62	Norepinephrine reuptake inhibitor	1	1.31	1.99
Profenamine	1094-08-2	4.64	Butyrylcholinesterase inhibitor	0.71	1.82	1.87
Vincristine	71486-22-1	3.46	Tubulin ligand	2.11	2.55	1.81
Patulin	149-29-1	10.47	Protein prenylation inhibitor	1.8	1.28	1.71
Pterostilbene	537-42-8	5.55	COX inhibitor	0.7	0.76	1.59
7,4'-dimethoxy-5-hydroxyisoflavone	34086-51-6	11.52	Derivative of genistein	1.22	0.92	1.51
Vinblastine	865-21-4	3.51	nAChRs inhibitor	5.65	4.6	1.41

Deoxyadenosine	16373-93-6	2.88	DNA nucleoside A	1.67	1.04	1.4
Saquinavir	127779-20-8	5.48	Protease inhibitor	0.33	0.69	1.35
Fast Green FCF	2353-45-9	4.06	Dye	1.43	4.45	1.31
Betulin	473-98-3	2.42	Sterol regulatory element binding protein (SREBP) inhibitor	1.22	0.95	1.3
3H-1,2-Dithiole-3-thione	534-25-8	3.69	Chemoprotective chemical	0.31	0.64	1.28
Quinapril hydrochloride	85441-61-8	3.11	Angiotensin-converting enzyme inhibitor (ACEi)	0.33	0.71	1.1
Lincomycin HCl	7179-49-9	5.07	Inhibitor of bacterial protein synthesis	0.65	0.99	1.04
Trimetozine	635-41-6	7.08	Sedative	0.33	0.7	1.04
Metoprolol	51384-51-1	2.16	Inhibitor of β1 adrenergic receptors	0.34	1.44	1.04
Fenbufen	36330-85-5	2.7	Anti-inflammatory drug	1.02	1.02	1.03
Podophyllotoxin	477-47-4	3.63	Tubulin polymerization inhibitor	3.06	4.06	1.02
Ursolic acid	77-52-1	3.96	STAT3i/JNKi	0.98	1.29	0.99
Benzethonium chloride	121-54-0	2.88	Inhibitor of nicotinic acetylcholine receptors (nAChRs)	1.5	1.5	0.88
Phenylmercuric Acetate	62-38-4	9.33	Serves as contraceptive gel and foam	1.03	0.75	0.76
Niclosamide	50-65-7	2.6	STAT3 and FRAP inhibior	2.45	1.71	0.69
Chlortetracycline HCl	64-72-2	5.39	Calcium ionophore antibiotic	0.99	0.33	0.67
Adapalene	106685-40-9	2.02	Retinoic acid receptor inhibitor	1.67	1.64	0.67
Clomifene citrate	50-41-9	8.04	Selective estrogen receptor modulator	0.63	1.23	0.65
Celastrol, tripterine	34157-83-0	14.42	Inhibitor of NF-κBi and Hsp90	0.71	0.08	0.6

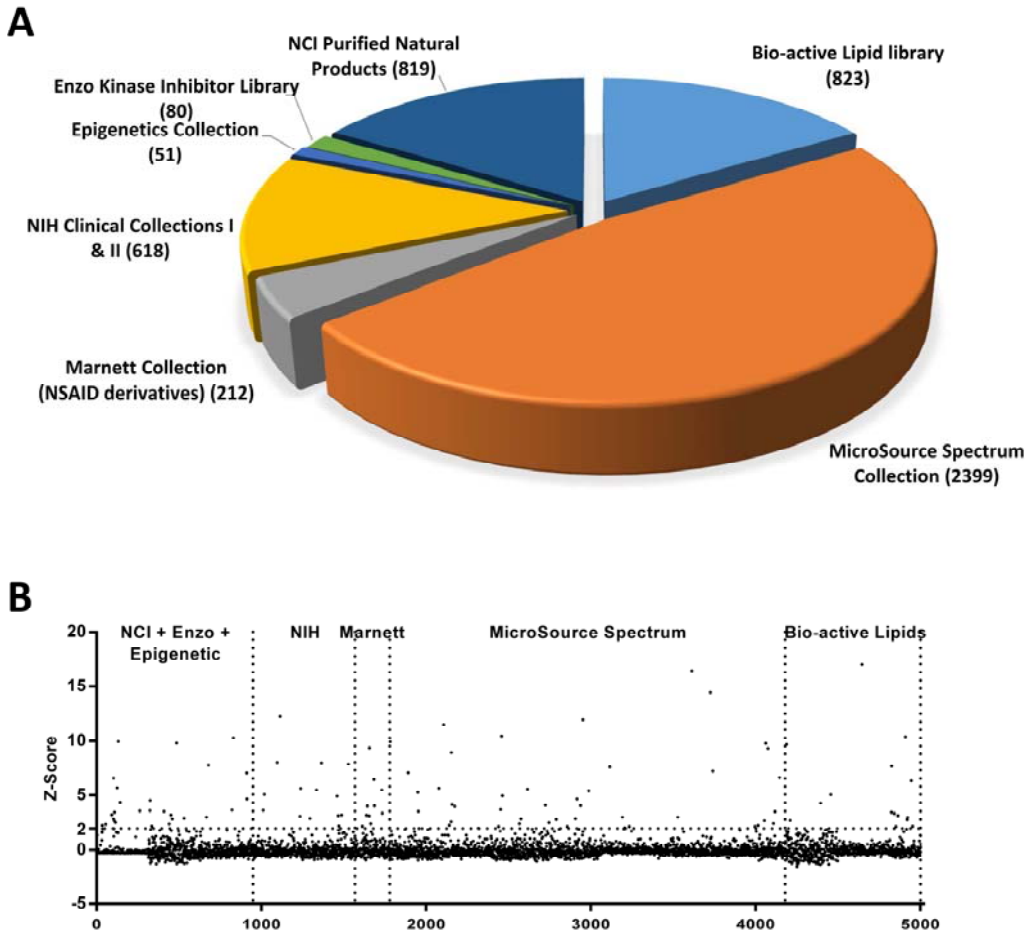

Fig. 4.1. High-throughput screening to identify host defense peptide-inducing compounds. (A) Identities of small-molecule compound libraries used in the screening. The number of compounds in each library is shown in the parentheses. (B) Z-scores of the 5,002 compounds in different libraries. The HTC/*AvBD9-luc* luciferase reporter cell line was stimulated in 384-well plates with 20 μM of each compound for 24 h, followed by luciferase assay. The alamaBlue dye was added 4 h before the luciferase assay to measure cell viability. The Z-score for each compound were calculated from luciferase activity normalized to cell viability.

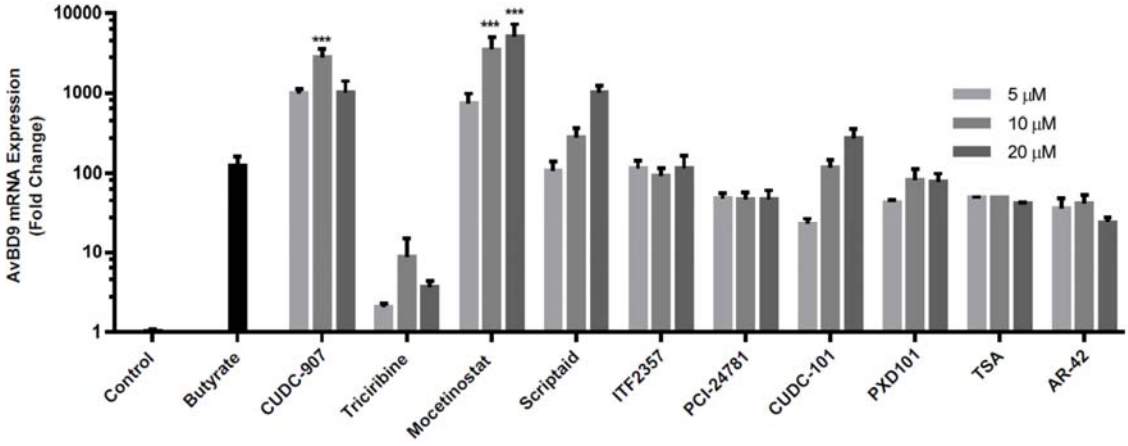

Fig. 4.2. Dose-dependent induction of the *AvBD9* mRNA expression in chicken HTC cells by newly identified HDP inducers. HTC cells were stimulated in duplicate with different concentrations of each compound for 24 h, followed by RT-qPCR analysis of *AvBD9* mRNA expression. Sodium butyrate (4 mM) was used as a positive control. The results are means ± SEM of three independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. ***$P < 0.001$ (relative to the unstimulated control).

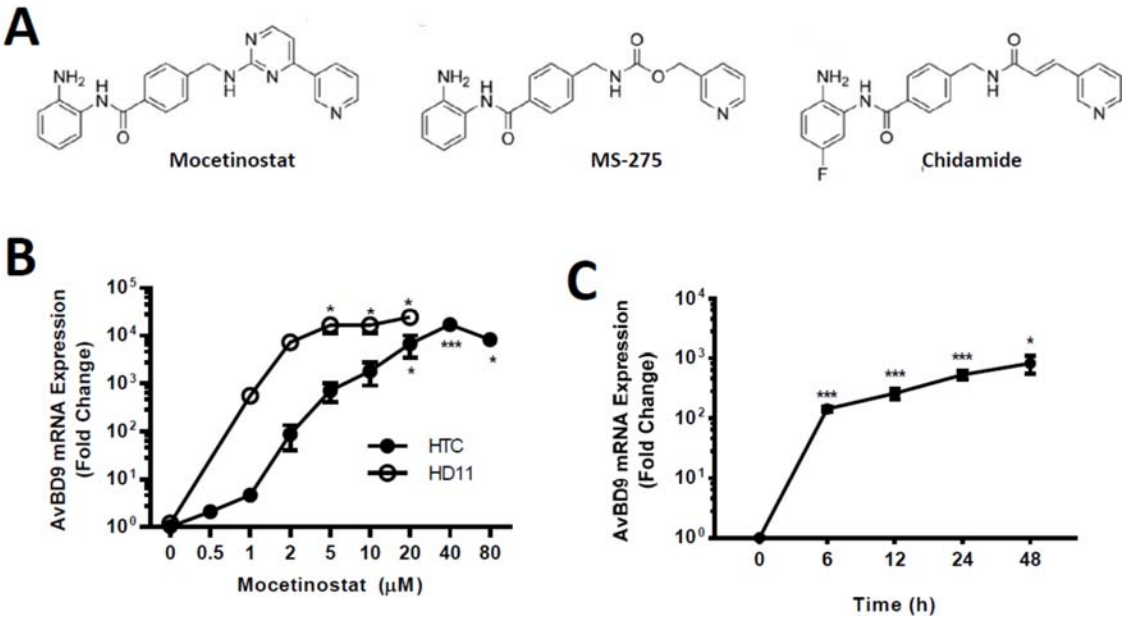

Fig. 4.3. Dose- and time-dependent induction of *AvBD9* mRNA expression by mocetinostat in two different chicken macrophage cell lines. (A) Chemical structures of mocetinostat and its two structural analogs, MS-275 and chidamide. (B) Dose-dependent changes of *AvBD9* mRNA expression in chicken HTC and HD11 macrophage cell lines in response to different concentrations of mocetinostat for 24 h. (B) Time-dependent induction of *AvBD9* mRNA in HTC cells in response to 2 μM mocetinostat for different lengths of time. AvBD9 mRNA expression levels were evaluated by RT-qPCR. The results are means ± SEM of three independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (relative to the unstimulated control).

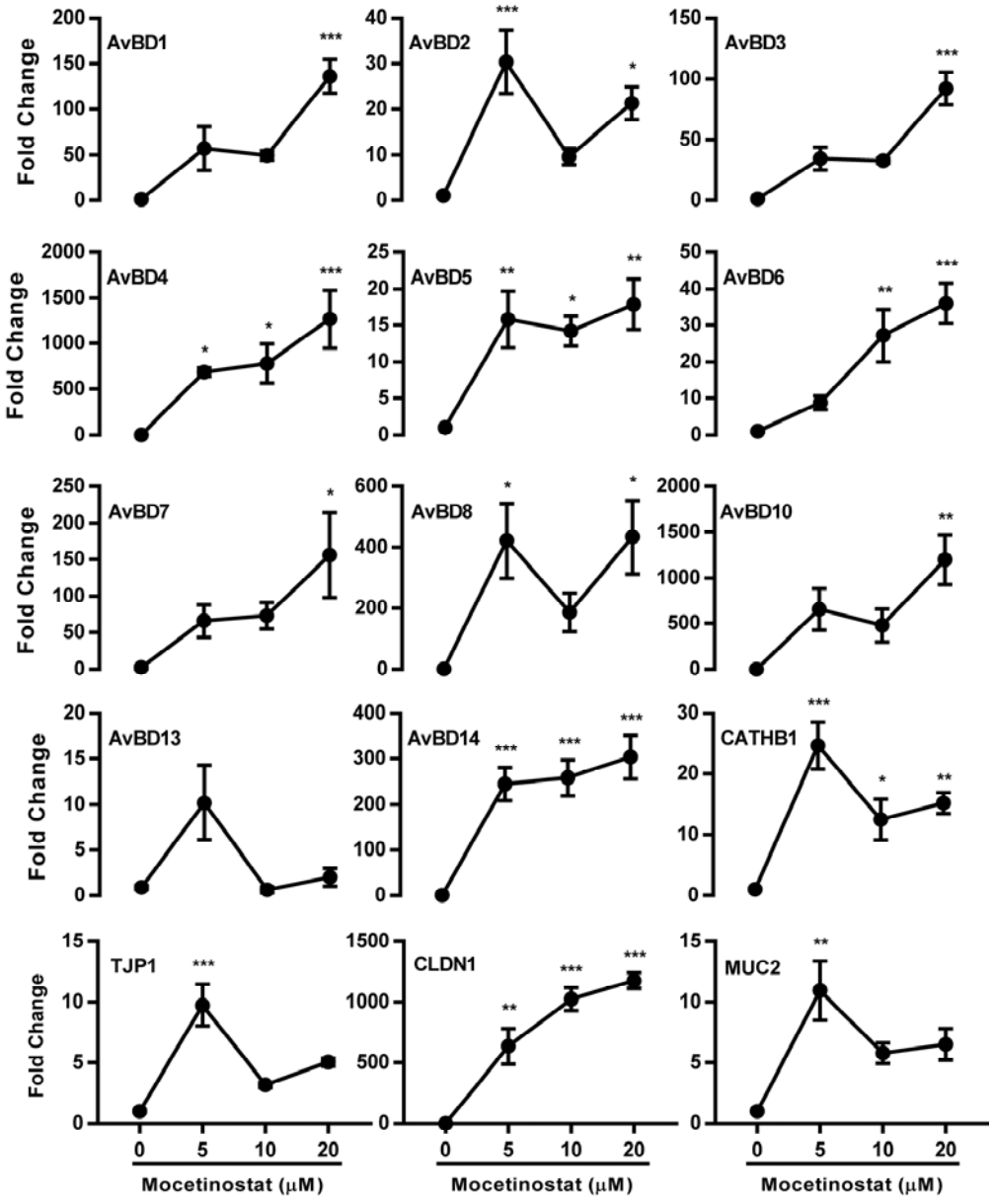

Fig. 4.4. Induction of multiple HDP and barrier functions genes in chicken HTC macrophages in response to mocetinostat. HTC cells were stimulated in duplicate with 5, 10, and 20 μM of mocetinostat for 24 h. HDP and barrier function gene expression levels were evaluated by real-time RT-qPCR. The results are means ± SEM of three independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (relative to the unstimulated control).

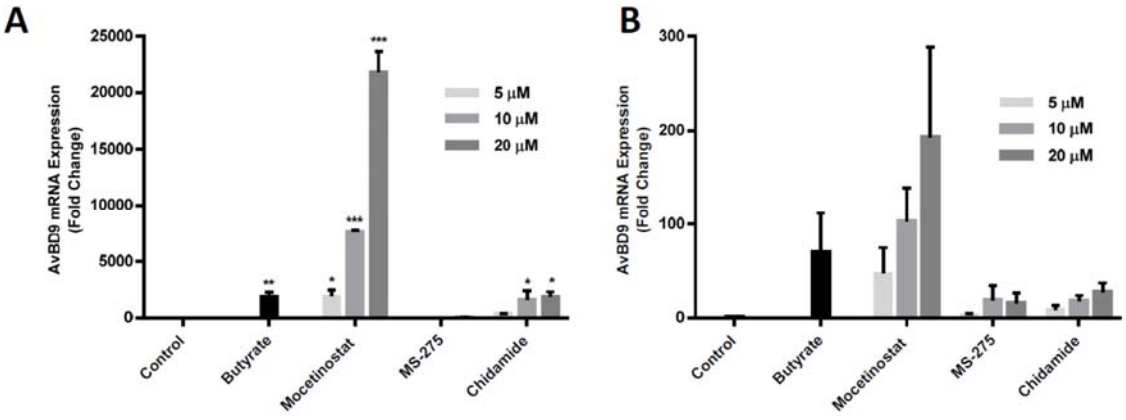

Fig. 4.5. Induction of *AvBD9* mRNA expression by mocetinostat, MS-275, and chidamide in HTC cells and chicken jejunal explants. HTC cells (A) or chicken jejunal explants (B) were exposed to 4 mM butyrate or three different concentrations of mocetinostat, MS-275 and chidamide for 24 h, followed by analysis of *AvBD9* gene expression by RT-qPCR. The results are means ± SEM of 2-3 independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (relative to the unstimulated control).

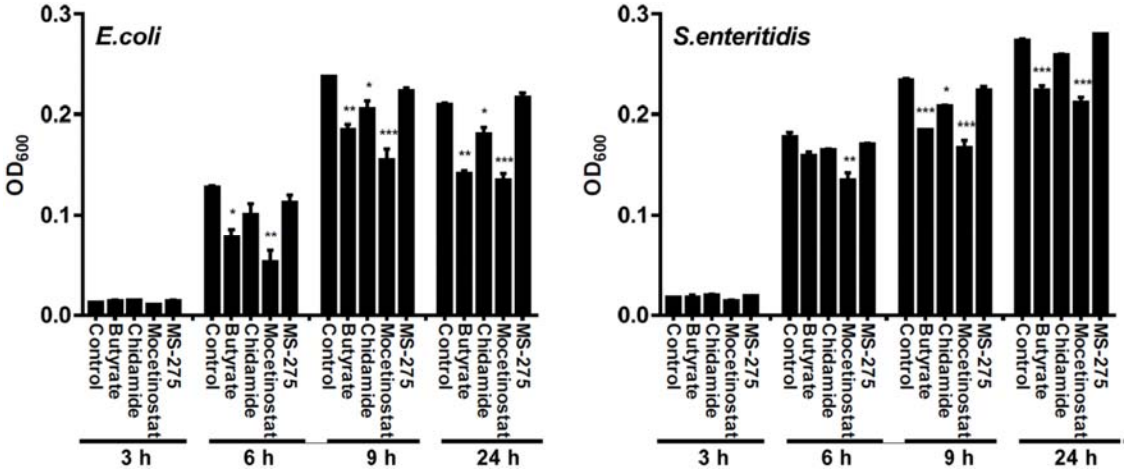

Fig. 4.6. Augmentation of the antibacterial activity of chicken HTC cells by mocetinostat, MS-275, and chidamide. Chicken HTCs were stimulated with 10 μM mocetinostat, MS-275, chidamide or 4 mM butyrate for 24 h, followed by cell lysis and incubation of the cell lysate with *Salmonella enteritidis* (ATCC 13076) or *Escherichia coli* (ATCC 25922) for various lengths of time. Bacterial turbidity was measured at OD_{600} as indication of bacterial growth. The results are means ± SEM of 2-3 independent experiments. ANOVA was performed, followed by Dunnett's multiple comparisons test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (relative to the unstimulated control).

CHAPTER V

CONCLUSION

A high throughput screening assay based on a chicken host defense peptide gene promoter-driven luciferase reporter cell line, HTC/*AvBD9-luc*, was successfully developed for the discovery of HDP-inducing compounds. After two separate screening efforts involving 584 natural product library and 5,002 small-molecule compounds, 21 and 110 hits were identified, respectively, with a minimum Z-score of 2.0. Among them, wortmannin was the most potent natural product, while mocetinostat was the most efficacious HDP inducer in the second larger-scale screening. Both wortmannin and mocetinostat were capable of inducing multiple HDP gene expression in different cell types. Importantly, wortmannin synergized with butyrate in enhancing HDP gene expression and the antibacterial activity. Mocetinostat showed a stronger *AvBD9*-inducing activity in chicken macrophages and jejunal explants than two of its structural analogs, MS-275 and chidamide. Wortmannin, the wortmannin/butyrate combination or mocetinostat has the potential to be developed as novel antibiotic alternatives for use in poultry and possibly other animal species including humans.

To further develop them as antibiotic alternatives for poultry use, these newly-identified HDP inducers will be supplemented in feed at different levels for their influence on chicken growth performance, bacterial clearance, intestinal development, barrier function, and microbiota. To achieve this goal, we will first assess the efficacy of these compounds in healthy chickens in

comparison with a commonly used in-feed antibiotic such as bacitracin methylene disalicylate (BMD). Weekly growth performance including average daily gain, average feed intake, and feed efficiency will be recorded and calculated. Besides, intestinal segments will be collected for HDP and tight junction protein gene expression and morphological studies. To further evaluate the efficacy of HDP-inducing compounds in bacterial clearance, we will feed chickens with BMD or different doses of each compound and then subject animals to necrotic enteritis. Chicken growth performance, intestinal lesion scores and pathologies will be assessed. Additionally, Intestinal segments will be collected for intestinal morphological studies. Moreover, intestinal contents will be collected in both studies for intestinal microbiota composition analysis.

It is our expectation that these newly-identified HDP inducers will reduce the intestinal pathology and bacterial colonization with no negative influence on growth performance. Therefore, the compounds will have potential for further development as antibiotic alternatives for disease control and prevention in chickens and/or other livestock species.

VITA

Wentao Lyu

Candidate for the Degree of
Doctor of Philosophy

Thesis: IDENTIFICATION OF HOST DEFENSE PEPTIDE-INDUCING COMPOUNDS AS ALTERNATIVES TO ANTIBIOTICS USING A CELL-BASED HIGH THROUGHPUT SCREENING ASSAY

Major Field: Animal Science

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Animal Science at Oklahoma State University, Stillwater, Oklahoma in December 2018.

Completed the requirements for the Master of Agriculture in Animal Science at China Agricultural University, Beijing, China in 2012.

Completed the requirements for the Bachelor of Engineering in Animal Science at Wuhan Polytechnic University, Wuhan, China in 2009.

Experience:

Graduate Research Assistant (2014-2018), Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma.

Graduate Teaching Assistant (2014-2017), Animal Genetics, Principle of Animal Nutrition, Pet and Companion Animal Management, Meat Animal and Carcass Evaluation at Department of Animal Science, Oklahoma State University, Stillwater, Oklahoma.

Research Assistant (2012-2014), Research & Development Department, Novus International Trade (Shanghai) Co., Ltd., Beijing, China.

Honors and awards:

Second Place in Oral Presentation, 15th Annual Research Symposium in Biological Sciences (2018), Oklahoma State University, Stillwater, Oklahoma, USA

Sitlington Enriched Graduate Scholarship (2014-2017), Oklahoma State University, Stillwater, Oklahoma, USA

Top-Tier Graduate Fellowship (2014-2015), Oklahoma State University, Stillwater, Oklahoma, USA

Professional Memberships:

Poultry Science Association

World's Poultry Science Association-USA