Chapter - 1

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Introduction

1.1 Historical Perspective

The principle of using organic compounds to fight infection is known since ancient times, although the mechanisms of antibiotic action were not scientifically understood until the late 20th century. Crude plant extracts were used medicinally for centuries, and there is anecdotal evidence for the use of cheese molds for topical treatment of infection. The first observation of what would now be called an antibiotic effect was made in the 19th century by French chemist Louis Pasteur, who discovered that certain saprophytic bacteria can kill anthrax bacilli.

The term antibiotic (in Greek, 'anti' = against; 'bios' = life) was defined by Vullemin in 1889 and redefined by Wakesman in 1945 as chemical substances of microbial origin which exerts antimicrobial activity in small amounts. The antibiotic era involved the work of several pioneering scientists. Pioneering work in systematic search of antibiotics was first done by Gratia and Bath in 1924, which later resulted in discovery of Actinomycetin in strains of *Actinomycetes.* In 1929, Alexander Fleming, of St. Mary's Hospital in London, published a paper in the *British Journal of Experimental Pathology* describing the isolation of penicillin from *Penicillium* mold and its potential use. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms. Penicillin in its original form could not be given by mouth because it was destroyed in the digestive tract and the preparations had too many impurities for injection. No progress was made until the outbreak of World War II which stimulated renewed research and the Australian pathologist Sir Howard Florey and German-British biochemist Ernst Chain purified enough of the drug to show that it would protect mice from infection.

In 1935, Gerhard Domagk at I.G. Farben, Germany synthesized the first synthetic antibacterial drug, Prontosil, whose active ingredient sulfanilamide the prototype for all sulfa drugs was later identified by the Pasteur Institute's Daniel Bovet. And in 1939, René Dubos in Oswald Avery's laboratory at the Rockefeller Institute for Medical Research in New York identified tyrothricin a mixture of the peptide antibiotics tyrocidin and gramicidin D from soil bacteria - widely regarded as the first antibiotic to be established as a therapeutic

substance. This substance is too toxic for general use, but it is employed in the external treatment of certain infections. Other antibiotics produced by a group of soil bacteria called actinomycetes have proved more successful. One of these, streptomycin, discovered in 1944 by American biologist Selman Waksman and his associates, was, in its time, the major treatment for tuberculosis.

In the early 1940s, the industrialization of penicillin production was quickly followed by the successful isolation and development of a large number of antibiotics (Watve et al, 2001) that have led to most of the major classes of antibiotics in use even to this day, namely, the tetracylines, lipopeptides, macrolides, aminoglycosides, cephalosporins, chloramphenicol, glycopeptides and rifamycins (Grunewald et al., 2004, Miao et al., 2006). As the majority of existing compounds originated from bacteria, the term 'antibiotics' has become almost synonymous with the more inclusive term of antibacterial agents (Yu et al., 1999).

Since antibiotics came into general use in the 1950s, they have transformed the patterns of disease and death. Many diseases that once headed the mortality tables—such as tuberculosis, pneumonia, and septicemia—now hold lower positions. Surgical procedures, too, have been improved enormously, because lengthy and complex operations can now be carried out without a prohibitively high risk of infection.

1.2 Classification

Antibiotics in its definition covers a large number of structurally dissimilar molecules acting selectively or nonselectively on large number of pathogenic bacteria affecting to cause various diseases. Further these molecules bring about the desired result by acting at varied targets at different concentrations. Due to involvement of so many variables, there are several ways by which antibiotics can be classified. The most common method classifies them according to their spectrum of effectiveness over a range of pathogens, the mode by which they accomplish their task and classification based on the chemical nature of the molecule.

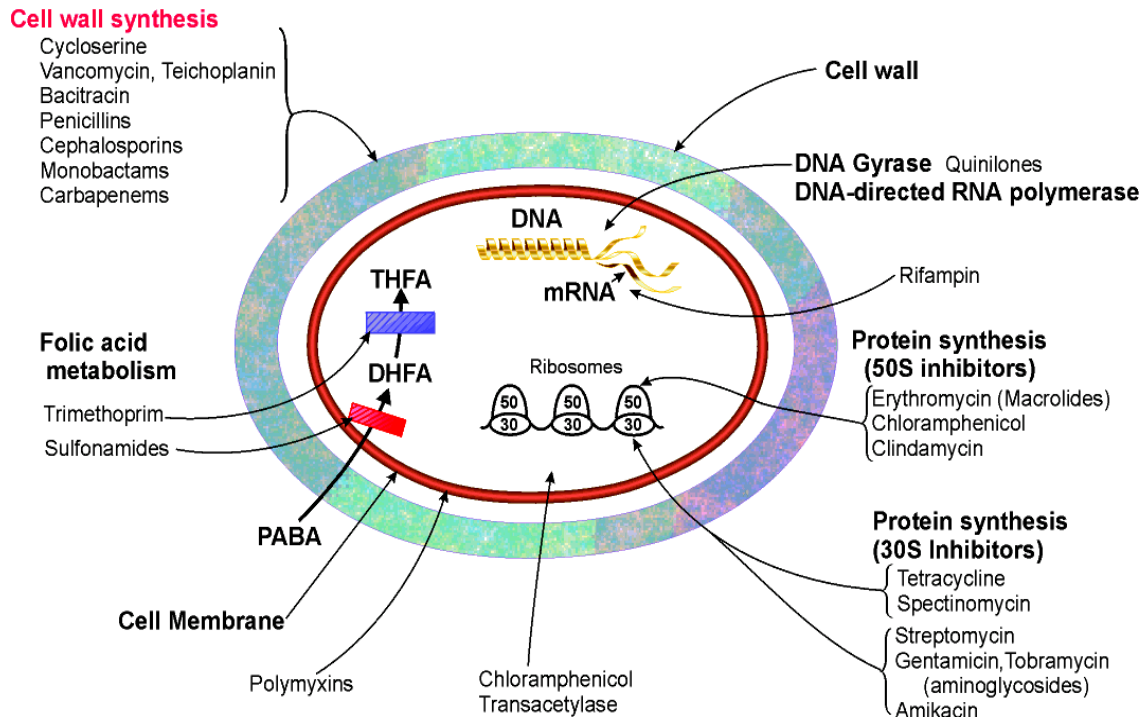## 1.2.1 Classification Based On Mode Of Action

Cell wall synthesis
Cycloserine
Vancomycin, Teichoplanin
Bacitracin
Penicillins
Cephalosporins
Monobactams
Carbapenems

Cell wall

DNA Gyrase Quinilones
DNA-directed RNA polymerase

DNA

THFA

mRNA

Rifampin

Folic acid metabolism

DHFA

Ribosomes

Protein synthesis (50S inhibitors)
Erythromycin (Macrolides)
Chloramphenicol
Clindamycin

Trimethoprim
Sulfonamides

50
30

50
30

50
30

Protein synthesis (30S Inhibitors)
Tetracycline
Spectinomycin

PABA

Streptomycin
Gentamicin,Tobramycin
(aminoglycosides)
Amikacin

Cell Membrane

Polymyxins

Chloramphenicol
Transacetylase

Fig -1.1 : Diagramatic representation of various antibiotic targets in a bacerial cell.

A) Cell Wall Inhibitors

Antibiotic agents usually diffuse easily through cell walls of Gram +ve bacteria, but in Gram –ve bacteria they need to go through narrow walls which is relatively difficult. Peptidoglycan is the critical attack site in cell wall inhibition because it is not found in eukaryotes, and it's loss would lead to cell death. It is a cross-linked complex made of polysaccharides and peptides. The cross linked structure is the secret of its great strength. Different antibiotics attack the cell wall at various stages of its synthesis. While fosfomycin and cycloserine inhibits precursor formation in the cytosol, Bacitracin interacts with the carrier of this precursor and thereby preventing its transport. Transglycosylases and

transpeptidases reticulate the peptidoglycan units. β–lactams binds competitively with transpeptidases making it unavailable for D-ala-D-ala. Similarly, glycopeptides inhibit both transglycosylases and transpeptidases by binding to the D-ala-D-ala termini making the site unavailable.

Glycopeptides (vancomycin), Bacitracin, Cycloserin, Fosfomycin, and the β–lactams (penicillins, cephalosporines, monobactams, carbapenems) are important some of the important drugns acting on bacterial cell envelope.

B) Cell Membrane Inhibitors:

These cause disorganization of the membrane. This disorganization leads to formation of leaky membranes that cause draining of ions out of the cells leading to cell death. Polymyxin B and colistin (polymyxin E) are low molecular weight octapeptides that inhibit Gram – ve bacteria with –vely charged lipids on the surface by cations to leak out of the cell so the cell dies. Fungal membranes have sterols while bacterial membranes don't. Polyene antibiotics bind to the sterols and make a pore in the membrane and the contents leak out. This does not work in prokaryotes.

C) Inhibitors of DNA replication

Fluoroquinolones block the action of DNA gyrase and DNA topoisomerase IV that control and modify the topological states of DNA in cells. They do this by relieving supercoils, which can form during the unwinding of DNA for replication or transcription. Quinolones block topoisomerases by binding to DNA gyrase and DNA. Nitroimidazoles nitro group is reduced by an electron transport protein in anaerobic bacteria, which causes the DNA strand to break. Host cells are unharmed because they don't have the enzyme required.

D) Inhibitors of RNA polymerase

Rifampins binds to a β-subunit of RNA polymerases and prevents initiation of DNA transcription. Mammalian mitochondrial RNA synthesis is not impaired significantly.

E) Inhibitors of nucleotide metabolism

Though not true antibiotics, acyclovir and flucytosine are known to inhibit nucleotide metabolism. Acyclovir inhibits viruses by getting converted to a tiphosphate and inhibiting the thymidine kinase and DNA polymerase of the herpes viruses. Flucytosine inhibits yeast by being converted to 5-fluorouracil which inhibits thymidylate synthetase so there are not enough thymine nucleotides to replicate DNA.

F) Protein Synthesis Inhibitors

This is the most popular target of antibiotic actions. A large number of antibiotics affect the process of protein synthesis at different steps. Aminoglycosides bind to specific ribosomal proteins and to a major deep grove in the rRNA. Streptomycin was the first studied and it has a different mechanism from other aminoglycosides. It binds to the S12 protein and causes the ribosome to misread the genetic code. Others also bind the L6 protein of the 50S ribosome. Eukaryotes are relatively unaffected but ribosomes in the mitochondria are sensitive to their effects. Tetracyclines inhibit binding of aminoacyl-tRNA into the A site of the bacterial ribosome. Macrolides, Ketolides and Lincinoids have large lactone rings. They bind to the peptidyl side of the 50S subunit, impair peptidyltransferase and interfere with the translocation of the peptide chain from A to P site, and promote dissociation of peptidyl-tRNA from the ribosomes. Chloramphenicol binds to a peptidyltransferase enzyme on the 50S ribosome. Streptogramin are relatives of macrolides and binds to 50S subunit of ribosomes. Oxazolidinones binds to 50S near the 30S interface which prevents the 30S initiation complex from forming the 70S complex, which blocks initiation of protein synthesis.
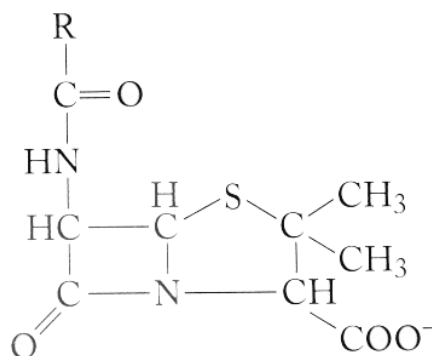
G) Metabolic Inhibitors

Bacteria synthesize folic acid while humans obtain it from their diet. Sulfonamides and trimethoprim block the biosynthesis of tetrahydrofolate, which is a carrier of 1C fragments and is necessary for DNA, RNA, and cell wall synthesis.

1.2.2 Classification based on chemical nature of molecule
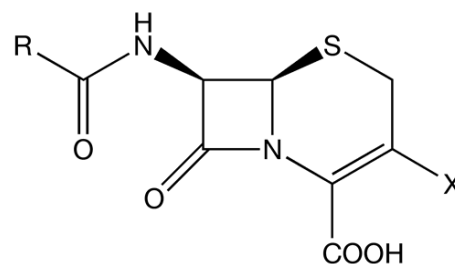
A) Penicillins

The penicillins are the oldest class of antibiotics. Penicillins have a common chemical structure which they share with the cephalopsorins. Penicillins are generally bactericidal, inhibiting formation of the cell wall. The natural pencillins are based on the original penicillin-G structure. Penicillin-G types are effective against gram-positive strains of streptococci, staphylococci, and some gram-negative bacteria such as meningococcus. Penicillinase-resistant penicillins are active even in the presence of the bacterial enzyme that inactivates most natural penicillins. Extended spectrum penicillins are effective against a wider range of bacteria. Aminopenicillins such as ampicillin and amoxicillin have an extended spectrum of action compared with the natural penicillins.
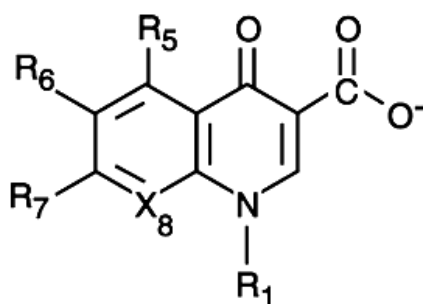
B) Cephalosporins

Cephalosporins have a mechanism of action identical to that of the penicillins. However, the basic chemical structure of the penicillins and cephalosporins differs in other respects, resulting in some difference in the spectrum of antibacterial activity. Like the penicillins, cephalosporins interfere with synthesis of the bacterial cell wall and so are bactericidal. Cephalosporins are among the most diverse classes of antibiotics, they are grouped into "generations" by their antimicrobial properties. Each generation has a broader spectrum of activity than the one before.The first generation cephalosporins (cephalothin, cefazolin, cephapirin, cephradine, cephalexin, cefadroxil.) possess generally excellent coverage against most gram-positive pathogens and variable to poor coverage against most gram negative pathogens. This limitation was worked out in second generation cephalosporins (cefaclor, cefamandole, cefonicid, ceforanide, cefuroxime) with an expanded gram-negative spectrum as well. The third generation cephalosporins (cefcapene,

cefdaloxime, cefditoren, cefetamet, cefixime, cefmenoxime, cefodizime, cefoperazone, cefotaxime, cefpimizole, cefpodoxime, ceftibuten, ceftriaxone) have the advantage of convenient dosing schedules. The fourth generation cephalosporins (cefclidine, cefepime, cefluprenam, cefozopran, cefpirome, cefquinome) are extended-spectrum agents with similar activity against gram-positive organisms as first-generation cephalosporins. They also have a greater resistance to beta-lactamases (bacterial enzymes that may destroy antibiotic before it can do its work) than the third generation cephalosporins. Many fourth generation cephalosporins can cross blood brain barrier and are effective in meningitis.
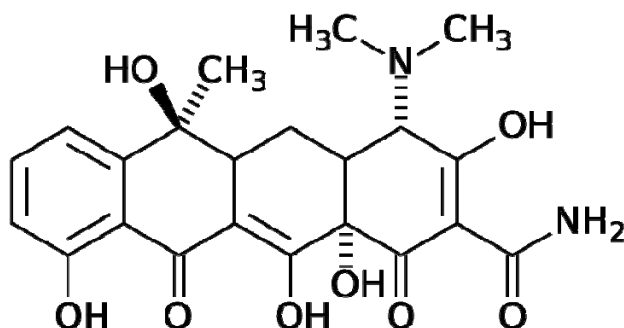
C) Fluoroquinolones

Fluoroquinolones are the newest class of antibiotics. Their generic name often contains the root "floxacin". They are synthetic antibiotics that belong to the family of antibiotics called quinolones. The older quinolones are not well absorbed and are used to treat mostly urinary tract infections. The newer fluoroquinolones are broad-spectrum bacteriocidal drugs that are chemically unrelated to the penicillins or the cephaloprosins. Because of their excellent absorption fluoroquinolones can be administered not only by intravenous but orally as well.Commonly used fluoroquinolones include ciprofloxacin, levofloxacin, lomefloxacin, norfloxacin, sparfloxacin, clinafloxacin, gatifloxacin, ofloxacin, trovafloxacin.

D) Tetracyclines

Tetracyclines got their name because they share a chemical structure that has four rings. They are derived from a species of *Streptomyces* bacteria. Tetracycline antibiotics are broad-spectrum bacteriostatic agents that inhibit bacterial protein synthesis.
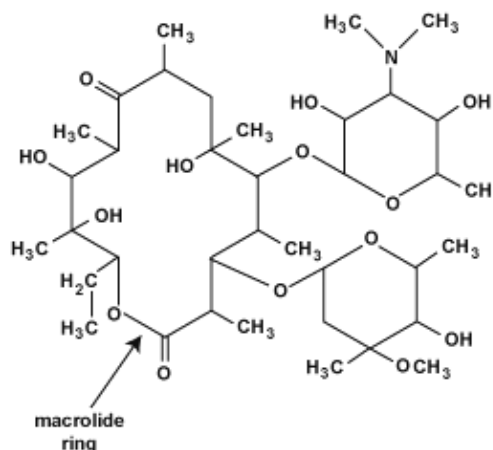
Tetracyclines are used in the treatment of infections of the respiratory tract, sinuses, middle ear, urinary tract, skin, intestines. Tetracyclines also are used to treat Gonorrhoea. Their most common current use is in the treatment of moderately severe acne and rosacea. The most commonly prescribed tetracycline antibiotics are: tetracycline, doxycycline, minocycline and oxytetracycline.

E) Macrolides

The macrolide antibiotics are derived from *Streptomyces* bacteria, and got their name because they all have a macrocyclic lactone chemical structure. The macrolides are bacteriostatic, binding with bacterial ribosomes to inhibit protein synthesis. Erythromycin, the prototype of this class, has a spectrum and use similar to penicillin. Macrolide antibiotics are used to treat respiratory tract infections (such as pharyngitis, sinusitis, and bronchitis), genital, gastrointestinal tract, and skin infections.The most commonly prescribed macrolide antibiotics are: erythromycin, clarithromycin, azithromycin, roxithromycin, troleandomycin.

1.3 Resistance

In 1979, the Surgeon General of United States said, " We can close the books on infectious diseases…" ...He spoke too soon. Infectious diseases were back, infact they had never left and many of them are now resistant to antibiotics.

The historical scourge known as the bubonic plague killed up to one-third of Europe's population in the 1300s. But in modern times, it has been controlled handily with the help of antibiotic drugs such as streptomycin, gentamicin and chloramphenicol. That is, until 1995, when a plague infection in a 16-year-old boy from Madagascar failed to respond to the usual antibiotic treatments. This first documented case of an antibiotic resistant plague, reported in the September 1997 New England Journal of Medicine, eventually succumbed to another antibiotic. (Dennis and Huges, 1997)

Globally, many infectious germs, including those that cause pneumonia, ear infections, acne, gonorrhea, urinary tract infections, meningitis, and tuberculosis, can now outwit some of the most commonly used antibiotics and their synthetic counterparts.

Antibiotic resistance isn't a new problem; resistant disease strains began emerging not long after the discovery of antibitocis, over half a century ago. Penicillin and other antibiotics, which were initially viewed as miracle drugs for their ability to cure such serious and often life threatening diseases as bacterial meningitis, typhoid fever and rheumatic fever, soon were challenged by some defiant strains. A stage has been reached where, resistance has no longer remained an isolated problem of few organisms turning resistant. Virtually all important human pathogens treatable with antibiotics have developed some resistance. To count a few, *Staphylococcus aureus* (MRSA) has developed resistance against most antibiotics available, by some or other mechanism, vancomycin being the last resort. A strain of *Streptococcus* (VRSE) causing pneumonia has gone a step further and developed resistance against vancomycin as well. *Neisseria gonorrhoeae, Salmonella, Mycobacterium* etc have also been reported to have developed resistance against many drugs in use. (Levy, 1998)

How do these tiny single celled organisms fight the might of man and overcome every weapon used by him? Bacteria use various mechanisms which are inheretently present in them or they might have developed them or obtained by acquization from other bacterium.

1.3.1 Types of Resistance:

A) Intrinsic resistance

Intrinsic resistance refers to bacteria that are insensitive, in their natural state, to an antibiotic without the acquisition of resistance factors. A common example is the reduced sensitivity of Gram-negative bacteria to penicillin. Gram-positive bacteria are surrounded by a thick, rigid, porous cell wall composed of peptidoglycans. It offers little resistance to the diffusion of small molecules such as antibiotics. Gram-negative bacteria have an additional outer membrane, composed of lipopolysaccharide, which is located around the cytoplasmic membrane and the thin peptidoglycan layer (Brody, 1994).

The outer, hydrophobic cell wall layer inhibits the diffusion of some unmodified penicillins conferring resistance to them (Williams, 1996). Some other examples of intrinsic
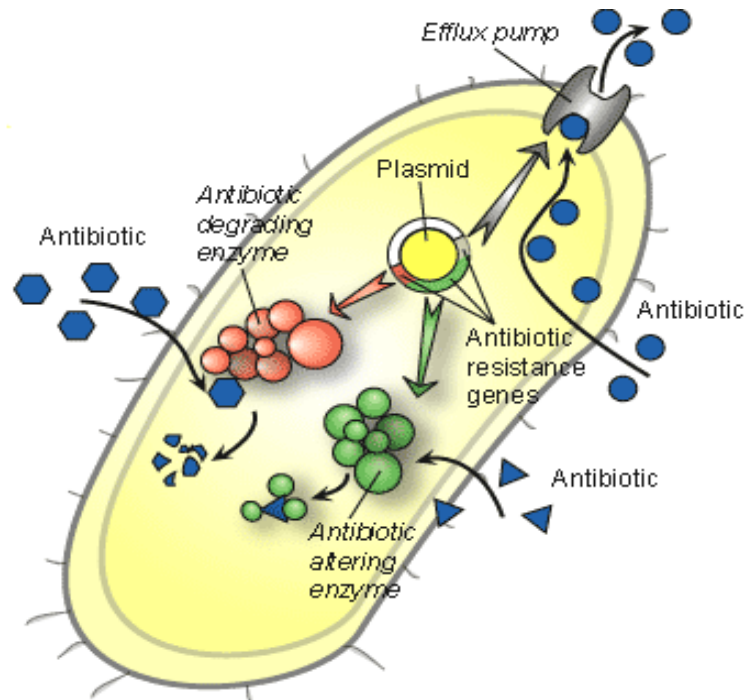

Fig- 1.2: Diagramatic representation of popular mechanisms of antibiotic resistance in bacteria

resistance include: *Haemophilus influenzae* and its ability to move between interstitial cells of the host where it cannot be reached by large, hydrophilic antibiotics such as gentamycin (Van Schilfgaarde, 1999) and the *Streptomycetes* that produce antibiotics as a means of self-protection, necessitating an intrinsic resistance to those antibiotics (Fouces, 1999; Benveniste, 1973).

B) Acquired resistance

Acquired resistance evolve via genetic alterations in the microbe's own genome or by horizontal transfer of resistance genes located on various types of mobile DNA elements (Normark *et al* 2002). Bacteria can acquire resistance to antibiotics as a result of mutation(s) in its genome, expression of latent chromosomal gene or via acquization of foreign DNA in form of plasmid, phage or transposon.

Different modes by which bacterium develop resistance has been discussed below:

i. Target Alteration

Changes in drug targets that interfere with or limit antibiotic interaction also prevent the antimicrobial agents and, thus, promote resistance. Many antibiotics inactivate a specific enzyme or, as in the case of a large number of protein synthesis inhibitors, the ribosome. One large class of resistant mutants is comprised of bacteria that, through a mutation, develop a target protein unable to bind the antibiotic, or less often, a target that retains its function even after formation of the complex. Frequently, this difference consists of substitution of a single amino acid in the protein chain (Lancini, 1995). The ribosome of *Staphylococci* can become insensitive to erythromycin following specific enzymatic modifications of rRNA (Davies, 1992). The most common mechanism of resistance to macrolides, for example, involves modification of their target site on the ribosome, specifically methylation of an adenine residue in domain V of the 23S rRNA (Weisblum 1995).

ii. Decreased influx

Many gram-negative bacteria show good susceptibility to some, beta-lactams and also to aminoglycosides, chloramphenicol, tetracyclines, quinolones, etc., which are not too large, are rather hydrophilic, and are therefore expected to diffuse rather rapidly through porin channels. On the other hand, these bacteria are resistant to a number of hydrophobic antibiotics and dyes (Nikaido, 1976) that are quite effective against gram-positive bacteria. For example, the permeability of *Pseudomonas aeruginosa* outer membrane to several cephalosporins is about two orders of magnitude lower than that of *E. coli* outer membrane (Nikaido and Hancock, 1986)

Antibiotics such as macrolides, novobiocin, the more hydrophobic beta-lactams, rifamycin, and actinomycin D form such group, that are selectively not permeated by some bacteria. These hydrophobic molecules cannot diffuse through the porin channels rapidly (Nikaido et al, 1983). The outer leaflet of the outer membrane bilayer, composed of lipopolysaccharide, appears to have an unusually low permeability and does not allow the diffusion of such hydrophobic agents (Nikaido and Vaara, 1985). Indeed, resistance to these agents is decreased drastically when the structure of the outer membrane bilayer is modified by mutational alteration (Nikaido, 1976), transient removal (Leive, 1974), or attachment of polycationic molecules (Vaara and Vaara, 1983) to its lipopolysaccharide component.

iii. Antibiotic efflux

Efflux is the pumping of a solute out of a cell. Efflux pump genes and proteins are present in both antibiotic-susceptible and antibiotic-resistant bacteria. Some systems can be induced by their substrates so that an apparently susceptible strain can overproduce a pump and become resistant. Efflux as a means of antibiotic resistance, is most commonly associated with the tetracycline group of antibiotics (e.g. TetA, TetB, TetK pumps) (Ginn et al, 2000) and the fluoroquinolones (Poole 2000) in both, Gram-positive as well as Gram-negative bacteria.

Antimicrobial resistance in an efflux mutant is either due to a.) expression of the efflux pump protein is increased or b.) the protein contains an amino acid substitution(s) that makes the protein more efficient at export. In either case, the intracellular concentration of the substrate antimicrobial is lowered and the organism becomes less susceptible to that agent. Efflux pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multiple drug resistance (MDR). Bacterial antimicrobial efflux transporters have generally been grouped into five super families, primarily on the basis of amino acid sequence homology. These include:

- MFS - Major Facilitator Superfamily (Marger, 1993; Griffith, 1992).
- ABC - ATP-Binding Cassette family (Higgins, 1992),
- RND – Resistance Nodulation Division family (Paulsen, 1996b)
- SMR - Small Multidrug Resistance protein family (Davies, 1998; Nikaido, 1994)
- MATE- Multidrug And Toxic compound Extrusion family (Paulsen, 1996a)
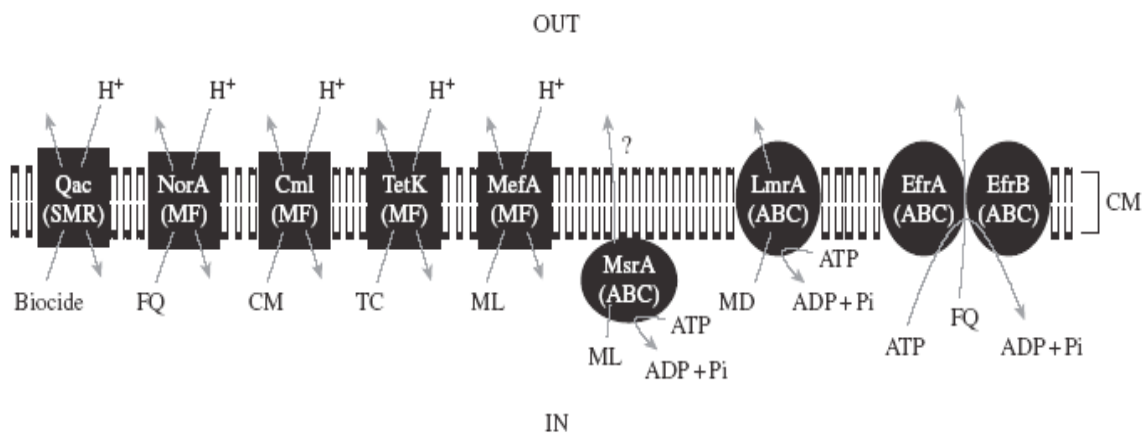

Fig-1.3: Schematic diagram of representative drug exporting systems in Gram-positive bacteria, highlighting different families of pumps involved in resistance.

(MD- multidrug, FQ- fluoroquinolone, CM- chloramphenicol, TC- tetracycline, ML- macrolides)

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Antibiotic efflux pumps fall into the RND, MFS, and MATE groups, with the RND and MATE families so far being unique to gram-negative bacteria. Thus, MFS-type transporters predominate as regards the efflux of antimicrobial agents in gram-positive organisms.

Bacterial antibiotic resistance resulting from drug extrusion was first identified in tetracycline resistant strains of *E. coli* (McMurry *et al*., 1980). Subsequently, several resistant strains were isolated by selection for growth on single toxic compounds that were cross resistant to a number of unrelated drugs and hence were identified as MDR mutants (Tennent *et al*., 1989). Mdt(a), a new efflux protein encoded by plasmid conferring multiple antibiotic resistance in *Lactococcus lactis* and *Escherichia coli* (Perreten et al, 2001).

iv. Enzyme inactivation of the drug

Enzyme inactivation is one of the most common biochemical processes that engenders resistance to a wide variety of antibiotic structural types in bacteria (Jacoby, 1991). As many of the antibiotics are produced by soil microorganisms, it is perhaps not surprising that bacteria living in the environment of antibiotic-producing organisms might produce enzymes that render antibiotics biologically inert.

Antibiotics can be inactivated either by enzymatic cleavage or by chemical modification such that they no longer interact with the target site or are no longer taken up by the organism rendering them inactive (Lancini, 1995). Chemical modification can confer clinical resistance to the amninoglycoside antibiotics, chloramphenicol, penicillins, cephalosporins and other β-lactams (Brody, 1994). The predominant mechanism of resistance to β-lactams remains β-lactamases, enzymes that inactivate the antibiotic by hydrolysing the β-lactam ring of the molecule. These enzymes can be encoded on genes that can be transferred by plasmids. (Bush et al, 1995)

v. Synthesis of resistant metabolic pathways

If a drug acts by inhibiting an enzyme that is critical for cell growth, then cells that produce greater amounts of the enzyme may be able to produce a sufficient amount of the metabolic product to survive in the presence of the inhibitory drug concentrations. (Pratt,1990). Mutants that produce more folic acid reductase are resistant to trimethoprim. Another example of this type of resistance is the thymidine-requiring streptococci that are not inhibited by trimethoprim and sulfonamides because they can produce adequate concentrations of thymidine nucleotides by an alternative pathway and therefore fail to undergo the thymineless death (Brody, 1994).

vi. Failure to metabolize the drug

When prodrugs need to be converted into the active form by the bacterium itself, failure to metabolize the drug will result in resistance (Pratt, 1990). For example, *Bacteroides fragilis* do not metabolize nitroimidazole, metronidazole to the active metabolite and are therefore resistant to this drug.

vii. Gene amplification

Gene amplification can be defined as the tandem duplication of the gene sequences to a copy number of more than hundred. It is recognized as a regulatory mechanism in both eukaryotes and prokaryotes. In eukaryotes, amplifications can be developmentally regulated or a response to metabolic stress. It is a common feature of the genome of the prokaryotic organisms. In prokaryotes, gene amplification is viewed as an adaptive response (i.e. a response to stress) (Romero et al, 1995) and as an important factor in the evolution of new genes; it has been studied extensively in gram-negative species. Gene amplification is 'adaptive' in the sense that it only occurs in response to the selective environment. Cells carrying the amplification are not hypermutated in unselected genes, and neither the SOS response nor *pol*-IV is required.

Over expression of gene expression through gene amplification may confer the phenotypic advantages needed for survival. From an energetic point of view, gene amplification should be very similar to the acquisition of a plasmid. It may be seen as an inefficient way to obtain resistance, since the organism must increase the size of its genome and needs to replicate additional genetic material, with considerable burden energy (Seoane and García-Lobo, 1991). Gene overexpression can also occur as a result of up-promoter mutations, attenuator mutations or IS element creating strong hybrid promoter. (Normark *et al* 2002).

However, gene amplification has an advantage over plasmid acquisition or promoter mutations because it is often a reversible process and the chromosome recovers the basal structure upon removal of the antibiotic from the culture medium, while the other mechanisms persist in the absence of the antibiotic (Seoane et al, 2003). Gene amplification in prokaryotes occurs through recombination between rRNA operons, IS sequences, or short DNA homologies. The rate limiting event in gene amplification is the formation of an initial duplication. Because of the remarkable potential for genetic adaptability that amplification can confer, it has long been postulated that amplification might be an adaptive response to selective conditions (Hastings et al, 2000).

1.3.2 Factors that encourage spread of resistance

As already discussed, resistance to antimicrobials is a natural biological phenomenon and there are different ways by which the pathogens achieve resistance, in nature. But besides these natural forces of intrinsic and extrinsic factors helping these pathogens to develop resistance, there are anthropogenic factors that encourage the spread of resistance in bacteria.

A strong view, that the overconsumption of antimicrobials is the critical factor in selecting resistance. Paradoxically, underuse through lack of access, inadequate dosing, poor adherence, and substandard anti-microbials may play as important a role as overuse. For

these reasons, improving use is a priority if the emergence and spread of resistance are to be controlled.

- urbanization with its associated overcrowding and poor sanitation, which greatly facilitate the spread of such diseases as typhoid, tuberculosis, respiratory infections, and pneumonia.
- pollution, environmental degradation, and changing weather patterns, which can affect the incidence and distribution of infectious diseases, especially those, such as malaria, that are spread by insects and other vectors;
- demographic changes, which have resulted in a growing proportion of elderly people needing hospital-based interventions and thus at risk of exposure to highly resistant pathogens found in hospital settings;
- the AIDS epidemic, which has greatly enlarged the population of immunocompromised patients at risk of numerous infections, many of which were previously rare;
- the resurgence of old foes, such as malaria and tuberculosis, which are now responsible for many millions of infections each year;
- the enormous growth of global trade and travel which have increased the speed and facility with which both infectious diseases and resistant microorganisms can spread between continents.
- the enhanced food requirements of an expanding world population have led to the widespread routine use of antimicrobials as growth promoters or preventive agents in food-producing animals and poultry flocks. Such practices have likewise contributed to the rise in resistant microbes, which can be transmitted from animals to man.

As the number of infections and the corresponding use of antimicrobials have increased, so has the prevalence of resistance due to phenomenon known as "selective pressure". The microbes which adapt and survive carry genes for resistance, which can be passed on.

1.3.3 Overcoming the predicament:

The battle against this predicament needs to be fought at two fronts. Firstly, adoption of strategies to reverse the phenomenon or atleast contain the further proliferation of it. Secondly, emphasis on research and development of new drugs, that can act on these pathogens more effectively and combat them. World Health Organization recognizes antimicrobial resistance as a global problem and has launced its first global strategy to combat it in September 2001, known as WHO Global Strategy for Containment of Antimicrobial Resistance. The strategy recognizes that antimicrobial resistance is a global problem that must be addressed in all countries. No single nation, however effective it is at containing resistance within its borders, can protect itself from the importation of resistant pathogens through travel and trade, SARS and Bird Flu being the lastest evidences of it. The strategy gives particular attention to interventions involving the introduction of legislation and policies governing the development, licensing, distribution, and sale of antimicrobial agents. The strategy is sufficiently flexible to be applied in poor and wealthy nations alike. Global principles for the containment of antimicrobial resistance in food-producing animals have already been issued by WHO in June 2000. Thus, efforts are on at political as well as practical levels to prevent further spread of antimicrobial resistance.

The other part of the battle against antimicrobial resistance involves mining new molecules from different biological sources. Investment in R&D into antibiotic discovery by the major pharmaceutical companies has declined dramatically in the last 15 years as a perception has taken hold that easily obtained natural products may have been fully exploited. Hence conventional screening of natural products for new drugs is no longer considered economically worthwhile. Unfortunately, the downturn in drug discovery has coincided with a dramatic worldwide increase in the incidence of resistance to all the antibiotics currently used in medicine.

According to Sir James Black's observations, "the most fruitful basis for the discovery of a new drug is to start with an old drug" (Raju, 2000). On these lines, new generation molecules have been developed from a single natural source, but now they are

also not responding to the notorious pathogens. Thus it is high time to go back to nature and find some new molecule that can counteract the antimicrobial resistance attack. There has been very encouraging analysis that strongly advocates about hugh potentials of discovering several new molecules from microbial sources. In 2001, Watve *et al.* estimated that from the first report of streptothricin in 1942 and streptomycin a year later, the order Actinomycetales had yielded ~3,000 known antibiotics (90% of those from *Streptomyces*, an Actinomycetales genus). On the basis of past experience, these authors proposed that if streptomycetes (exclusively) were screened as widely as they had been in 1995, 15–20 antibiotics would be discovered each year for the next 50 years. Over the subsequent five decades, these ~1,000 new molecules would yield 20–40 new antibiotics for human clinical use, assuming that the historical trend of one marketed antibiotic for 25–50 novel molecules remains the same. Recently Baltz (2005) also estimated that less than one part in 1012 of the earth's soil surface has been screened for actinomycetes.

1.4 *Streptomyces*

Streptomycetes are Gram-positive, aerobic, filamentous soil bacteria that undergo morphological differentiation during their life cycle. They produce extensive branching vegetative (substrate) mycelium and aerial mycelium bearing chains of arthrospores. The *Streptomycetes* are able to utilize a wide range of organic compounds as a carbon source, including complex biological materials, such as cellulose and lignin, and can also utilize an inorganic nitrogen source. *Streptomycetes* are common in soil, but also found in composts, fodder and aquatic habitats. Due to their characteristic life cycle, they are good survivors under the fluctuating growth conditions predominating in nature.

On agar plates, they form lichenoid, leathery or butyrous colonies. The GC-content of the DNA is 69-78 % (Wright and Bibb, 1992). L-diaminopimelic acid is the characteristic compound present in the cell wall peptidoglycan of *Streptomycetes*. They normally occur as spores, but in the presence of sufficient moisture and nutrients, the spores can germinate and form vegetative mycelium (Williams et al., 1989). In response to environmental signals, such as shortage of nutrients or water, the process of differentiation begins, and spores

resistant to desiccation and starvation are formed again. Streptomyces is a potential source of large number of polyketides that are bioactive in nature (Wietzorrek and Bibb, 1997).

1.5 Polyketides:

The polyketides are "most probably the largest single family of natural products" (Robinson, 1988), which share the basic principle of their biosynthesis. Collie was first to coin the term Polyketide in 1907, which represented natural products containing multiple carbonyl or hydroxyl groups, each separated by one carbon atom

$$- \underline{CH_2} \, \mathbf{C(=O)} \, \underline{CH_2} \, CH(OH) \underline{CH_2} \, \mathbf{C(=O)} -$$

Polyketides are group of secondary metabolites, exhibiting remarkable diversity both in terms of their structure and function. It is the diversity and complexity of these secondary products that make it difficult to precisely define them. With time, knowledge about this interesting group of compounds increased making it more and more difficult to define. Based on their understanding of the construction process, Bentley and Bennett in 1999, have provided a possible comprehensive definition, in biosynthetic terms rather than those of structural chemistry.

These metabolites are ubiquitous in distribution and have been reported from organisms as diverse as bacteria, fungi, plants, insects, dinoflagellates, mollusks and sponges. The wide spectrum of activity of polyketides makes them economically, clinically and industrially the most sought after secondary metabolite. Polyketide natural products are known to possess a wealth of pharmacologically important activities, including e.g. antibacterials (streptomycin, tetracycline, chloramphenicol), antifungal (nystatin), antiviral (tunicamycin), antiparasitic (avermectin), immunosuppressive (rapamycin), antitumor (actinomycin, mitomycin C, anthracyclines), enzyme inhibitory (clavulanic acid), diabetogenic (bafilomycin, streptozotocin) these are a few more of the thousands of polyketides discovered so far (Wang et al., 2000).

Polyketides are very diverse in structure and may be divided into four Classes, aromatics (e.g., doxorubicin and tetracycline), macrolides (e.g., erythromycin and rapamycin), polyethers (e.g., monensin and salinomycin), and polyenes (e.g., amphotericin and candicidin), the last three are grouped together as complex polyketides.

Although the structures of polyketides vary enormously, they are all synthesized, in their initial stages, by a mechanism that is very similar to fatty acid biosynthesis: simple acyl precursors such as acetyl and malonyl units are condensed in a sequential fashion to give a long carbon chain, catalyzed by the polyketide synthase (Hopwood, 1993). Polyketide synthase (PKS) are a family of enzymes that catalyses the biosynthesis of structurally diverse and pharmaceutically important class of natural products just described, the polyketides. They are divided into two classes following the convention of fatty acid synthases (FASs), according to their enzyme architecture and gene organization. Type I PKSs are multifunctional proteins consisting of domain for individual enzyme activities and have been found in bacteria as well as in fungi and plants. Type II PKSs are multienzyme complexes consisting of discrete proteins that are largely monofunctional and have so far only been found in bacteria.

1.5.1 Relation between FAS and PKS

Since the beginning of the polyketide hypothesis (Robinson, 1955, quoted in Lynen and Tada, 1961) it has been postulated that polyketide biosynthesis is a variant of fatty acid biosynthesis. It has also been postulated that the cellular machinery synthesizing polyketides has evolved from fatty acid synthases (O'Hagan, 1990). On the other hand, for hypothesis of a single common origin for typical FASs and PKSs, an early ancestor of present day bacteria and eukaryotes might have evolved a primitive condensing enzyme that recruited other functions to become more efficient; addition of an acyl carrier protein and acyl transferases could have given rise to a rudimentary PKS, perhaps followed by recruitment of the reductive cycle to convert it to a FAS. The resulting primordial multifunctional synthase would have become further improved and diversified by subsequent mutation, recombination between diverged gene sequences, and gene duplication, opening the way for

PKSs to evolve the ability to generate chemically distinct products, while the (by then) essential function of fatty acid biosynthesis could be retained by the organisms' FAS (Hopwood, 1997).

The biosynthesis of fatty acids is catalyzed by an enzyme system known as fatty acid synthase (FAS). It follows a default pathway (Fig: 1.4) wherein, an acetyl unit that acts as a "starter" is transferred by an acetyl transferase from acetyl coenzyme A (CoA) onto the thiol
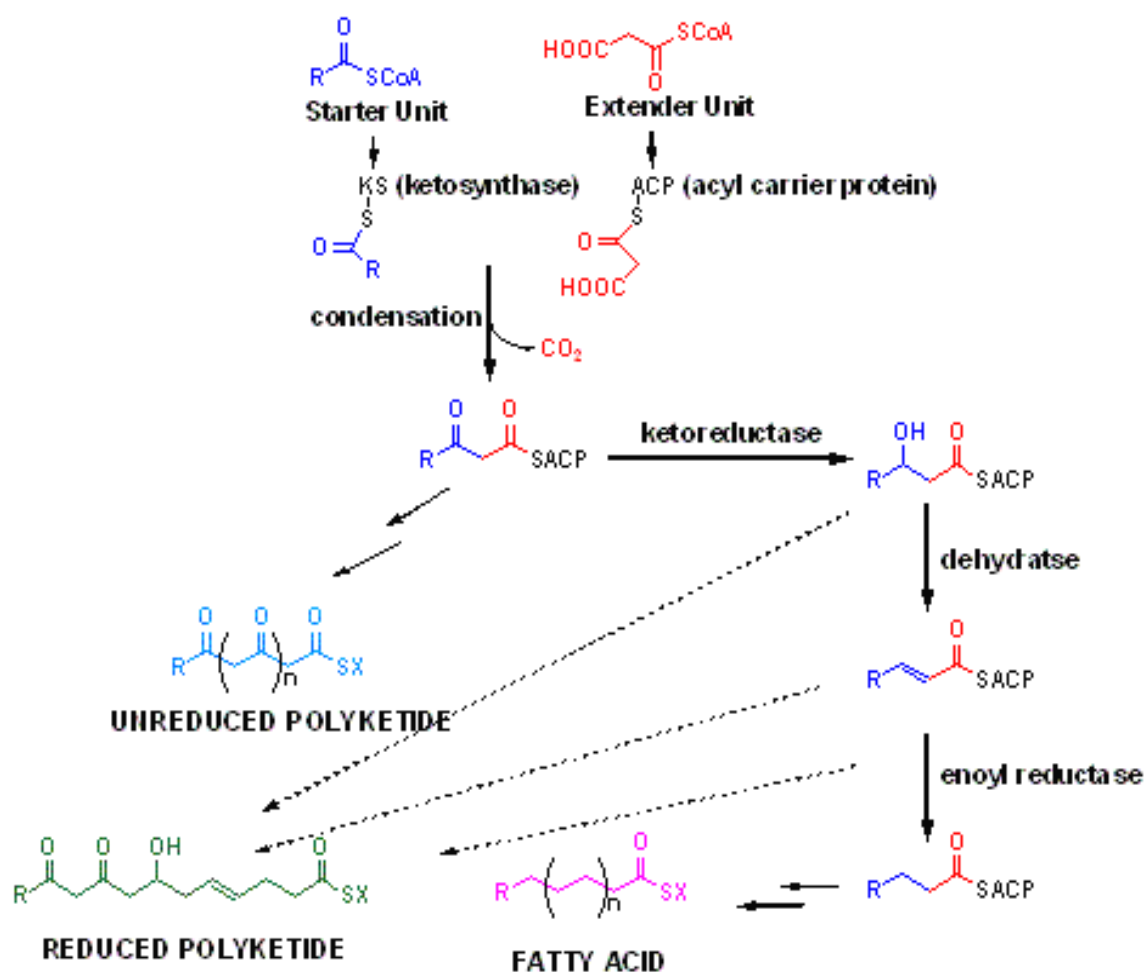

Fig- 1.4: Generalized pathway of Fatty acid biosynthesis and Polyketide biosynthesis, suggesting flexibility at various levels in formation of polyketide molecules.

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group of acyl carrier protein (ACP), and further onto the thiol group of ketoacyl synthase (KS); the enzymatic activity responsible for the latter reaction has not been unambiguously assigned. A malonate "extender" unit is transferred onto the thiol group of ACP from malonyl-CoA by acyltransferase (AT). Thereafter, the KS catalyzes the condensation of the two acid residues, with elimination of CO_2, to produce a -ketoacyl residue on the thiol group of the ACP. This is the basic chain extension reaction. bond, and enoyl reductase (ER), also using NADPH as cofactor, reducing the double bond to a saturated carbon chain. After transfer of the extended acyl group onto the thiol group of KS, the cycle can repeat beginning with the acyl transfer reaction. When the fatty acid has reached it's predetermined length, it is detached from the synthase complex either by a thioesterase, producing free fatty acid, or by a transferase (e.g. palmityl transferase to produce palmityl-CoA).

In analogy with FAS, the enzyme system in PKS passes on with the same basic procedure. But it is highly flexible and all the above steps can be a source of variation in the polyketide formation:

1) *Prevelance of the clusters*:

The FAS system is omnipresent from bacteria to mammals and angiosperms. PKS system though is diversely present but its prevelance is selectively restricted.

2) *Variation in the "starter" unit*:

It is the unit with which the chain biosynthesis begins. In fatty acid biosynthesis this is always acetyl-CoA; in polyketide biosynthesis this may also be propionyl-CoA, or, indeed, "there is little reason to doubt that examples may be found of initiation of the chain by almost any acyl coenzyme A found in nature" (Birch, 1967).

3) *Variation in the "extender" units*.

In fatty acid biosynthesis the carbon chain is always extended with acetate units derived from malonyl-CoA; in polyketides propionate units from methylmalonyl-CoA and butyryl units from ethylmalonyl-CoA are also incorporated. The latter give rise to

methyl and ethyl side chains, respectively, in the carbon chain, and also introduce a chiral center.

4) *Variation in the number of ketide units*.

Usually, the chain length of fatty acids ranges from 6 to 12 acetate units (C12-C24 fatty acids). In polyketides 3 to 10 ketide units are usual, but up to 25 units have been observed.

5) *Variation in the reactions following chain elongation*.

Fatty acid biosynthesis, follows a stringent order of reactions to form saturated C2-unit, whereas PKS shows high level of flexibility wherein any step (and those following it) can be skipped to obtain varying functionality in the carbon chain which can be different from C2-unit. Appropriately modified chain-elongation intermediates have been isolated from blocked mutants of polyketide producers.

6) *Reactions following carbon chain biosynthesis*.

Fatty acids are coupled with glycerol and sphingosine derivatives to produce various lipids. The fate of the polyketide carbon chain is more varied. It can be cyclized to aromatic compounds such as in anthracycline and tetracycline biosynthesis; it can be lactonized to form macrolides. New functional groups can be introduced by specific enzymes, such as the 1- and 11-hydroxyls in anthracyclines. In several polyketides specific hydroxyls are glycosylated, producing a further mode of variation. By combining these modes of variation, a vast number of structures can be generated.

7) *Product and its fate:*

FAS product is primary metabolite which is retained by the cell whereas that of PKS is a secondary metabolite which is usually leached out by the cell.

1.5.2 Types of Polyketide Synthases (PKSs)

i. Type I systems

It consist of very large multifunctional proteins which can be either processive (for example the unique modular systems responsible for synthesis of macrolides like erythromycin, rapamycin, rifamycin etc.) or iterative (for example the lovastatin nonaketide synthase). Iterative Type I synthases are analogous to vertebrate fatty acid synthases. These are typically involved in the biosynthesis of fungal polyketides such as 6-methylsalicylic acid and aflatoxin. These PKSs are large multidomain proteins carrying all the active sites required for polyketide biosynthesis.

ii. The iterative Type II

This systems consist of complexes of mono-functional proteins exemplified by the actinorhodin PKS from *Streptomyces coelicolor*. In these synthases, active sites are distributed among several smaller, typically monofunctional polypeptides. Type II synthases catalyse the formation of compounds that require aromatization and cyclization, but not extensive reduction or reduction/dehydration cycles. These PKSs are analogous to bacterial FAS and are involved in the biosynthesis of bacterial aromatic natural products such as actinorhodin, tetracenomycin and doxorubicin

iii. Type III polyketide synthases

are responsible for the synthesis of chalcones and stilbenes in plants. Chalcone synthase like proteins are comparatively small proteins with a single polypeptide chain and are involved in the biosynthesis of precursors for flavonoids. Unlike all other PKSs, these proteins do not have a phosphopantetheinyl (P-Pant) arm on which the growing polyketide chains are tethered. Type III polyketides are prevelant in higher plants and are very different from the bacterial types. Thus, its mention has been avoided.

Table- 1.1: Differences between Type II and Type I polyketides

TYPE II	TYPE I
It is considered to be primitive	It is relatively advanced version of PKS
It resembles prokaryotic FAS	It resembles eukaryotic FAS
They are exclusively iterative in nature	They can either be modular or iterative
They are found only in bacteria	The iterative type is prevelant in eukaryotes whereas bacterial form show modular mode of synthesis.
Each protein has a single active site of action	Proteins carry more than one active sites
Each protein may be repeatedly used in building a molecule	Each domain appears once in formation of the molecule.
Minimal PKS first generates a carbon skeleton which is then furnished by other proteins	The assembly and maturation process of extension units is accomplished simultaneously.
Chain Length Factor (CLF) determines the length of carbon backbone	Chain Length Factor is absent.
Malonyl Co-A is found to be the extender unit	They can have varied extender units like malonyl, methylmalonyl, or more complex Extenders like ethylmalonyl unit in tylosin and spiramycin or glycerol-derived extenders in soraphen.
Simple (actinorhodin) or no (tetracynomycin) reductive changes to the B-keto groups of the growing chain	Reductive cycles can generate five functionalities at each round of chain building.

1.6 Type II polyketide synthase:

1.6.1 Regulation:

The role of the highly phosphorylated guanosine nucleotide (p)ppGpp in triggering antibiotic production in *Streptomycetes* has received considerable attention, especially due to its likely participation in the growth rate control of gene expression in unicellular bacteria (Gralla, 2005). The ribosome-associated ppGpp synthetase (RelA) is required for antibiotic production under conditions of nitrogen limitation in *Streptomyces* (Chakraburtty and Bibb, 1997). Whether ppGpp was directly involved in promoting transcription of antibiotic biosynthetic genes or whether the latter was an indirect consequence of a reduction in growth rate prompted by ppGpp-mediated inhibition of rRNA synthesis was unclear. However, use of modified RelA provided the most convincing evidence for a direct role for ppGpp in activating the transcription of antibiotic biosynthetic genes.

While RelA is absolutely required for antibiotic production in *S. coelicolor* upon nitrogen starvation, it is dispensable under conditions of phosphate limitation, where a ppGpp-independent signalling mechanism must operate to initiate secondary metabolism (Chakraburtty and Bibb, 1997). An excessive level of inorganic phosphate in the culture medium prevents the production of many structurally diverse secondary metabolites (Martin, 2004), and in atleast some cases this reflects repression of transcription of biosynthetic gene clusters (Gil and Campelo-Diez, 2003). Mutation of the two-component regulatory system PhoR-PhoP of *Streptomyces lividans* resulted in reduced levels of alkaline phosphatase activity and phosphate transport at low phosphate concentrations, and in a marked increase in the level of Actiorhodin and undecylprodigiosin (Red) production (Sola-Landa et al, 2003).

Many of the pathway-specific regulatory proteins that control secondary metabolism in streptomycetes belong to the SARP family (Wietzorrek and Bibb, 1997). These transcriptional activators contain a winged helix-turn-helix motif towards their N-termini that is also found in the OmpR family of proteins, and at least some of the SARPs appear to recognize heptameric repeats within the promoter regions of genes that they regulate

(Lombo et al, 1999; Sheldon et al, 2002). They have been found associated with secondary metabolic gene clusters that encode aromatic polyketides (Sheldon et al, 2002; Pang et al, 2004; Ichinose et al, 2003), ribosomally and non-ribosomally synthesized peptides (Ryding et al, 2002), undecylprodiginines (Cerdeno et al, 2001), Type I polyketides [Sun et al, 2003; Oliynyk et al, 2003], b-lactams [Nunez et al, 2003] and azoxy compounds [Garg et al, 2002]. While genes encoding phylogenetically diverse classes of bacterial regulatory proteins occur in many secondary metabolic gene clusters, the SARP family of proteins have only been found in actinomycetes, and most of them within the streptomycetes (other genera include Mycobacterium, Nocardia, Thermobifida and Lechevalieria).

A LAL family of regulatory protein has been found to be present in regulation of atleast 13 molecules of type I polyketide synthases. Besides these, there have been reports of some pleotropic regulators and even extracellular signaling molecules like gamma-butyralactones (Horinouchi and Beppu, 1992; Choi et al, 2003) of which A-factor from *S. griseus* (Yamazaki et al, 2004; Kato et al, 2004) is well characterized and PI factors(Recio et al, 2004) have been shown to play role in regulating polyketide synthesis in Streptomyces.

1.6.2 Chemistry of biosynthesis in Type II PKS:

Tradionally actinorhodin, tetracyclines and anthracyclines were classified as octaketides, nonaketides and decaketides. Formarly, polyketides were grouped on the basis of their final chemical structure rather than the mechanism of biosynthetic assembly. With the construction of CH999, the gates for understanding pathway of polyketide synthesis were opened. Various genes from any PKS cluster could be inserted on a plasmid, seemingly in any desired order and combination, transformed into this polyketide non-producing host, and the resulting *in vivo* PK product, if any, obtained and identified. This led to faithfully decoding the pathways for various groups of polyketide antibiotics.

For production of any polyketide action of three genes namely KS (Ketosynathse), CLF (chain length factor) and ACP (acyl carrier protein) is essential without which a product cannot be expected. Thus this set of 'minimal PKS' genes promote the assembly of

the correct length of chain, catalyse and direct the regiochemistry of the first cyclisation, or at least deter other non-enzymatic cyclisations from occurring. Most bacterial 'aromatic polyketides' are reduced where the growing acyl chain 'turns the corner', and is folded. The molecular reason for this is little understood, but most are reduced at the keto group nine carbons inward, from the final position of the thioester carbonyl, by a KR1(9). The frequently encountered exceptions are the tetracenomycins and aureolic acids, where no such reduction takes place. This reduction has an important role in the activity and role of later proteins. Unfortunately, the tetracenomycins and 'C-9 reduced' decaketides are frequently both referred to as anthracycline antibiotics, despite having quite different acetate-folding patterns. When the chain length, position of the first reduction relative to thioester, and regiochemistry of the first cyclisation are considered, there are only a few common basic folding types, as shown in fig 1.5, which encompass most bacterial Type II aromatic polyketides.

In the C-9 *reduced systems*, the minimal PKS controlled first cyclisation (CYC) usually occurs between carbons 7 and 12 *i.e.* CYC1(7/12) to give SEK4 (in the actinorhodin minimal PKS, an alternative presumed non-enzymatic first cyclisation also occurs, CYC1(10/15) to give SEK4b). This is then followed by an enzymatically triggered aromatisation or dehydration (ARO1) to form the first aromatic ring. In these KR1(9)CYC1(7/12) systems, the first cyclisation can be partially regiochemically directed and catalysed by just the minimal PKS. However, the trifunctional enzyme traditionally labelled 'ARO', is also thought to help promote and direct CYC1, aromatise the first ring ARO1 (acting as a dehydratase) as well as aromatise the second ring (but only after the action of a cyclase labelled CYC2/3). These processes, involving the minimal PKS, KR1, ARO and CYC2/3 are referred to as the 'early PKS' system, with, OOX(6), CYC4 *etc*. to form the first readily isolable 'wild type' intermediates as 'later PKS', and subsequent steps as 'post PKS'. The vast majority of metabolites in this section are KR1(9)CYC1(7/12), varying only in chain length (usually 16/18/20), starter unit ('X'), and the later steps (Fig-1.5). These labels have been abbreviated to *e.g.* 7,9,12-octaketides, and 7,9,12-decaketides, with nonreduced systems such as tetracenomycin labelled as 9,14-decaketides.
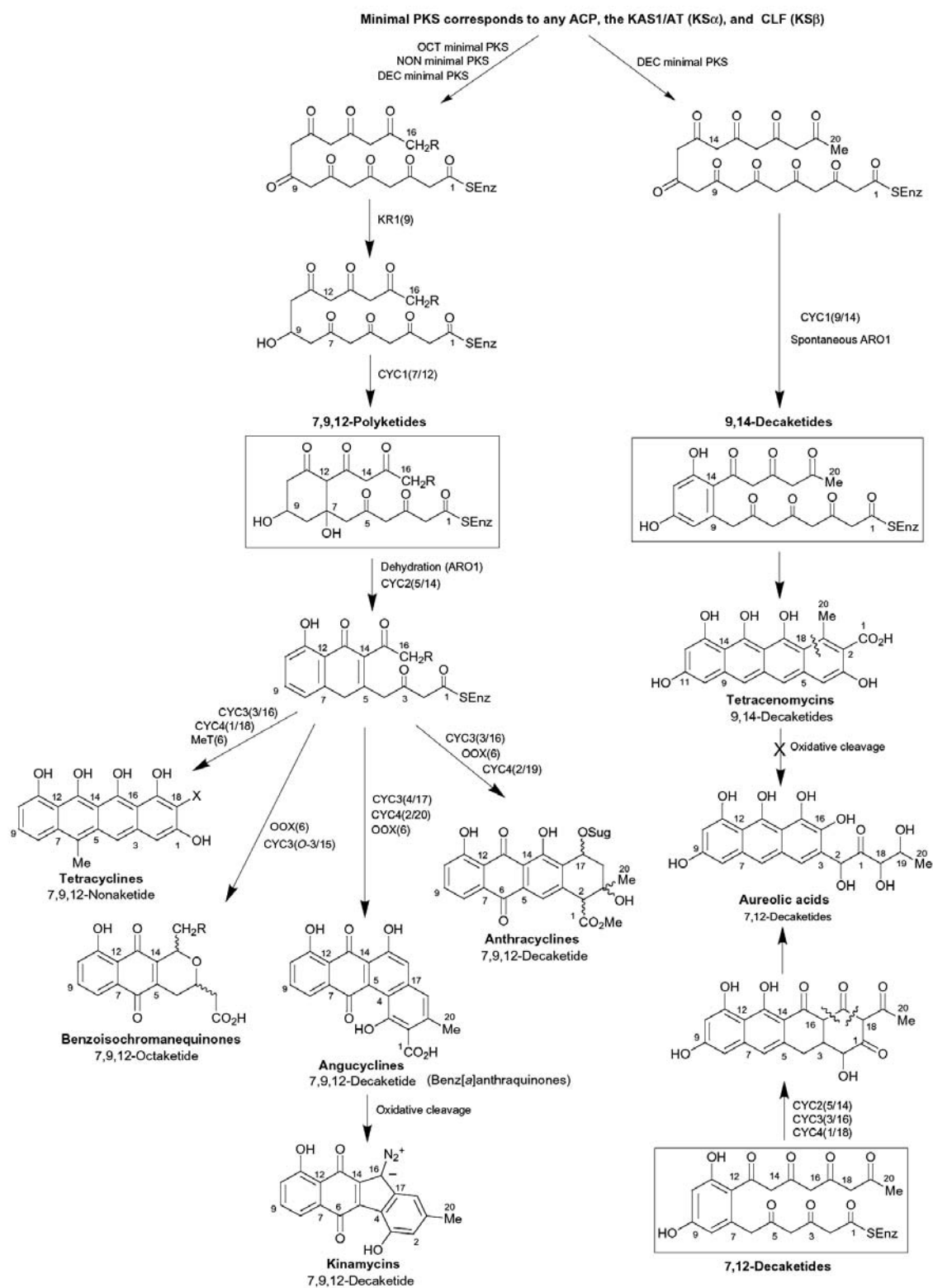
Fig -1.5: Schematic presentation of biosynthetic pathways of some polyketides

In the non-reduced systems, such as tetracenomycin, this first aromatisation is thought to occur spontaneously after ring closure without the need for an ARO enzyme activity. The multifunctional 'AROc' protein responsible for CYC1/ARO1 (TcmN) affects the regiochemistry of the first cyclisation, and is thought to play a role in later cyclisations (CYC2 and 3). Subsequent 'later PKS' enzymes, that convert the unstable 'early PKS' intermediate into an easily isolable product, seem to perform a wider variety of reactions than previously thought, possibly including oxidation to form quinoid systems, SAM methylation, and *O*-cylisation to form pyran rings. Methyl transferases, isoprenyl transferases etc. may be an integral part of these gene clusters, and such reactions may be occurring before the full ring system is formed.

1.7 Aureolic acid group

Members of the aureolic acid family are tricyclic polyketides with antitumor activity which are produced by different *Streptomycete* species. The first member of this family of compounds, mithramycin, was described in the 1950s and was also known as aureolic acid, plicamycin, antibiotic LA-7017, and PA-144 as a consequence of its isolation by different groups (Grundy et al. 1953; Sensi et al. 1958; Rao et al. 1962). It is produced by several actinomycetes, like *Streptomyces argillaceus* American Type Culture Collection (ATCC) 12956, *Streptomyces plicatus* ATCC 12957, *Streptomyces tanashiensis* ATCC 31053, and *Streptomyces atroolivaceus* ATCC 27627. The family also includes chromomycins (Sato et al. 1960), produced by *Streptomyces griseus* subsp. *griseus* ATCC 13273 and *Streptomyces cavourensis* ATCC 27732; olivomycins (Brazhnikova et al. 1962), produced by *Streptoverticillum cinnamoneum*; chromocyclomycin (Blumauerova et al. 1976), produced by *S. atroolivaceus*; UCH9 (Ogawa et al. 1998), produced by *Streptomyces* sp.; and durhamycin A (Jayasuriya et al. 2002), produced by *Actinoplanes durhamensis* (Fig -1.6).

All these compounds are glycosylated aromatic polyketides with an intense yellow color and fluorescence under uv- light, which is responsible for the name of the family. With the exception of chromocyclomycin, which is a tetracyclic compound, the aglycons of this

family show a tricyclic ring system fused to a unique dihydroxy-methoxy-oxo-pentyl aliphatic side chain attached at C-3. In some cases a small alkyl residue (methyl, isobutyl) is attached at position C-7. Some initial suggestions postulating the involvement of two different polyketide chains to form these aglycon systems have been finally ruled out, and nowadays the involvement of only one polyketide synthase (PKS) and one polyketide chain has been unequivocally established.
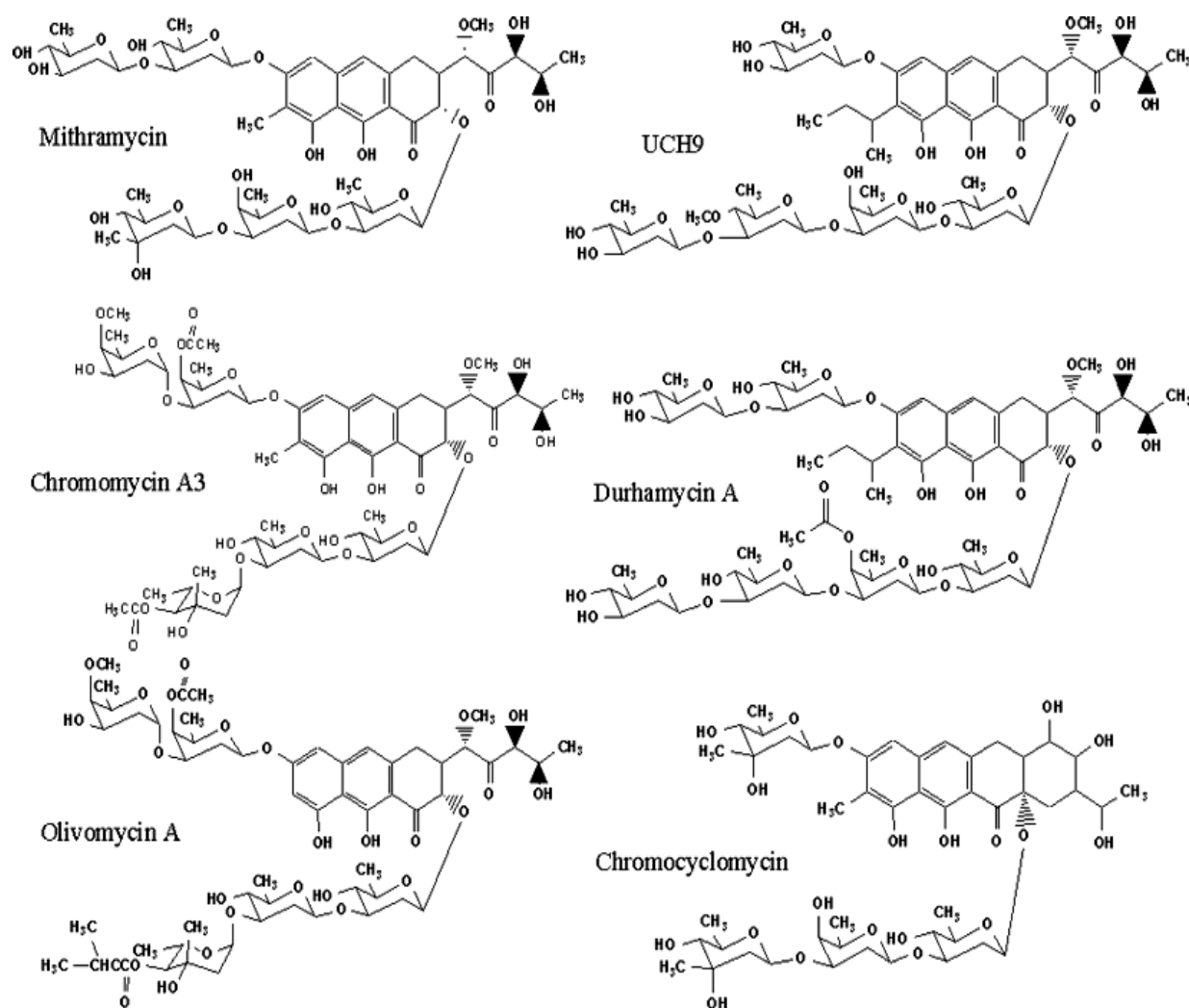

Fig- 1.6: Chemical structures of members of the aureolic acid family

In all members of the family, two oligosaccharide chains are bound to the aromatic polyketide moiety. In the case of mithramycin, chromocyclomycin, chromomycins, and olivomycins, these chains contain two and three deoxysugars. There were several structural disagreements regarding the positions and linkages of the deoxysugars in the case of mithramycin. Its correct structure was first introduced in a DNA interaction paper (Sastry and Patel 1993) and later confirmed by NMR and mass spectrometry (Wohlert et al. 1999). UCH9 and durhamycin contain a tetrasaccharide and a mono- or disaccharide, respectively. All sugars belong to the 2,6-dideoxysugar family and they comprise different combinations of D-olivose, D-oliose, D-mycarose, L-chromose B, and *O*-methylated or *O*-acetylated derivatives. These deoxysugars are connected via β-(1,3) glycosidic bonds.

1.7.1 Biological activities and mode of action

Initially, the members of this family of natural products were isolated due to their antibiotic activity against Gram-positive bacteria. However, they are not active against Gram-negative bacteria due to permeability problems. Their main pharmacological interest resides in their antitumor activity.

The members of this family interact with the DNA helix minor groove in regions with high GC content and in a nonintercalative way (Waring 1981; Katahira et al. 1998). This binding is carried out by complexes of dimers together with Mg^{2+} ion. During these interactions, several H-bonds are created among the aglycon hydroxyl groups and the guanine amino protons (Sastry and Patel 1993). The deoxysugars are necessary for stabilizing this complex with the DNA (Sastry et al. 1995; Keniry et al. 2000), and its structure influences the sequence specificity. Consequently, the acetyl and methyl groups in the chromomycin deoxysugars make these oligosaccharides less flexible, which induces higher DNA-sequence specificity than in the case of mithramycin, and a more stable minor groove binding (Majee et al. 1997; Chakrabarti et al. 2000–2001).

Interaction with double helix causes a DNA-dependent inhibition on RNA synthesis, which gives this family of compounds a strong antitumor activity against a variety of cancer cell lines (Wakisaka et al. 1963; Ward et al. 1965; Ogawa et al. 1998). Based on this

antitumor activity, mithramycin has found clinical application in the treatment of some cancers, such as testicular carcinoma (Du Priest and Fletcher 1973).

The specificity for GC-rich regions along the DNA makes these compounds good inhibitors of specific promoter regions, preventing the binding of regulatory proteins. This effect has been described for the *c-myc* and *c-Ha-ras* (Campbell et al. 1994), *c-myb* (Vigneswaran et al. 2001), and *MDR1* genes (Tagashira et al. 2000). Mithramycin binds at the C-fos-depending Sp1 regulatory regions, and therefore, it prevents transcription due to this transcriptional factor, generating a global inhibition mechanism (Ryuto et al. 1996).

Mithramycin also inhibits calcium resorption in osteoclasts, and it has been used for treating cancer-associated hypercalcemia processes (Hall et al. 1993). This effect is based on transcription regulation in these cells. The exact mechanism of action involves binding of the drug to the promoter regions of a gene, which is necessary for osteoclasts promotion, the *c-src* gene, abolishing the binding of Sp1 transcription factors (Remsing et al. 2003a).

Antiviral activity has also been described for some members of the family, as the inhibitory effect of durhamycin A on HIV Tat replication protein (Jayasuriya et al. 2002). Chromomycin also causes inhibition on the binding of the transcription factor Sp1 to its target sequences in the HIV-1 long terminal repeat regions, thus abolishing the activation of the HIV-1 provirus (Bianchi et al. 1997).

Some aureolic acids have been shown to prevent resistance to other antitumor agents by a number of mechanisms, including the down regulation of proteins such as MDR1 (Mir et al. 2003; Tagashira et al. 2000). Chromomycin and mithramycin are also potent inhibitors of neuronal apoptosis (Chatterjee et al. 2001). These two compounds also bind, in Mg^{2+}-independent manner, to the erythrocyte cytoskeletal protein spectrin with affinity constants comparable to those for the association of spectrin with other cytoskeletal proteins like F-actin or ankyrin (Majee and Chakrabarti 1995; Majee et al. 1999).

1.7.2 Aureolic acid biosynthesis gene clusters

Currently, two aureolic acid biosynthesis gene clusters have been isolated and characterized: those involved in the biosynthesis of mithramycin and chromomycin A_3. Both gene clusters have been sequenced, intermediates in the mutant strains have been purified and subjected to structural elucidation. Despite the high structural similarity between mithramycin and chromomycin A_3, the genetic organization of both gene clusters is highly different, which favors the hypothesis of convergent evolution for the generation in both antitumor compounds, instead of divergent evolution from a common ancestor (Menéndez et al. 2004a).

The putative borders of the chromomycin cluster are genes coding for a cyclase and an aromatase. In the case of mithramycin, genes encoding regulatory and resistance functions would be the ends of the cluster. One surprising characteristic of the mithramycin gene cluster is the presence of a perfectly 241-bp repeated sequence at each end of the cluster. This could have implications in how the producer strain, *S. argillaceus*, acquired the gene cluster through evolution. This cluster could have been part of an extrachromosomal element in which a copy of this repeated sequence would be present. Through Campbell-type recombination to an identical repeated sequence that would have been present in the chromosome of an ancestor nonmithramycin-producing strain of *S. argillaceus*, the extrachromosomal element could have been incorporated into the *S. argillaceus* chromosome, and as a consequence, two repeated sequences are now flanking the mithramycin cluster (Lombó et al. 1999).

1.8 Generation of hybrid compounds

The knowledge generated within the mithramycin biosynthetic pathway has allowed the design of combinatorial biosynthesis experiments to generate novel hybrid compounds. In some cases, mithramycin genes contributed to modifying other aromatic polyketide routes, whereas in others, genes from different biosynthesis gene clusters were introduced
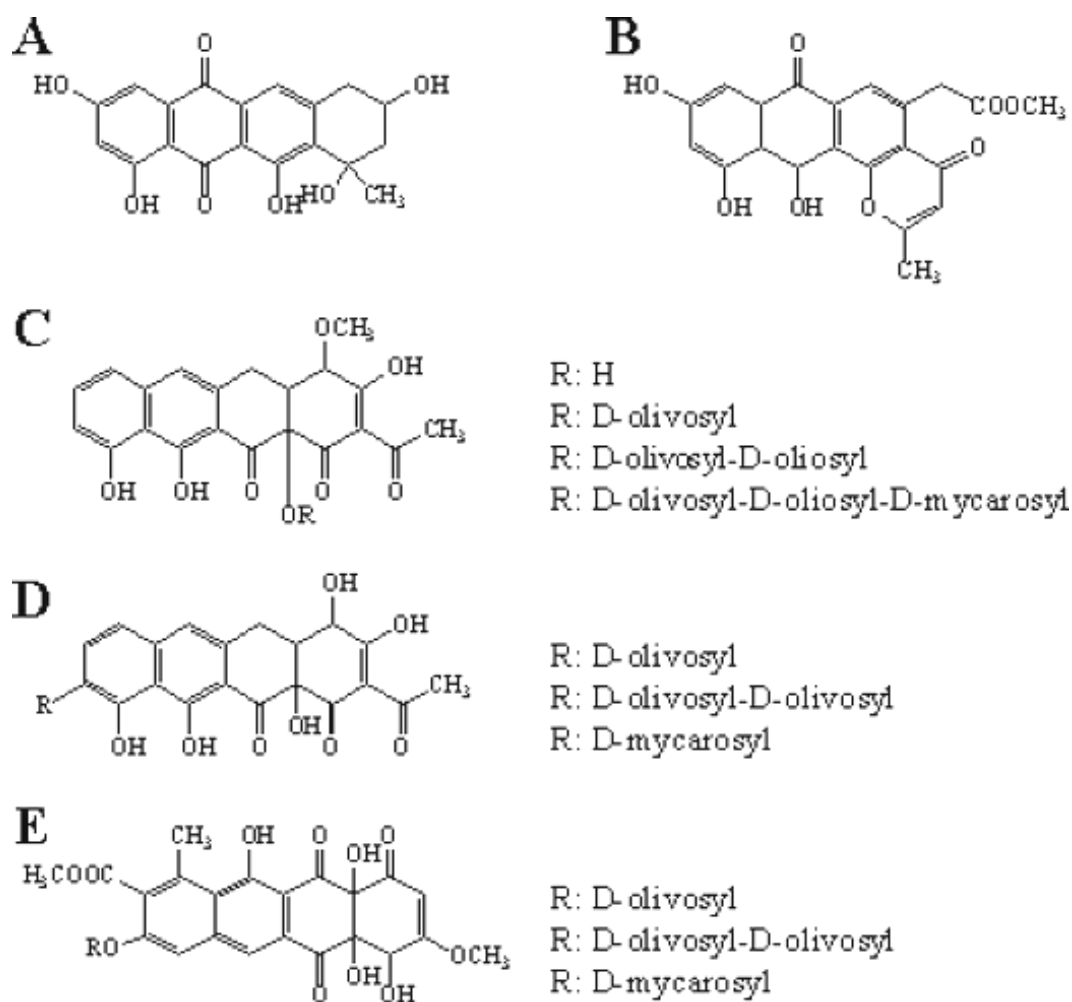
A

B

C

R: H
R: D-olivosyl
R: D-olivosyl-D-oliosyl
R: D-olivosyl-D-oliosyl-D-mycarosyl

D

R: D-olivosyl
R: D-olivosyl-D-olivosyl
R: D-mycarosyl

E

R: D-olivosyl
R: D-olivosyl-D-olivosyl
R: D-mycarosyl

Fig. - 1.7 **:** Novel antitumor compounds generated by combinatorial biosynthesis.

      A. Tetracenomycin M, produced by expressing the *mtmPKS* (minimal PKS) and the *mtmX* cyclase and *mtmTI* ketoreductase in the tetracenomycin C producer *S. glaucescens* Tü49.

      B. PMC H, produced by expressing the *tcmH* monooxygenase from the tetracenomycin cluster in *S. argillaceus* M7D1.

      C. Formation of glycosylated premithramycin derivatives by expressing the nogalamycin minimal PKS genes (*snoABC*), the *snoD* ketoreductase, and the *snoaE* aromatase in *S. argillaceus*.

      D. Formation of glycosylated PMCs by expressing *urdGT2* (glycosyltransferase from the urdamycin cluster) alone or together with *lanGT1* (glycosyltransferase from the landomycin cluster) in *S. argillaceus* M3G4.

      E. Formation of novel glycosylated tetracenomycins by expressing cos16F4 in *S. argillaceus*

into selected *S. argillaceus* hosts, altering the mithramycin pathway and therefore leading to the formation of new derivatives.

Introduction of genes encoding the mithramycin minimal PKS (*mtmPKS*), along with the putative cyclase *mtmX* and the ketoreductase *mtmTI*, into the producer strain of tetracenomycin C, *Streptomyces glaucescens* Tü49, was expected to modify some of the tetracyclic tetracenomycin C intermediates. In fact, a new hybrid compound, tetracenomycin M was generated (Künzel et al. 1997). Its structure revealed that this compound had suffered a fourth ring closure involving an intramolecular aldol addition, quite different from the typical intramolecular aldol condensation of tetracenomycins (Shen and Hutchinson 1993a). Formation of tetracenomycin M threw light on the function of MtmX, which was proposed to be the mithramycin fourth ring cyclase responsible for this aldol addition (Künzel et al. 1997). Some tailoring enzymes as oxygenases have enough substrate flexibility to be used as appropriate tools in combinatorial biosynthesis. The four-ring intermediate tetracenomycin F1 is the substrate for the monooxygenase TcmH during tetracenomycin C biosynthesis (Shen and Hutchinson 1993b). This compound shows some structural similarity to PMC, a tetracyclic biosynthetic intermediate in the mithramycin pathway. Based on this, an experiment was rationally designed to try to modify PMC with TcmH monooxygenase.

The *tcmH* gene was expressed in the mutant strain *S. argillaceus* M7D1, which accumulates PMC, resulting in the formation of a new hybrid antitumor compound, PMC H (Fig. 5b; Lombó et al. 2000). This new compound resulted from the spontaneous cyclization of an anthraquinone, derived from an early and unstable tricyclic anthrone PMC intermediate, which is oxygenated at the second ring by TcmH, generating a quinone system.

By altering the polyketide structure, it is possible to modify the glycosylation pattern. In this way, by expressing several genes (PKS, aromatase, ketoreductase) involved in the biosynthesis of the polyketide moiety of nogalamycin in *S. argillaceus*, three new glycosylated hybrid compounds were generated (Fig. 5c; Kantola et al. 2000). All these compounds share the premithamycinone aglycon lacking a hydroxyl group at C-8, resulting from the action of the nogalamycin ketoreductase and aromatase together with the

mithramycin aglycon genes. This new aglycon gets glycosylated at position 12a (as in wild-type *S. argillaceus*) with oligosaccharides of different lengths containing the natural mithramycin deoxysugars: D-olivosyl, D-olivosyl–D-oliosyl, and D-olivosyl–D-oliosyl–D-mycarosyl. Further glycosylation toward a fully glycosylated mithramycin was impossible because the elimination of the C-8 hydroxyl group in this aglycon by the action of the nogalamycin ketoreductase/aromatase abolished the disaccharide binding position (Kunnari et al. 2002).

Making use of the known substrate flexibility of some glycosyltransferases, it is possible to alter the glycosylation profile of bioactive compounds. UrdGT2 is a flexible glycosyltransferase responsible for the C-binding of a D-olivose moiety to the urdamycin aglycon (Faust et al. 2000). Precursors of this tetracyclic angular aglycon share some common features with the linear mithramycin intermediate PMC. A plasmid containing the *urdGT2* gene was introduced into a *S. argillaceus* mutant lacking all the mithramycin glycosyltransferases (Prado et al. 1999b), and also in mutant M3G4, lacking only the first PMC glycosyltransferase, *mtmGIV* (Blanco et al. 2000). This recombinant strain produced two new hybrid compounds which contained either D-olivose or D-mycarose attached to the C-9 position of PMC (or its 4-demethyl precursor) by a C—C bond (Trefzer et al. 2002). This demonstrated that UrdGT2 was flexible enough to attach D-mycarose, a branched-chain deoxysugar moiety very different with respect to its natural substrate, D-olivose. Furthermore, UrdGT2 was able to recognize a linear polyketide as aglycon, although its natural substrate is an angucycline (Künzel et al. 1999). One of these two new compounds, 9-*C*-olivosyl-PMC, was further modified by expressing the landomycin glycosyltransferase gene *lanGT1* together with *urdGT2*, into these two *S. argillaceus* mutants. LanGT1 is responsible for the attachment of the second D-olivose moiety of the trisaccharide during landomycin biosynthesis (Trefzer et al. 2001). As a consequence of this experiment, a novel hybrid compound was generated, in which the PMC aglycon contained, attached at position C-9, a diolivosyl moiety (Trefzer et al. 2002).

A last example of the possibilities of combinatorial biosynthesis consists in the use of the mithramycin deoxysugar biosynthetic machinery and the flexible glycosyltransferase ElmGT of the elloramycin cluster to modify the glycosylation pattern of this antitumor

compound. Cosmid 16F4 (Decker et al. 1995) is a cosmid containing the genes for the generation of the elloramycin aglycon (8-demethyl-tetracenomycin C) and the glycosyltransferase ElmGT which attaches L-rhamnose to this aglycon. Expression of cos16F4 in the mithramycin producer led to the generation of three new hybrid compounds, 8-demethyl- 8-β-D-olivosyl-tetracenomycin C, 8-demethyl-8-β-D-mycarosyl-tetracenomycin C, and 8-demethyl-8-β-D-diolivosyl-tetracenomycin C (Fig. 5; Wohlert et al. 1998; Blanco et al. 2001). In this case, the elloramycin aglycon gets glycosylated at its natural position, but by three different sugar moieties, including a disaccharide, which are synthesized by the mithramycin producer.

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