The Generating Factors of Antimicrobial Peptide's Production in Human

A project submitted

by

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Dedication

This work is dedicated to my parents and my sibling to whom I owe my achievements.

Certification statement

This is to certify that, this project titled ‘The generating factors of antimicrobial peptide’s production in human’ submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Sinayat Mahzabeen, Lecturer, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor

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Acknowledgement

I would like to begin my gratitude to Almighty Allah for the help in the completion of this research and preparation of this paper.

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Jerin Mahbub

Abstract

Antimicrobial peptide (AMP) has been recognized as a noble antibiotic against wide range of bacteria now a days. Isolated AMPs from various sources are used to control a number of diseases in humans. To reinforce their expression, some factors can be used to work on specific parts of human body. In this study, we accumulate seven factors which assist production of cathelicidin and defensins family of human AMPs. Vitamin D3 and bile salt prompt up cathelicidin expression in various epithelial cell lines through vitamin D receptor and farnesoid X receptor as respectively. On the other side, beta defensins can be induced by epigallocatechin-3-gallate, isoleucine, sulphoraphane and platelet release growth factor in targeted areas. Lastly, phenylbutyrate works on both cathelicidin and beta defensin 1. Most of them work effectively in dose and time dependent manner. In some cases, the combination therapy of two or three of them have been turned out positively than their individual action which is also highlighted here. In spite of their usefulness, some obstacles are still lagging behind them from implementation which need further investigation. This study clearly indicates that all the elements successfully elevate antimicrobial peptide expression within the body and also improve innate immunity to fight against infections in both primary and critical stages.

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Abbreviation

AMP	-	Antimicrobial peptide
HBD	-	Human beta defensin
EGCG	-	Epigallocatechin-3-gallate
PBA	-	Phenylbutyrate
SFN	-	Sulphoraphane
HDAC	-	Histone Deactylase
VDR	-	Vitamin D Receptor
PRGF	-	Platelet Release Growth Factor

1. Introduction:

Antimicrobial peptides (AMPs) are the part of first line innate immune system that defend the host from various pathogenic attacks. Besides virus, fungi and cancerous cells, AMPs are highly effective against both gram positive and gram negative bacteria. They have been isolated not only from human but also from single celled organisms, fish, bird, insects, invertebrates and other mammals. Basically, their expression within the body increases from infectious stimuli. These AMPs or broad spectrum antibiotics were first discovered in 1939 by Dubos when he extracted an antimicrobial agent from a soil bacillus strain to use it for protection of mice from pneumococci infection (R.J, 1939). AMPs, this small peptides generally consist of 12 to 50 amino acids. Their net charges vary from -3 to +20. Variability in their structures is one of the benefits of them to use as an antimicrobial agent. Antibiotic resistance, the burning issue in the medical sector, can be minimized or controlled with the help of antimicrobial peptides. Moreover, they possess other functions such as, immunomodulator, wound healing and apoptosis etc. Recently, they have been used as a marker in order to detect many cancerous cells which also neutralized by their apoptosis activity.

From the very earlier, an extent research work has been done on AMPs activity. The structure, characteristics, classification and their mechanism of action have been identified. The effective activity concentration has been examined against bacteria, fungi, virus and other pathogens. These peptides play greater role in healing and preventing many fatal diseases. In spite of, they are still lacking behind for clinical implication in some areas due to low bioactivity over available drugs. However, some synthetic peptides which are prepared from these natural ones significantly available in treating diseases. The factors responsible for inducing their production in human body also have been identified. They are highly potent to induce antimicrobial peptides which can be taken either as food or nutrients. So, the proper use of them can lead to induce AMPs activity as well as innate immunity in order to keep us away from being infected by any diseases easily. The aim of this review is to accumulate these factors with their potentiality and also further improvement could be done in these area.

2. Classification of Antimicrobial Peptide:

The majority of AMPs are cationic in nature due to presence of arginine and/or lysine but anionic AMPs are minor in number. Till now four classes of AMPs have been proposed based on their structure and they are α-helical, β-sheet, loop and extended peptides (Hancock RE, 1998).

- The α-helical peptides family is most common and cationic in nature. This class contains 250 peptides which composed of less than 40 amino acids other than cysteine (KA., 2005).
- The β-sheet peptides are more stabilized because of the disulfide bridges (KA., 2005). These bridges and cyclic structures have greater impact on their action and also essential for antibacterial activity (Matsuzaki K, 1997) (AG., 1999).
- The extended peptides are linear in shape but have an unusual composition where over expression of one or more amino acids can be found. This class contains 90 peptides and very flexible in solution.

According to a study, four minimum possible reasons had been found for this structural diversity among AMPs (Hancock RE, 2000).

1. All classes of antimicrobial peptides cannot be active against every pathogen. So, diversity in structure can help them to kill specific microbes.
2. Different structures of AMPs can work together with synergism.
3. Those structures are helping them to do other non-antibacterial activities such as, chemotactic or pro-inflammatory activities.
4. Different AMPs are produced by different cell types which is another cause to be different in structure.

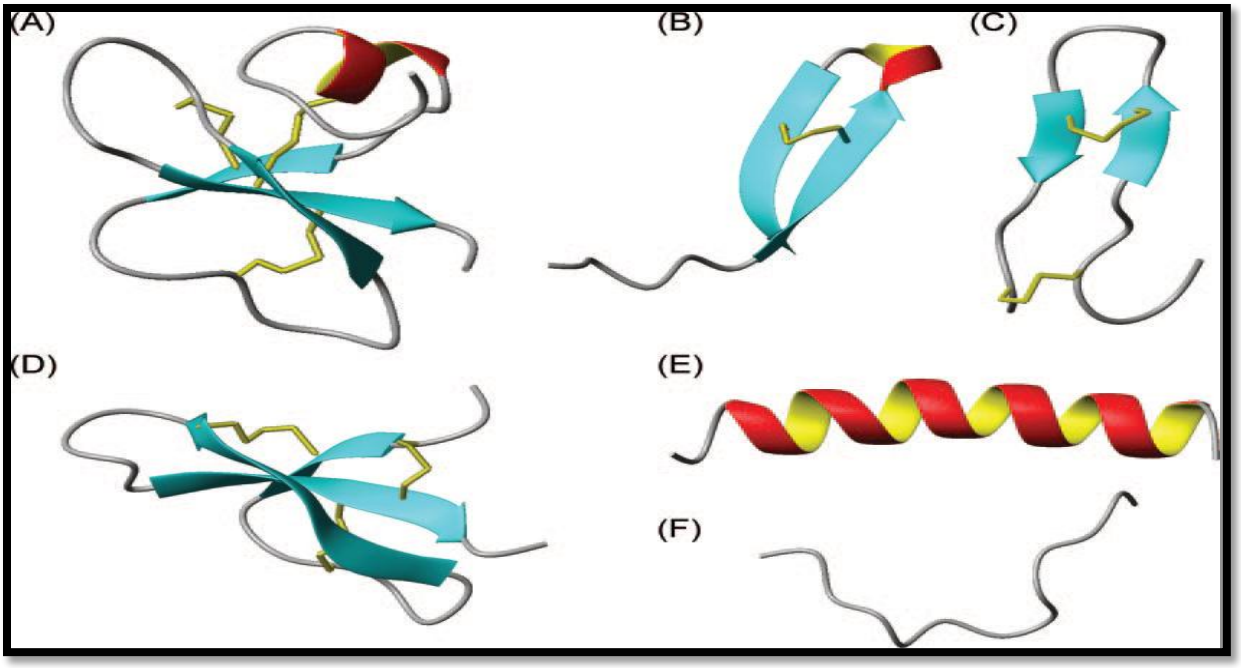

Figure 2.1: Different types of antimicrobial peptide. (Håvard J, 2006)

In the above picture, various classes of antimicrobial peptides have been shown (Håvard J, 2006). They are given below.

A) Mixed structure of human β-defensin-2;

(B) Looped thanatin;

(C) β-sheeted polyphemusin;

(D) Rabbit kidney defensin 1;

(E) α-helical magainin-2;

(F) Extended indolicidin.

3. Antimicrobial Peptides in Human:

Many antimicrobial peptides can be found in different species but among them a few can be isolated from the human. Among them, two are very much important and they are cathelicidins (LL-37) and defensins (alpha and beta).

3.1 Cathelicidin

Cathelicidins are varying in amino acid sequence, structure and size. They range in size from 12-80 amino acid residues and have a wide range of structure (Gennaro R, 2000). About 30 cathelicidin family members have been idenfied in mammalian species but LL-37 is the only one cathelicidin found in human. LL-37 which begins with two leucine residues at its N-terminus, and is 37 a residues long, with a molecular weight of 18 kDa (Gudmundsson GH, 1995). LL-37 is disordered in aqueous solution but can quickly transfer into α-helix while binding with bacterial cell wall (Nijnik A, 2009).

Location: Cathelicidins are a family of polypeptides and mostly found in metropolis, epithelia of the skin, guts and lungs, monocytes, natural killer cells and mast cells.

Mechanism of action: Though the actual mechanism of action of cathelicidin is not clear, it is hypothesized that they bind to the cytoplasmic membranes of microbes and cause a disruptive effect ((Lee CC, 2011).

Activity: It can work effectively as antimicrobial agent at a physiological concentration of approximately 2µg/ml but can increase eventually during infection (Bals R, 1999) (Schaller-Bals S, 2002). It is strongly active against various bacterial strains such as, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Neisseria gonorrhoeae etc. It prohibits the formation of P. aeruginosa bacterial biofilm which is a bacterial growth pattern related to the antibiotic resistance and it happens at 0.5µg/ml concentration only (Overhage J, 2008). This inhibition is done by lowering the attachment of bacterial cells, provoking quorum sensing system and stirring twitching motility which ultimately affecting the gene regulation for biofilm formation (Overhage J, 2008). This α-helical peptide also gives immune response against Mycobacterium tuberculosis. It has been observed that mycobacterial infection stimulates the production of this peptide in A549 epithelial cell, alveolar macrophages, neutrophils and Toll-like receptors (Liu PT,

2006) (Rivas-Santiago B, 2008). Moreover, it had been found that LL-37 gene expression is influenced by MEK 1/2 and p38 MAPK signaling pathways in A549 cells infected with Mycobacterium bovis bacillus Calmette-Guerin which is a tuberculosis vaccine mostly used in worldwide (Mendez-Samperio P, 2008). As an antiviral agent, LL-37 has been found to give local protection against HIV-1 infection by epithelial expression (Bergman P, 2007). It was found that the immunological response of this peptide has been changed in infected patients with papillomavirus (Conner K, 2002). As an antifungal agent, it is also active against Candida albicans. The activity is depending on the culture condition whether it will effective as a superficial barrier to the invasion (den Hertog AL, 2006) (Lopez-Garcia B, 2005).

3.2 Defensins:

Defensins are also small, cysteine-rich and cationic peptide which are consisting of 18-45 amino acids with 6-8 cysteine residues and they form disulfide bridges. Due to the differences in alignment of disulfide bond and molecular structure, two subclasses can be found in human. They are alpha defensins and beta defensins. Human alpha defensins are also called human neutrophil peptides and have four subtypes such as, HNP1, HNP2, HNP3 and HNP4 which are 29-35 amino acids long. First three subtypes increase the expression of interleukin-1 and tumor necrosis factor α in human monocytes (Chaly YV, 2000). Another two α-defensins (HD-5 & 6) are known as enteric defensins. The C terminal part in their structure is responsible for antimicrobial activity (Contreras, 2005). They can take part in the oxygen independent killing of phagocytosed microbes in neutrophil. Furthermore, human beta defensins (hBD) can also be classified into four and they are hBD-1, hBD-2, hBD-3 and hBD-4, expressed by different intestinal epithelial cells (K.D. Smet, 2005).

Location: HNP1, HNP2, HNP3 and HNP4 are found in azurophilic granules of neutrophils as mature peptides (Nizet, 2003). Again, HD-5 & 6 are found in the granules of peneth cells of small intestine and epithelial cells of female urogenital tract (Jones DE, 1992). On the other hand, HBD-1, 2, 3 & 4 can be found in several epithelial cells of respiratory tract, gastrointestinal tract, cornea, skin and kidney etc.

Mechanism of Action: Defensins electrostatically bind to membranes, causing the formation of multimeric pores and the leakage of essential minerals and metabolites (Lehrer R I, 1989). They also cause membrane depolarization, decreased cytoplasmic ATP levels, and inhibited cellular respiration (Cociancich S, 1993). The entrance of defensins into cells has caused DNA damage (Gera J F, 1991) (Lehrer R I, 1985).

Activity: HBD-1 and 2 are more bactericidal against gram negative bacteria rather than gram positive. On the other hand, HBD-3 kills broad ranges of microbes and opportunistic pathogenic yeast like Candida albicans. Other than antimicrobial activity, defensins also show their potentiality in stimulation of cell proliferation, chemo attraction of immune cells, induction of cytokine and cell proliferation (Contreras, 2005).

Table 3.1: Structure and sequences of human cathelicidin and defensins

Peptide	Structure	Amino acid sequence
LL-37	α-helix	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
HNP-1	β-sheet	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-4	Same	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD
HD-5	Same	ARATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HD-6	Same	RAFTCHCRRS-CYSTEYSYGTCTVMGN-HRFCCL
HBD-1	Same	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
HBD-2	Same	DPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
HBD-3	Same	QKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
HBD-4	Same	LDRICGYGTARCRKK-CRSQEYRIGRCPNTYA CCLRKPWDESLLNRTK

Reference: Kris De Smet & Roland Contreras (2005) Human antimicrobial peptides: defensins, cathelicidins and histatins. J. Springer. Biotechnology Letters 27: 1337-1347

4. Clinical Importance of AMPs:

Antimicrobial peptides have a greater clinical importance in medical sector.

4.1 Natural antibiotics:

Antimicrobial peptides is such natural antibiotic which have greater importance in killing multidrug-resistant microorganisms that bring it into high spot in treating diseases. Now a days, antibiotic resistance has become a major issue in the field of treatment. As we know, antibiotics do not only useful in killing bacteria but also in surgery, chemotherapy, chronic infections and patients suffering from diabetes, end-stage renal disease, rheumatoid arthritis and organ transplant etc (J., 2016) (The lancet infectious diseases comission, 2013). From recent review on antimicrobial resistance, it has been known that about 700,000 annual deaths are currently attributable to drug-resistant pathogenic infections (Gould IM, 2013). The overuse of antibiotics, environmental factors and also uncontrolled sales in many low or middle income countries are the possible reasons behind antibiotic resistance development (Radek K, 2007). Penicillin, tetracycline, gentamycin, vancomycin and levofloxacin etc. antibiotic have been already resistant to the human body in past few decades. People are not using medicines schedule wise and not completing course as well which is starting a base line for resistance and making easier for microbes to grow again with more virulence. Besides human body, antibiotics are polluting environment because of their introduction into human wastes, animals and the pharmaceutical industry (Martinez, 2012). To handle this critical situation we can use natural antibiotics which can kill microbes with same efficiency and does not produce resistant so quickly because of their various mode of action against pathogens.

4.2 Immunomodulator:

These peptides have ability to boost immunity by behaving as immunomodulator. Human cathelicidin, LL-37 is a chemoattractant for mast cells, monocytes, T lymphocytes and neutrophils (Yang D, 2000). LL37 contributes to host defense against microbial invasion, by participating in the recruitment of leukocytes to sites of infection. This mechanism is potentially important in vivo, because the chemotactic activity of LL37, unlike its antimicrobial action, is not significantly inhibited by the presence of human

serum. The activation of FPRL-1 requires relatively high concentrations of LL-37 (10−5M) when compared to other classical chemoattractant agents, suggesting a low-affinity peptide-receptor interaction (De Y, 2000). LL-37 modulates cellular immune responses by stimulating chemokine production. It activates airway epithelial cells through the activation of mitogen activated protein kinase (MAPK) and increases the release of potent chemoattractant IL-8 (De Y, 2000). In keratinocytes, LL-37 induces IL8, and promotes migration and wound healing, and these activities depend on ADAM family metalloproteinase, EGFR and FPRL1 (Carretero M, 2008).

4.3 Anti-inflammatory agent:

They are widely used in preventing skin diseases. Studies have shown that LL-37 and HBD-2 are greatly increased in patients with inflammatory skin conditions (Stolzenberg ED, 1997) (Gallo RL, 1940) (Frohm M, 1997). LL-37 is induced in human keratinocytes during psoriasis, lupus erythematosus and contact dermatitis (FROHM M, 1997). It is also able to induce angiogenesis, a process important for wound healing and tissue repair (KOCZULLA R, 2003).

4.4 Anti-infective agent:

They are used as anti-infective agents in the medical sector (Hancock, R. E.W., 2006). Though the major importance of antimicrobial peptides are as antibiotics but they also effectively act as antiviral and antifungal. Now a days, various manipulations with chemical structure have been done to create designer synthetic peptides for the development of AMPs as a new class of drugs to prevent and treat systemic and topical infection.

Antimicrobial peptides can be used as intestinal infections. Paneth cells, which are a characteristic epithelial lineage of the small intestine and localize to the bottom of the intestinal crypts, secrete α-defensins in response to bacterial antigens including lipopolysaccharide and muramyl dipeptide (Ayabe T, 2000). HD-5 plays multiple roles in inflammation and infection. It is found in metaplastic Paneth cells in the colon of IBD patients and presumably it serves as a protective role in response to bacterial

challenges during colitis (Cunliffe RN, 2001). The cationic proteins extracted from colonic mucosal biopsies of PPARγ deficient mice showed a lack of bactericidal activity against Candida albicans, Bacteroides fragilis, Enterococcus faecalis and E. coli, suggesting that HBD-1 may play a role in colonic inflammation and infection ((Peyrin-Biroulet L, 2010).

4.5 Cancer biomarker:

AMPs can be used as anticancer agent against various types of cancer. A recent report showed that LL-37 activated p53 dependent but caspase independent activation of apoptosis in human cancer cells including HCT116 and LoVo. In addition, LL-37 expression in human colonic tumors is down-regulated while LL-37 is expressed strongly in normal colonic tissues (Ren SX, 2012). On the other hand, alpha defensin may serve as a biomarker for colorectal cancer which is based on a study with 100 colorectal cancer patient ((Kemik O, 2011).

5. Factors Generating Antimicrobial Peptide:

As AMPs are part of our body defense, the peptides are regulated by numerous factors which can be controlled. All the listed factors work on either Cathelicidin or Defensins. Following is a table belongs to the list the factors and some basic information to express specific antimicrobial peptides in brief.

Table 5.1: List of factors, targeted AMP, targeted site and related experiments.

Factors	**Targeted AMP**	**Targeted site in Human**	**References**
Vitamin D3	Cathelicidin	Keratinocytes of human skin	Svensson D, Nebel D, Voss U, Ekblad E & Nilsson B. Vitamin D-induced up-regulation of human keratinocyte cathelicidin anti-microbial peptide expression involves retinoid X receptor α. 2016.
Phenylbutyrate	Cathelicidin and beta defensin 1	Human bronchial epithelial cell line, renal carcinoma cell line, colonic adenocarcinoma cell line and leukemic monocyte lymphoma cell line	Steinmann J, Halldorsson S, Agerberth B, and Gudmundsson G H. Phenylbutyrate Induces Antimicrobial Peptide Expression. Institute of Biology, University of Iceland, Reykjavik, Iceland, and Medical Biophysics and Biochemistry, Karolinska Institutet, Stockholm, Sweden. 2009. Vol. 53, No. 12.
Bile Salt	Cathelicidin	Human liver	D'ALDEBERT E, BIYEYEME M J, MERGEY M, WENDUM D, FIRRINCIELI D, COILLY A, FOUASSIER L, CORPECHOT C, POUPON R, HOUSSET C and CHIGNARD N. Bile Salts Control the Antimicrobial Peptide Cathelicidin Through Nuclear Receptors in the Human Biliary

			Epithelium. Gastroenterology 2009; 136:1435–1443.
Epigallocatechin-3-Gallate	Beta defensin	Porcine intestinal epithelial cell	Murphy L. Y. Wan, K. H. Ling, M. F. Wang and Hani El-Nezami., Green tea polyphenol epigallocatechin-3-gallate improves the epithelial barrier function by inducing the production of antimicrobial peptide pBD-1 and pBD-2 in monolayers of porcine intestinal epithelial IPEC-J2 cells. 2016. Mol. Nutr. Food Res. 60, 1048–1058.
Isoleucine	Beta defensin	Bovine kidney epithelial cell	Pascale Fehlbaum, Meena Rao, Michael Zasloff, and G. Mark Anderson. An essential amino acid induces epithelial beta-defensin expression. Magainin Research Institute and Magainin Pharmaceuticals Inc. 2000.
Sulphoraphane	Beta defensin 2	Human colorectal cancer cell lines	Schwab M, Reynders V, Loitsch S, Steinhilber D, Schroder O and Stein J. The dietary histone deacetylase inhibitor sulforaphane induces human β-defensin-2 in intestinal epithelial cells. 2008. Immunology, 125, 241–251.
Platelet Release Growth Factor	Beta defensin 2	Primary keratinocyte	Andreas Bayer, Justus Lammel, Franziska Rademacher, Justus Groß, Markus Siggelkow, Sebastian Lippross, Tim Kluter, Deike Varoga, Mersedeh Tohidnezhad, Thomas Pufe, Jochen Cremer, Regine Glaser, and Jurgen Harder. Platelet-released growth factors induce the antimicrobial peptide human beta-defensin-2 in primary keratinocytes. Experimental Dermatology, 2016, 25, 460–465.

5.1 Epigallocatechin-3-Gallate

Tea is one of the popular beverages consumed by almost everyone. Green, black and Oolong tea are the most common form all around the world. Among all of them, the consumption of green tea has significant effects on human health (Cabrera C, 2006). Generally green tea composed of protein, amino acid, lipids, minerals, carbohydrates and many others compounds. Green tea also contains polyphenols which may present up to 30% of dry weight. It is evident that polyphenols protect against a wide range of oxidative stress related disorders, such as cardiovascular diseases, cancers, inflammation, neurodegenerative diseases, and diabetes (Scalbert, 2005). Most of the green tea polyphenols are flavonols, commonly called catechins. There are four types of catechins mainly find in green tea among them one of is epigallocatechin-3-gallate (EGCG) (Sano M, 2001). A cup of green tea may contain 100-200 mg of EGCG (Zaveri, 2006).

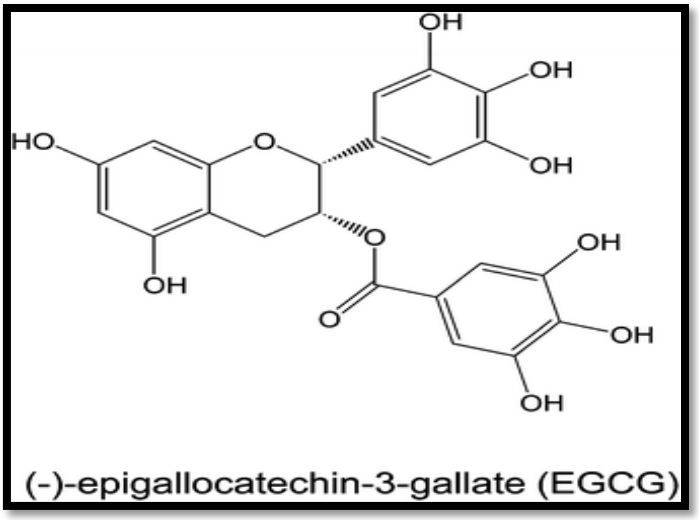

Figure 5.1: Epigallocatechin-3-gallate (EGCG)

Epidemiological surveys show that regular green tea consumption is related to lower chance of cardiovascular diseases, cancers, and obesity (Bell, 2002) (McKay, 2002). It is assumed that the ability of EGCG to bind many biological molecules and provoke the activity of various enzymes and signal transduction pathways at the micromolar and nanomolar level is the reason behind this beneficial effect of green tea (Lee MJ, 2002). As EGCG is a food-derived component, intestinal

epithelial cells are continuously and directly exposed to EGCG. This is the reason it can exert beneficial effects to the intestine. A previous study has been done demonstrating that the antimicrobial activity of AMPs, such as beta-defensins, may be associated with bacterial populations in the intestine, which naturally present a barrier limiting undesirable gut mucosal infections and preventing against bacterial translocation (Veldhuizen, 2008). Another study has also shown that EGCG induced defensin secretion on gingival epithelial cell (Lombardo Bedran, 2014).

A study was conducted based on the hypothesis that EGCG has protective response on intestinal barrier functions against bacterial translocation. The experiment was done by using porcine jejunal epithelial cells, IPEC-J2. IPEC-J2 cells isolated from neonatal piglet mid-jejunum. The cell line is unique in that it is derived from small intestinal tissue. IPEC-J2 cell line was chosen for the current study because it is a better model of normal IEC line than transformed cell line such as Caco-2 to study pathogen–host interactions (Geens, 2011). It conserves its epithelial nature, and is well documented to be able to express and secrete AMPs such as porcine beta-defensins 1 and 2 (pBD-1 and 2) as part of the innate immune response (Mariani, 2009).

EGCG had reduced bacterial translocation at a noncytotoxic concentration. It enhanced epithelial immunological barrier but not epithelial physical barrier. It may also be attributed to the secretions of defensins which was examined by ELISA with the amount of 12, 25 and 50μM of EGCG. Compare with control group, 25μM induced higher level of pBD-1 and 2 secretion significantly. In case of expression of pBD-1 and 2, EGCG successfully enhanced the expression of pBD-2 by 50% but no significant result for pBD-1.

This test was done by specific MAPK pharmacological inhibitor which shown that inhibition to p38 MAPK reduced the expression of pBD-2 and protein secretion. The induction of pBD-1 expression plays a potential role in surveillance and maintenance of a homeostatic state of gut micro biota on the mucosal epithelium (Veldhuizen E. H., 2006) (Elahi, 2006), whereas elevated pBD-2 expression will likely inhibit pathogenic bacteria (Zhang, 2011).

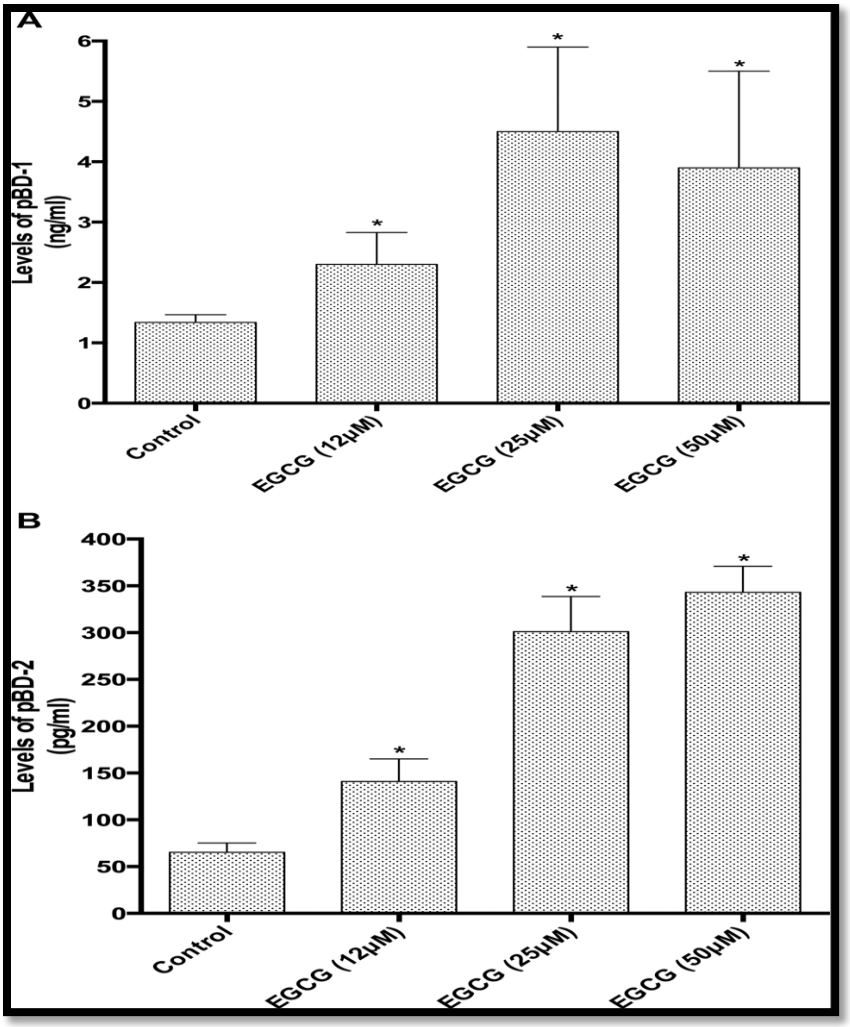

Figure 5.2: Showing result of pBD 1 & pBD 2 level after treating with EGCG (Murphy L. Y. Wan, 2016).

It was the first study to illustrate the protective capacity of EGCG on intestinal cells. The obtained results were satisfied enough to prove the fact that how much beneficial effect can be taken by adding green tea into our daily diet chart. Moreover, it helps to increase the production of mRNA and protein in both defensins. EGCG may be useful for prevention of intestinal disorder or bacterial infection in animals/humans (Murphy L. Y. Wan, 2016).

5.2 Vitamin D3

Vitamin D is essential for the calcium, iron, magnesium and phosphate absorption through intestine more efficiently. The basic function of vitamin D is promoting healthy bones. Osteoporosis, osteomalacia or rickets can be occurred from the deficiency of this class of vitamin. Among several types, D2 and D3 are the major forms of vitamin D which are collectively known as calciferol (Dorland's Illustrated Medical Dictionary, under Vitamin (Table of Vitamins)). Vitamin D3 can also be called cholecalciferol. Firstly it remains inactive and then forms calcitriol by converting into two active forms through two hydroxylation process in the liver and kidney as consequently.

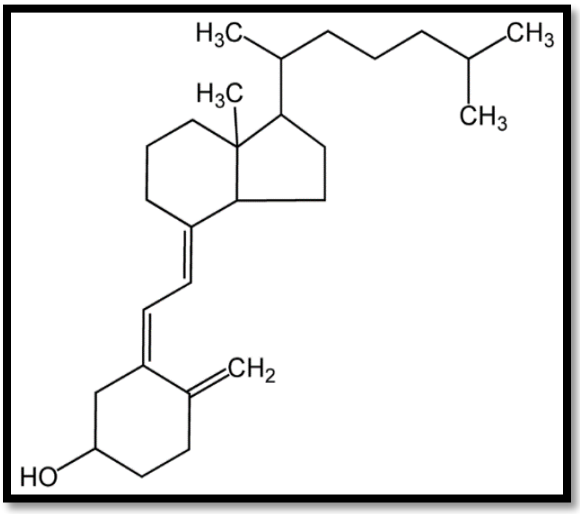

Figure 5.3: Cholecalciferol

One study has shown that vitamin D triggers regulation of human keratinocyte cathelicidin expression involves retinoid X receptor α (RXR). It has been said that the biologically active metabolite of vitamin D 1 α, 25-dihydroxyvitamin D3 (1,25D3), binds to the intracellular vitamin D receptor (VDR) belonging to the steroid receptor family. This complex is thought to form a heterodimer with the retinoid X receptors (RXRs), and the VitD3/VDR/RXR complex then binds to the vitamin D responsive element of vitamin D target genes and regulates their transcriptional activity, although RXR-independent mechanisms have also been reported (Haussler et al. 2013; Carlberg and Campbell 2013; Carlberg et al. 1993). The aim of the study was to assess Vitamin D

receptor signaling in human gingival and skin biopsies and to investigate the importance of RXRs for 1,25D3-induced CAMP gene activity in human keratinocyte HaCaT cells. The HaCaT cell line is a spontaneously immortalized human keratinocyte cell line from adult skin showing complete epidermal differentiation capacity, and thus these cells are most probably representative of human keratinocytes in vivo (Boukamp et al, 1988). Eight students were selected for the experiment and biopsies were collected from skin and gingiva by using a sterile biopsy punch. After going through the process, the obtained result was analyzed by various tests to ensure the effectivity of the study.

VDR immunoreactivity was tested from those individuals where positive cells were observed within the spectrum of both skin and gingiva. The mRNA expression for CAMP was about eight times higher ($P < 0.01$) in skin compared with gingiva. The expression of mRNA for VDR was identical in human skin and gingival biopsies confirming the VDR protein expression data. On the other hand, the RXRα expression was about three times higher ($P < 0.01$) in skin vs. gingiva, whereas the RXRβ expression was similar in skin and gingival biopsies.

Another study was conducted at 2008 and based on administration of oral vitamin D induces cathelicidin production in atopic individuals that basically a skin problem. Atopic dermatitis is a type of inflammation which results in itchy, red, swollen and cracked skin. It affects 10% to 20% of children and 1% to 3% of adults (Schultz-Larsen FV, 2002). Individuals with atopic dermatitis are at an increased risk for cutaneous infections with Staphylococcus aureus, herpes simplex, and the small pox or vaccinia virus (Boguniewicz M, 2006). It has been shown that defects in the capacity to increase the production of cathelicidin, may account for this increase in infections (Ong PY, 2002) (Schauber J, 2008). The experiment was done on 14 normal controls and 14 atopic individuals suffering from moderate to severe atopic dermatitis. Firstly, their biopsies were taken and baselines of calcium and vitamin D were obtained. 4000 IU/day of oral vitamin D3 was given to the subjects for 21 days. After the finite time, biopsies were collected again along with serum calcium and vitamin D level. Those samples had gone under analysis with proper methods and techniques.

From the result of this experiment, AD lesional skin showed a significant increase in cathelicidin expression from a median of 3.53 relative copy units (RCU) before supplementation to a median of 23.91 RCU post supplementation and for nonlesional skin median value was from 1.50 RCU to 1.75 RCU. On the other side, normal skin showed a modest increase from a median of 1.0 RCU to

1.78 RCU. Immunofluorescence staining of duplicate skin biopsies from lesional skin confirmed results from RT-PCR and demonstrated an increase in cathelicidin protein after oral vitamin D supplementation (Tissa R. Hata, 2008). The intake of oral supplement also increased the level of 25-hydroxyvitamin D level (Vieth R, 2001). During this time, their serum 25-hydroxyvitamin D levels rose from a median of 24.5 mg/mL to 37 mg/mL. Similarly, atopic subjects' serum calcium levels also decreased from a median of 9.6 mg/dL to a median of 9.4 mg/dL post supplementation, and their serum 25-hydroxyvitamin D levels rose from a median of 22.5 mg/mL to 35.5 mg/mL. Analysis of previous data has shown that response to wound or disruption of the epidermal barrier, cathelicidin is induced (Schauber J D. R., 2007). But this data had shown that there is a small (but not statistically significant) increase in cathelicidin in AD lesional skin before treatment with vitamin D indicative of some ability of the atopic subject to induce cathelicidin with disruption of the epidermal barrier (Tissa R. Hata, 2008).

5.3 Sodium Phenylbutyrate

Sodium phenylbutyrate or 4-phenylbutyrate or phenylbutyrate (PBA) is a salt of an aromatic fatty acid and chemical formula is $C_{10}H_{11}NaO_2$. Basically it is converted into phenylacetate by β-oxidation (Spira AI, 2003). It is produced from dietary fibers by bacterial fermentation in the colon.

PBA is a histone deacetylase inhibitor which can be used as a mood stabilizer and anti-epileptic (Iannitti & Palmieri, 2011).

Phenylbutyrate has several clinical applications in which some of them are already applied and some are still under research. One of the important role of phenylbutyrate has been found that it can stimulate the production of LL-37 which is the only antimicrobial peptide of cathelicidin family in human body. To establish the fact, PBA was treated with various cell lines (Jonas Steinmann, 2009). It also has been said that vitamin D3 worked synergistically with PBA to boost up antimicrobial peptide expression at mRNA level. Phenylbutyrate induces cathelicidin in four cell lines and those are human bronchial epithelial cell line (VA10), renal carcinoma cell line (A498), colonic adenocarcinoma cell line (HT-29) and leukemic monocyte lymphoma cell line (U937). Same dose and time duration was given to all of the cells and increased level of cathelicidin was related to the dose and increased with time. It increased gradually with a period

of 48hours and PBA concentration was up to 4mM. At the opposite side, this gene expression by PBA can be hampered when treated with cyclohexidine and MAP kinase inhibitors because from previous studies it revealed that MAP kinase signaling pathway has effect on PBA enhanced CAMP expression (Schauber, 2003).

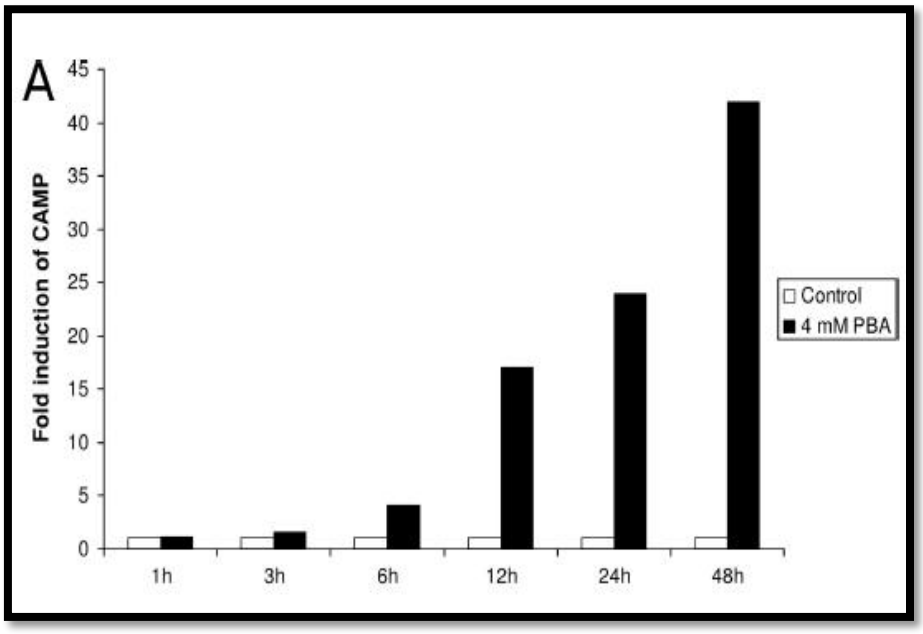

Figure 5.4: Showing cell stimulation with 4mM PBA and solvent alone (Jonas Steinmann, 2009).

The PBA concentration used in the experiment was approximately close to the reachable plasma level after oral administration. Also, it can be brought into medical field to boost up innate immunity as it has control over AMPs expression. Unfortunately, phenylbutyrate has limitation due to rapid degradation (Gilbert, 2001). To defeat the problem, some synthetic analogues of PBA has been used that shown half -lives longer stability than original in vivo (Pace, 2002). Moreover, vitamin D3 and phenylbutyrate works synergistically to improve CAMP gene expression. This can help tuberculosis patient to settle down vitamin D3 level by taking PBA as a medicine (Jonas Steinmann, 2009). From another study, an optimum dose has been found for PBA and vitamin D3 combination to boost up AMPs activity in tuberculosis which is 500 mg PB b.d. plus 5000 IU vitamin D3 o.d. taken orally (Akhirunnesa Mily, 2013). This combination can proceed intracellular killing of Mycobacterium Tuberculosis because of the induction of LL-37 expression. It also has been said that intracellular killing is mediated by autophagy and apoptosis.

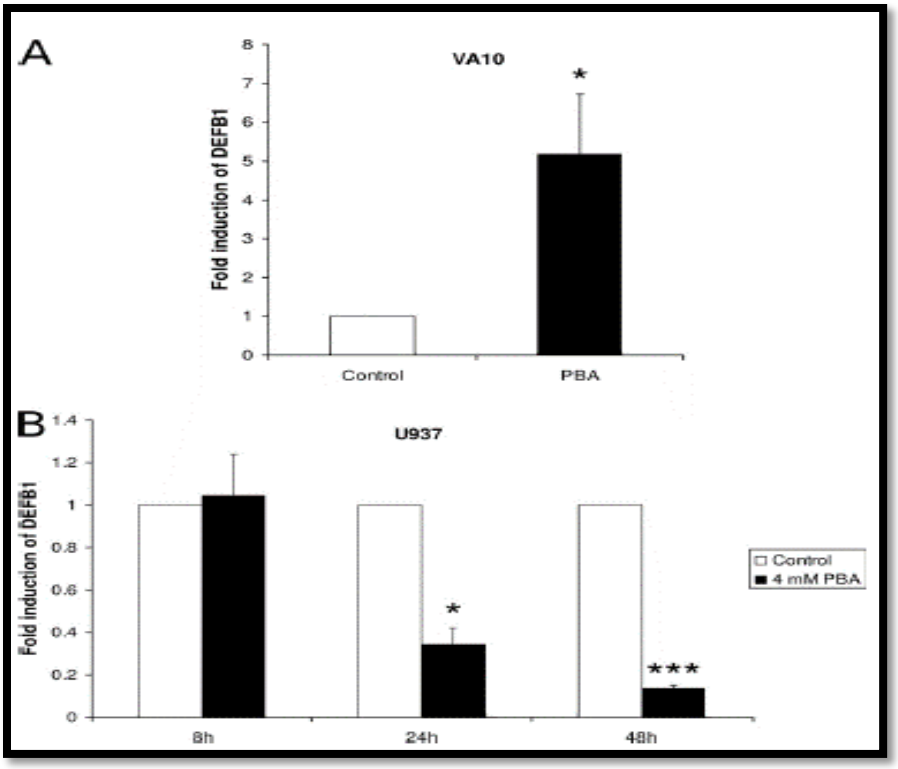

Figure 5.5: A) Showing result for expression of beta defensin 1 in VA10 cells; B) Showing results for expression of beta defensin 1 in U937 cells (Jonas Steinmann, 2009).

On the other hand, phenylbutyrate does not induce defensin family rather than beta defensin-1. At the same dose, PBA only enhances beta defensin-1 but not beta defensin-2, 3 or 4 in VA10 cells. But it gave negative result in U937 cells. So, it varies from cell to cell type.

5.4 Isoleucine

Isoleucine is an essential, nonpolar, uncharged and branched chain aliphatic amino acid. It is the isomer of leucine and encoded like ATT-ATC-ATA (Center for Biological Sequence Analysis, University of Denmark). In the chemical structure, two groups and one side chain has been found which are α-amino group, α-carboxylic acid group and hydrocarbon side chain as consecutively. As it is essential amino acid, daily diet or supplements can fill up the gap of isoleucine in human body. The diet source for this element are meat, fish, cheese, eggs, lamb, soy protein and soy seeds (List is in order of highest to lowest of per 200 Calorie serving of the food, not volume or weight).

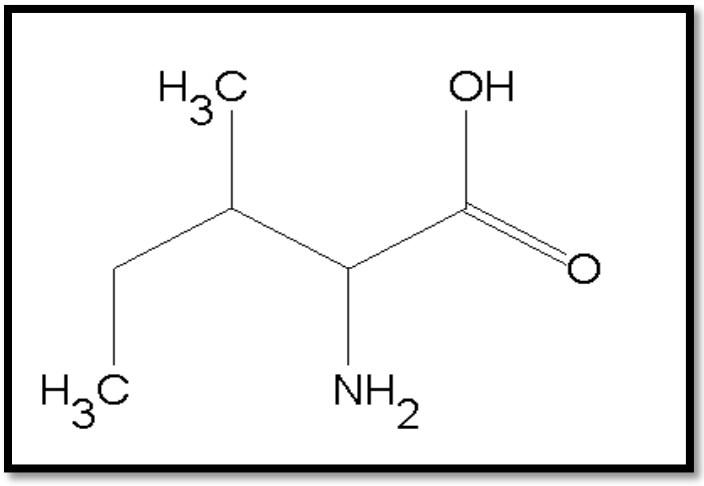

Figure 5.6: Chemical structure of isoleucine.

Isoleucine has various physiological functions including protein synthesis, induction of muscle protein synthesis, increase glucose uptake, detoxification, stimulate immune function and wound healing etc. It can be synthesized from plants and microorganisms following some steps which starts with pyruvic acid and alpha-ketoglutarate (Kisumi, Komatsubara, & Chibata, 1977).

In the middle of two enantiomers (L & D) of isoleucine, L-isoleucine is the potent inducer of epithelial beta defensin expression. The amount of isoleucine needed to reach at the peak level is between 3.12 - 12.5 µg/ml in MDBK (Madin–Darby bovine kidney) cells (Pascale Fehlbaum, 2000). On the other hand, D-isoleucine require more concentration which is approximately 200µg/ml. Moreover, the lower defensin activity has been observed with high concentration of this enantiomer. The micromolar quantity of L-isoleucine can serve as a marker for detecting microbes

and activate the antimicrobial action. It works through binding with receptor or enzyme intracellularly but the nature of receptor was unknown (Pascale Fehlbaum, 2000). It is also revealed that the inactivation of NF-κB by pharmacologic inhibitor causes down regulation of isoleucine mediated defensin production. So, isoleucine can be used as an immunostimulant for this particular AMP production.

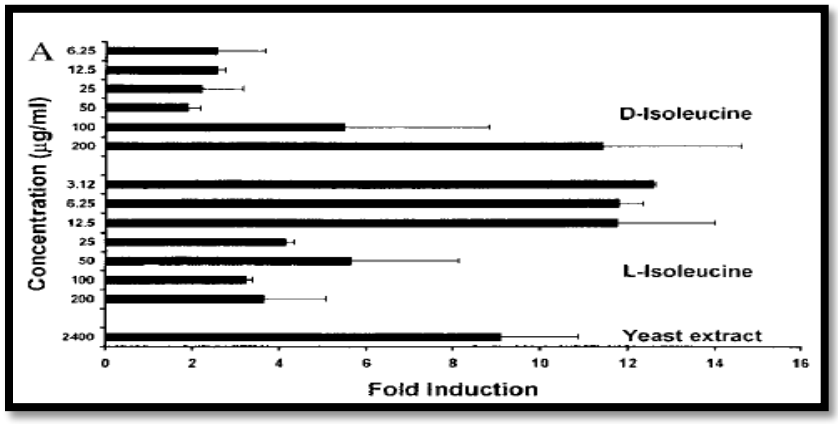

Figure 5.7: Relative expression of epithelial beta defensin in treatment with both L & D- isoleucine.

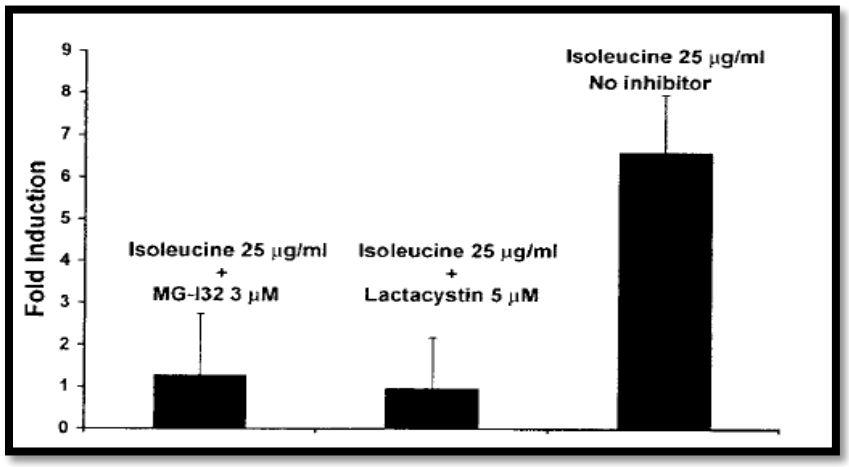

Figure 5.8: Proteasome inhibitors MG132 and Lactacystin block isoleucine activation of the defensin promoter.

Being inspired from the positive result, L- isoleucine was used in a murine model with progressive pulmonary tuberculosis to see the level of beta defensins expression (C. E. Rivas-Santiago, 2011). Isoleucine was injected into the mice after 8 weeks of infection. Significantly, both beta defensin 3 & 4 were induced with less tissue destruction even in mice infected with multidrug resistance bacilli. Though it could not fully cure the disease but might be used as a control therapy against this infectious one.

5.5 Sulphoraphane

Sulforaphane (SFN) is a phytochemical agent containing isothiocyanate group in the chemical structure. The main source of sulforaphane is cruciferous vegetable such as, broccoli, Brussels sprout, cauliflower and cabbage etc. It is also known as broccoli extract because of their highly abundance in broccoli sprouts in the form of sulforaphane glucosinolate. It appears to be well absorbed from the intestine after oral administration and bioavailability is about 74% (Petri N, 2003). Sulforaphane works as an anticancer agent by epigenetic changes through inhibiting histone deacetylase (HDAC) activity. In colorectal cancer, it restricts cell proliferation and stimulates apoptosis and also does in prostate cancer (Myzak MC, 2006). This histone deacetylase inhibitor sulforaphane is responsible for inducing human beta defensin 2 in intestine epithelial cell.

In order to explore the above fact, an experiment was conducted which was based on colorectal cancer (Markus Schwab, 2008). In the experiment, human colorectal cancer cell lines Caco-2, HT-29 and SW480 were used and processed to make ready for doing various tests. From the result, it had been known that SFN can directly induce HBD-2 mRNA and protein in colonocyte. In HT-29 cells, HBD-2 mRNA was upregulated after 24 and 48hrs of treatment in a dose-dependent manner significantly in ≥ 5 μM concentration.

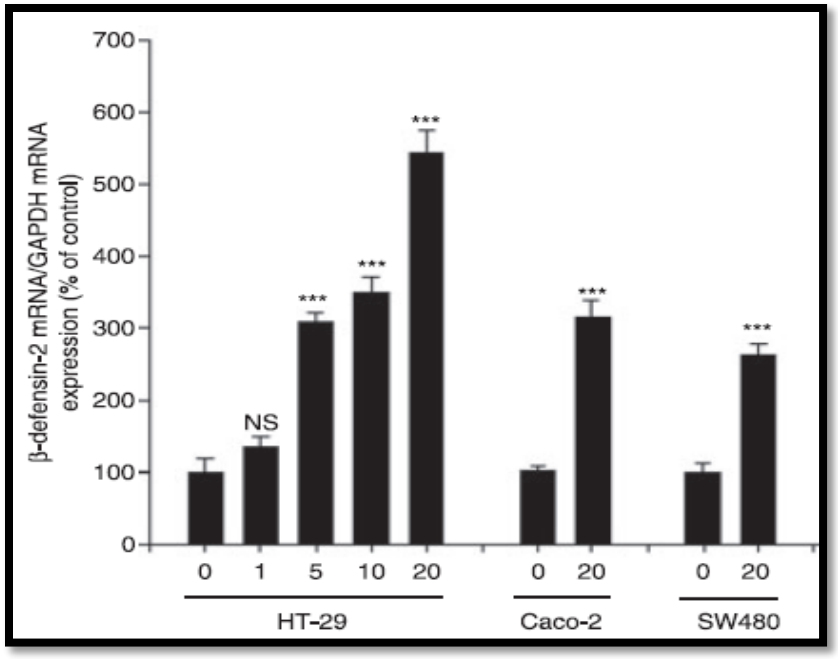

Figure 5.9: Dose dependent expression of sulphoraphane (1-20 µM) beta-defensin 2 in colorectal cell lines.

Also, HBD-2 gene was expressed upward in Caco-2 and SW480 after 24hrs. Following that, protein level was measured by ELISA where increasing HBD-2 level has been show. SFN influences the production of AMPs via vitamin D receptor (VDR) protein/ extra cellular-regulated kinase 1/2 and nuclear factor-κB signaling pathway. Upon the blocking of VDR, SFN mediated HBD-2 expression was down regulated. But in case of PPARɣ and p38 MAPK, it remains unchanged. At the opposite side, NF-κB pathway is recognized as important factor for HBD-2 in colon epithelial cells (Wehkamp J, 2004). So, SFN can be useful in the therapy of colonic Crohn's disease.

5.6 Bile Salt

Bile salts are the derivatives of bile acids which contains sodium and potassium salts. Bile acids are steroid in nature which can be found in the bile of animals. There is basically three types of bile acids: primary, conjugated and secondary bile acids. Among them chenodeoxycholic acid is a primary bile acid which is synthesized in the liver. It has various function such as dissolve gallstones, treatment of cerebrotendineous xanthomatosis and constipation etc (Thistle JL, 1973) (Berginer VM, 1984).

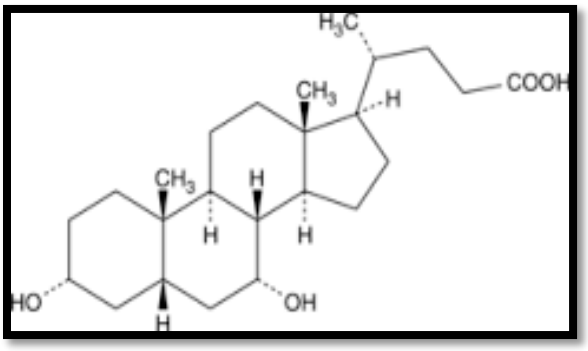

Figure 5.10: Chemical structure of chenodeoxycholic acid.

Ursodeoxycholic acid is a secondary bile acid which is metabolized from primary bile acids by intestinal bacteria. It has some significant medical uses for example reduction in gallstone formation, treatment of primary biliary cholangitis and improve bile flow in cystic fibrosis etc.

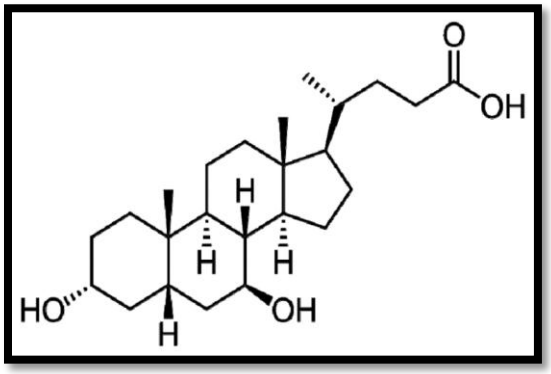

Figure 5.11: Chemical structure of ursodeoxycholic acid.

Bile salts may contribute to the cathelicidin expression in human biliary epithelium. In case of pathogen attacks, cathelicidin always expresses in the lumen of the biliary tract to defeat them. Again, we know that cathelicidin has affinity for VDR in biliary epithelial cells. Study showed

that both CDCA and UDCA induce the expression of AMP through two different nuclear receptors and they are farnesoid X receptor and vitamin D receptor (EMILIE D'ALDEBERT, 2009). Farnesoid X receptor is activated by CDCA and consequently act upon cathelicidin expression. Again, UDCA increases VDR protein expression in biliary epithelial cells resulting in AMP production. So, UDCA is dependent on VDR but CDCA is not.

It also has been found that combination therapy of CDCA, UDCA and VD3 can give better result in inflammatory biliary diseases, like primary biliary cirrhosis (Poupon RE, 1991) (Corpechot C, 2005).

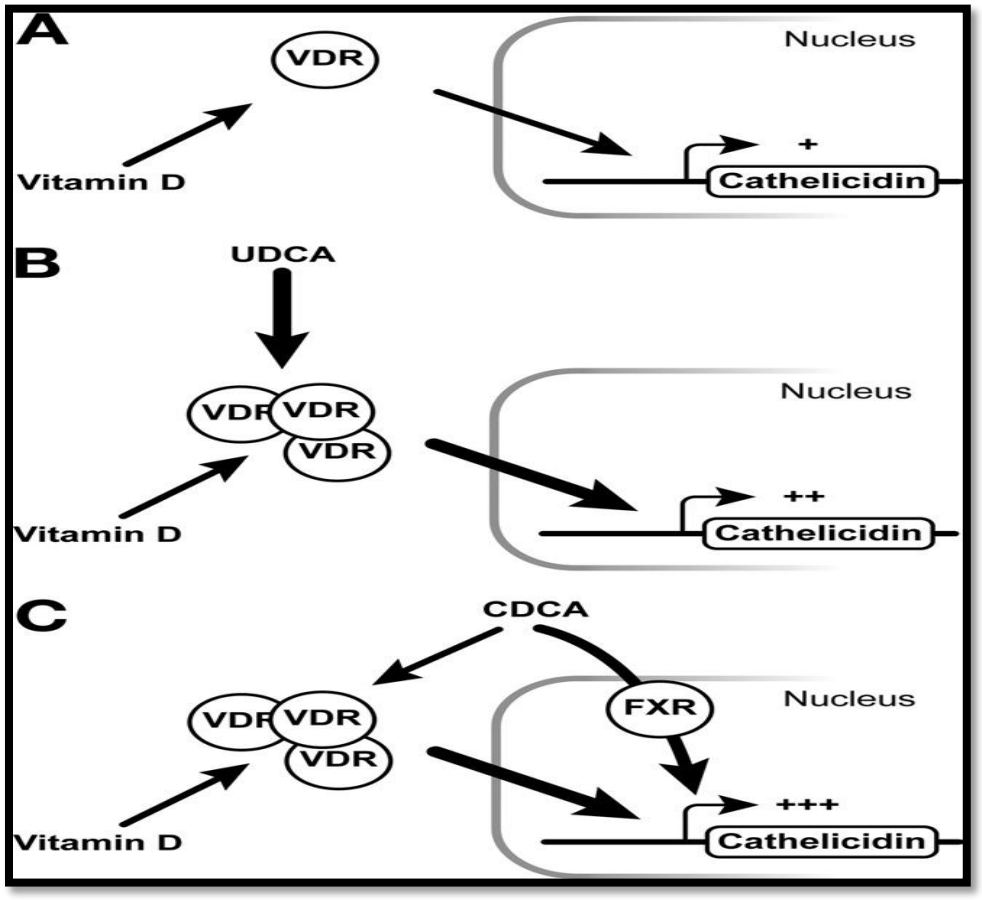

Figure 5.12: Showing possible effect of CDCA and UDCA along with vitamin D on cathelicidin's activity (EMILIE D'ALDEBERT, 2009).

5.7 Platelet Release Growth Factor

Platelet release growth factor is a constituent of whole blood serum which is purified from human platelets (Heldin C.H, 1999). It works as a mitogen for fibroblast, smooth muscle cell and glial cells. It helps in cell growth, division and angiogenesis. Studies shown that recombinant PRGF used to treat bone loss and chronic ulcer (GE, 2013).

The induction of HBD-2 occurs with the introduction of PRGF and in primary keratinocyte but in dose and time dependent manner (Andreas Bayer, 2016). When PRGF was treated with sample in vitro, HBD-2 gene expression was shown to increase after 24, 48 and 72 hours of PRGF stimulation. On the other hand, Vivostat PRF® also show positive result towards proliferation of HBD-2 in keratinocytes in the treatment of cutaneous wounds.

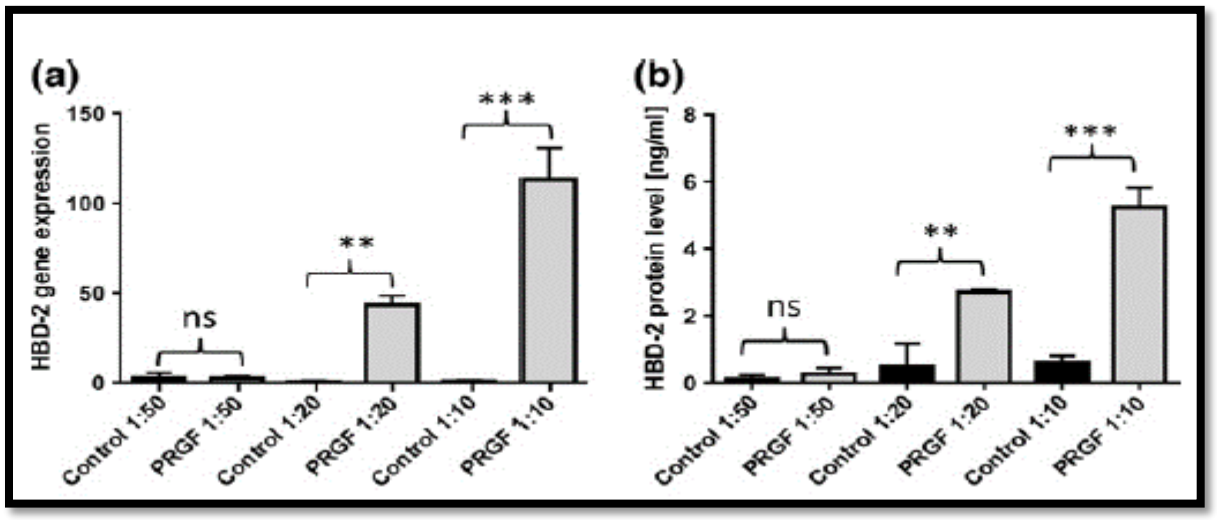

Figure 5.13: a) Showing HBD-2 gene expression with different concentration of PRGF; b) Showing protein level of HBD-2 with same concentration of PRGF as before (Andreas Bayer, 2016).

On the other hand, Vivostat PRF® also show positive result towards proliferation of HBD-2 in keratinocytes in the treatment of cutaneous wounds. In control part, wounds were treated with NaCl 0.9% and another part was treated with Vivostat PRF®. After ten days they were examined to see the HBD-2 level and the result was so much satisfactory.

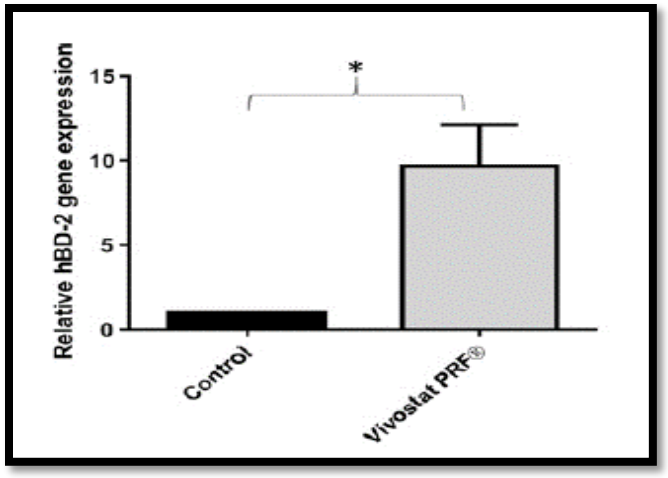

Figure 5.14: Showing the level of HBD-2 gene expression after treatment with Vivostat PRF® (Andreas Bayer, 2016).

In addition, epidermal growth factor receptor (EGFR) is potentially involved in this induction mechanism. This fact had been confirmed when an antibody (Cetuximab) is used to inactivate EGFR and HBD-2 level was drastically fallen down as a consequence. Moreover, induction of interleukin 6 (IL-6) was found in PRGF mediated experiment after 4hours stimulation. IL-6 is able to further increase activated protein 1 activity (Chuang J-Y, 2014).

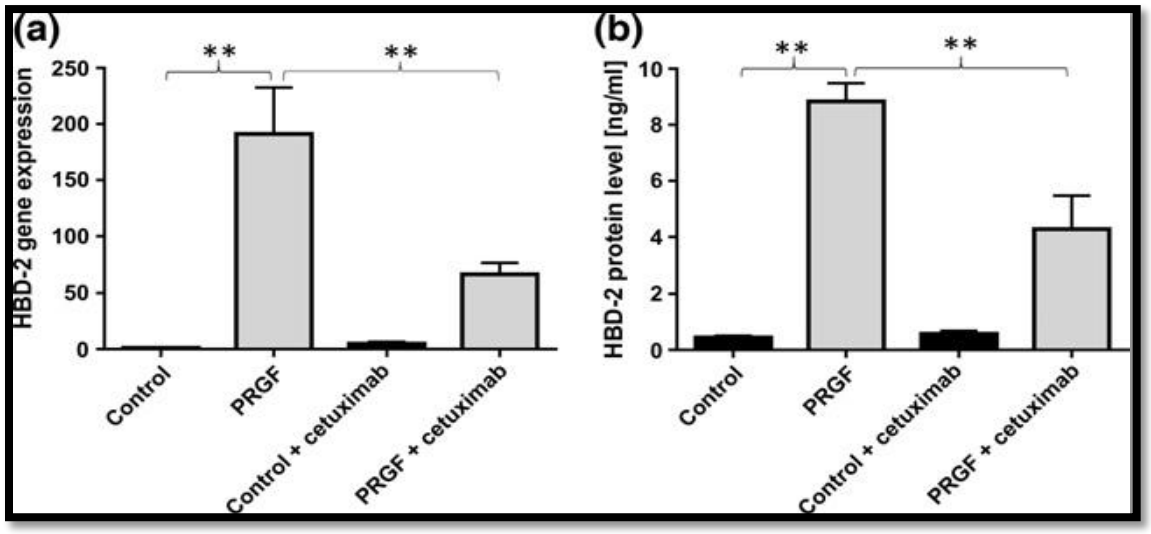

Figure 5.15: a) Showing HBD-2 gene expression upon activation of EGFR with cetuximab; b) Showing protein level of HBD-2 over that condition (Andreas Bayer, 2016).

6. Discussion:

In this study, some important factors are discussed in regulating antimicrobial peptide's production in human body. All of them work on cathelicidin and/or beta defensins. Epigallocatechin-3-gallate, isoleucine, sulphoraphane and platelet release growth factor act upon beta defensins family. On the other hand, vitamin D3 and bile salt act upon cathelicidin (LL-37). Meanwhile, phenylbutyrate effects on both cathelicidin and beta defensin 1.

Epigallocatechin-3-gallate which is a polyphenol present in green tea improve the immunological activity of intestinal epithelial cell. Approximately, 25μM is needed to get significant expression in both beta defensins. This effect can be useful to treat Crohn's diseases and inflammatory bowel diseases (Wehkamp, 2005). L-isoleucine can increase the level of beta defensins within a specific limit. This isoleucine can work as microbial marker to increase defensin activity and they act through a receptor which is still unknown. Sulphoraphane can directly induce gene expression of beta defensin 2. It also has dose dependent activity which is more than 5μM concentration. Platelet release growth factor also increase beta defensin 2 in keratinocytes in vitro. Again, Vivostat PRF® helps to regulate hBD-2 and treat chronic wounds.

Vitamin D3 is a factor which act upon cathelicidin expression. Though we know vitamin D3 binds with vitamin D receptor to become activate but in case of this regulation it must be bind with retinoid X receptor α. Moreover, the CAMP expression will increase where RXR activity is highest which is in skin. So, this vitamin D3 mediated cathelicidin expression can be used to treat atopic dermatitis. Moreover, supplementation of vitamin D injection can increase LL-37 level in ulcerative colitis patients (Sharifi A, 2016). Bile salts also induce cathelicidin expression in human biliary epithelium through farnesoid X receptor and vitamin D receptor. But a combination therapy of bile salts and vitamin D3 can give satisfactory result in primary biliary cirrhosis.

Phenylbutyrate is such a factor which can increase both cathelicidin and beta defensin 1. It induces cathelicidin expression in bronchial epithelial cell line. It works in dose and time dependent manner. Various synthetic analogs is used in medical field to avoid degradative nature of original one. Moreover, tuberculosis patient might be helpful by taking vitamin D3 and phenylbutyrate combination as a medicine because it reinforces the activity of phenylbutyrate. On the contrary, beta defensin 1 is induced by phenylbutyrate which again depend on cell to cell type. But no activity had been observed in beta defensin 2 expression with this factor.

Though all the factors are identified as the effective one but still some lackings are lying there. Many of them are not gone for human study or other animal study rather than one. So, the opportunity to get new medicines in relative diseases is dying. Phenylbutyrate is not stable in normal condition which is a big fault for introducing them as a drug or supplementary. Few of them need more intensive study to understand their proper mechanism of action. If all the drawbacks are taken into account and solved them in most possible way then these factors become more effective.

In the conclusion, it can be said that antimicrobial peptide has become a need for patient suffering of resistance issue from various antibiotics. It is the natural killer which can diminish microbial colonization in the body and keep free from being infected without developing resistance. Proper handling of these factors can make them useful for our benefits.

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