Aureolic acids are a group of polyketides, defined as linearly-fused and tricyclic aromatic polyketides. It has been revealed that the biosynthesis of compounds of aureolic acid family go through an intermediate of tetracycline and naphthacene[1]. Chromomycin A₃ and mithramycin are type II polyketides belonging to the class of aureolic acids with antitumor activities. These polyketides have properties of inhibiting growth and multiplication of many tumor cell lines[2]. Chromomycin A₃ and mithramycin have been shown to have a stimulatory effect on K562 cell erythroid differentiation[3] and have also been found as neurological therapeutics[4] and in treatment of HIV-1[5].

Chromomycin A₃ is the major constituent of a fermentation mixture synthesized by Streptomyces griseus while mithramycin is produced by different Streptomyces strains[2]. A same aglycon pattern is found in chromomycin A₃ and mithramycin but the former differs in its glycosylation pattern. Mithramycin is the best studied example among aureolic acids[6]. It was first proposed that mithramycin was derived from a tetracenomycin-like scaffold, based on putative last-ring cyclase heterologous expression from biosynthetic pathway of mithramycin in S. glaucescens Tu49[7]. A trisaccharide of d-olivose, d-oliose and d-mycarose, and a disaccharide of d-olivose have found in mithramycin while a trisaccharide of d-olivose (sugar C), d-oliose (sugar D), and 4-O-acetyl-l-chromose B (sugar E),
and a disaccharide of 4-O-acetyl-d-oliose (sugar A) and 4-O-methyl-d-oliose (sugar B) were attached at positions 2 and 6 of the aglycon, respectively were found in chromomycin A₃[8]. Although, chromomycin A₃ and mithramycin both fall in aureolic acid class of polyketides yet they show more identity with other classes of polyketides for the sequences of their respective KSα genes involved in their biosynthesis. The current study was therefore, planned to point out the dissimilarities between genetic organizations of biosynthetic gene clusters of two aureolic acids i.e. chromomycin A₃ and mithramycin. The gene sequence of KSα was used for preliminary classification of bacterial strains on the basis of their genetic abilities to produce different aromatic polyketides[9]. 3D models of KSα proteins of both aureolic acids were also predicted along with two other closely related polyketides i.e. chlortetracycline and polyketomycin.

MATERIALS AND METHODS

Sequence retrieval for genetic organizations:

Chromomycin A₃ and mithramycin were the only members of aureolic acid class of polyketides found in DoBISCUIT (Database Of BioSynthesis clusters CUrated and InTegrated)[10]. The sequence files for gene clusters of chromomycin A₃ from S. griseus and mithramycin from S. argillaceus were downloaded in GenBank format from NCBI database with accession numbers AJ578458 and X89899, respectively.

Genetic organization study:

For gene clusters analysis, the web tool antiSMASH (antibiotics and secondary metabolite analysis shell)[11] was used and the editing of genetic organizations of both aureolic acids was done manually. Mauve application v 2.3.1 was used for further visualization[12].

Sequences retrieval for 3D structure predictions:

Sequences of KSα subunits of type II PKSs were collected from DoBISCUIT and NCBI for selected polyketides i.e. chlortetracycline, chromomycin A₃, mithramycin and polyketomycin and these sequences are found under accession numbers (GenPept: BAB12566, CAE17527, CAA61989 and ACN64834, respectively) in NCBI. Multiple sequence alignment of selected polyketides along with their template was performed through Geneious[13].

Models building by homology modelling:

To predict 3D structures of chlortetracycline, chromomycin A₃, mithramycin and polyketomycin homology modelling was used, which is the most suitable method for building protein models[14]. CPH model server was used to select template[15]. Modeller v9.11 was used for template and query alignments[16] using align2d command and output file in PIR format was used for building five models against each query. The analyses for model evaluation and quality for all four models were done by ProSA-web Z-score[17], Qmean plot[18] and PROCHECK Ramachandran plot[19]. Root mean squared deviation (RMSD) and superimposition of each query and template structure were performed using UCSF Chimera 1.10 workbench[20].

RESULTS AND DISCUSSION

The biosynthetic gene cluster of chromomycin A₃ was compared to that of mithramycin for genes involved only in polyketide biosynthesis and post-polyketide
A great difference in the genetic organizations of both polyketides was observed. Ketosynthase (KS), chain length factor (CLF) and acyl carrier protein (ACP) are involved in the biosynthesis of minimal PKS. In the gene cluster of mithramycin, all these three genes are located together in the central region of the cluster whereas KS and CLF genes are located together and ACP is located more than 8 kb distant downstream in the gene cluster of chromomycin A₃. One more difference in polyketide biosynthesis of two polyketides is the location of bifunctional cyclase/aromatase gene. The gene is similar to various aromatases and involved in the biosynthesis of type II polyketides such as in mithramycin[21]. The location of aromatase in the gene cluster of chromomycin A₃ is pretty unusual as it is present at one end of the cluster that is far from KS gene whereas it is located very close to KS in the gene cluster of mithramycin. Talking about post polyketide steps, three ketoreductase genes were found in the gene cluster of chromomycin A₃ but two in that of mithramycin.

The organizations of genes involved in the biosynthesis of chromomycin A₃ and mithramycin were also shown through Mauve alignment (fig. 2). Similarities and differences in both biosynthetic gene clusters could easily be visualized in Mauve alignment and it was in fully accordance with the gene cluster analysis performed through antiSMASH.

Modeller v9.11 was used for homology modelling and the template was selected (PDB:1TQY) on CPH server. Alignment of all four queries along with their template was shown in fig. 3. Five models for each query were developed using Modeller v9.11 and the best model of each query was selected on the basis of their structural evaluations through ProSA-web Z-scores and PROCHECK Ramachandran plots. Z-score values of 7.75 for chromomycin, 9.3 for chlortetracycline, 10.52 for mithramycin and 9.99 for polyketomycin respectively, confirmed that both target proteins and template have similar folds. Ramachandran plots were obtained from PROCHECK server and they showed that 90.5% of residues for chlortetracycline, 86.7% for chromomycin, 91% for mithramycin and 92.6% for polyketomycin were in most favoured regions (fig. 4). Superimpositions of each model with template (reference structure) using UCSF Chimera v1.10 program (fig. 5) showed very low RMSD values of 0.399 Å for chromomycin and chlortetracycline, 0.191 Å for mithramycin and polyketomycin, and 0.395 Å for chromomycin and mithramycin, respectively. Very low values of RMSD proved that there are high similarities between each query and template. The statistics of sequence alignments of template and queries are given in Table 1.

We compared the biosynthetic gene clusters of two aureolic acids to find out the dissimilarities between them. Genetic organizations of biosynthetic clusters in many cases are pretty similar for structurally related bacterial polyketides. However, this similarity was not reflected at biosynthetic genes level for chromomycin A₃, a polyketide that is closely related in its chemical structure to mithramycin and a very different genetic organization was observed in both biosynthetic gene clusters. The genes involved in polyketide biosynthesis were grouped in the central part of the biosynthetic gene cluster of mithramycin while they scattered throughout
the cluster in chromomycin A\textsubscript{3}. Form this observation, it was suggested that bacterial aromatic polyketide biosynthetic clusters among different \textit{Streptomyces} species might have been transferred horizontally and therefore, quite similar polyketide biosynthetic gene clusters can be observed in distantly related bacterial species\cite{22}. Evolution could be responsible if this transfer has occurred in case of chromomycin A\textsubscript{3} and mithramycin that has probably rearranged the genes and caused differences in gene organizations of both clusters\cite{2}.

This is the first study in which 3D models of KS\textalpha{} proteins of both aureolic acids were also prepared and superimposed with closely related polyketides of other families to study similarities between them. RMSD plays an important role to measure the similarity in 3D structures after optimal rigid body superimposition. A very large value of RMSD means the structures, which are superimposed are dissimilar and zero RMSD means structures are identical in their confirmation\cite{23}. Because mithramycin and chromomycin A\textsubscript{3} belong to the same group of polyketides i.e. aureolic acid, here an interesting observation was found that RMSD value of 0.191 Å for mithramycin and polyketomycin showed better superimposition hence more structural similarities as compared to mithramycin and chromomycin A\textsubscript{3} superimposition with an RMSD value of 0.395 Å. The RMSD values for superimpositions of chromomycin A\textsubscript{3} and chlortetracycline, and that of chromomycin A\textsubscript{3} and mithramycin were almost identical i.e. 0.399 Å and 0.395 Å, respectively, which revealed that chromomycin A\textsubscript{3} has nearly equal structural similarities for both polyketides. These observations were again justified by the alignment scores. Mithramycin and polyketomycin for 420 atom pairs gave better alignment score of 1757.6 whereas mithramycin and chromomycin for 360 atoms gave 851.1 alignment score. Similarly, chromomycin A\textsubscript{3} with chlortetracycline and mithramycin gave nearly equal alignment score i.e. with chlortetracycline gave 794.8 alignment score between 374 atoms and with mithramycin 851.1 between 360 atoms. Similar results were also found by Feng \textit{et al.}\cite{24} who conducted phylogenetic studies for different classes of polyketides. They generated a phylogenetic tree for KS\beta{} subunit of type II PKSs and found that chromomycin A\textsubscript{3},
Fig. 4: 3D model evaluations
A) Chlortetracycline, B) chromomycin A₃, c) mithramycin, d) polyketomycin. †Ramachandran plot analyses for predicted models. The plot statistics are: residues in the most favoured region (red); residues in allowed (yellow) and in generously allowed (light yellow) region. ††Z-score plots from ProSA-web server showing the quality of predicted models in NMR region (dark blue). †††Energy plots showing all residues of predicted models at very stable positions (dark green lines)
showed more similarities towards tetracycline class of polyketides rather than for mithramycin i.e. aureolic acid. The current study has also justified the findings of Zhang et al.\cite{25} that aureolic acids were more likely to be derived from a cyclization pathway of tetracycline-like and in fact a number of highly homologous enzymes are shared by the biosynthetic pathways of mithramycin and oxytetracycline such as cyclases and tailoring enzymes. Later, it was identified that premithramycin B was transformed into an aureolic acid structure by oxygenase MtmOIV through fourth ring Baeyer-Villiger oxidative cleavage\cite{26}. The enzyme homologue to cyclase MtmOIV was also identified in the biosynthetic gene cluster o of chromomycin A\textsubscript{3}\cite{2} derived from prechromomycin B, which is a tetracylic intermediate\cite{27}.

KSs are the most conserved domains and found as essential part of each PKS gene cluster as they have been used to identify PKS genes from individual bacterial strains\cite{28} and environmental DNA\cite{29}. Each catalytic site is encoded on a distinct protein by type II PKSs, which also called iterative type of PKS. Two discrete KS domains are encoded by type II PKSs i.e. KS\textalpha{} and KS\textbeta{}. The former domain performs condensation reaction while KS\textbeta{} that also known as CLF defines the number of iterative condensation steps\cite{30}. As the arrangement of genes of two pathways is totally different in the organizations of both aureolic acids but synthesis of similar intermediates is still accomplished in the most effective way therefore, it can be suggested that arrangement of genes does not influence the arrangement of protein at all\cite{2}. The findings of this study suggested that care should be taken while classifying different bacterial polyketides on the basis of their KS\textalpha{} genes.

Chromomycin A\textsubscript{3} and mithramycin are members of the aureolic acid family of antitumor antibiotics and are effective against Gram-positive bacteria as they inhibit growth and multiplication of several cancer cell lines\cite{31}. This inhibiting activity of aureolic acids comes through interaction in an Mg\textsuperscript{2+}-dependent manner with regions of GC-rich in the minor groove of DNA\cite{32}. Zn\textsuperscript{2+} metalloenzymes including alcohol dehydrogenase (ADH) can also be inhibited by both aureolic acids through binding at zinc centers and disruption of quaternary structure of the metalloenzyme complex. This property makes these aureolic acids potential therapeutic agents against neurodegenerative disorders and metal dyshomeostasis\cite{33}.

In conclusion, for the analysis of growing volume

<table>
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<th>Extinction coefficient (mean)</th>
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<td>44.498</td>
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Fig. 5: Superimpositions of predicted models for KS\textalpha{} subunits done by Chimera v1.10

(A) superimposition of KS\textalpha{} from chromomycin A\textsubscript{3} (green) and chlortetracycline (red), (B) superimposition of KS\textalpha{} from mithramycin (magenta) and polyketomycin (blue), (C) superimposition of KS\textalpha{} from chromomycin A\textsubscript{3} (green) and mithramycin (magenta)
of DNA sequence data new bioinformatics tools are needed. This is especially true in case of biosynthesis of secondary metabolites where major challenges for accurate sequence assembly and analysis are created by highly repetitive nature of associated genes. The current study has proven that biosynthetic gene clusters of an antitumor antibiotic chromomycin A₃, synthesized by S. griseus has different genetic organization as compared to mithramycin that is a closely structurally related polyketide of the same class i.e. aureolic acid. Moreover, the 3D structures of KSₐ subunit of type II PKS of both aureolic acids have shown less degree of structural superimposition as compared to other classes of polyketides. Mithramycin has shown a better structural superimposition with polyketomycin i.e. a tetracyclic quinone rather than chromomycin A₃. These incongruences are due to different rates of evolution of bacterial biosynthetic genes and more importantly to the process of horizontal gene transfer (HGT) that has been now widely recognized as a major force driving bacterial evolution. The dissimilarities are clearly indicating that HGT for both aureolic acids has gone through different directions. As the classification of bacterial polyketides plays a vital role to identify and study biosynthetic pathways of novel polyketides therefore, findings of this study will surely help in correct organization and classification of different classes of polyketides in future.

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Conflicts of interest:

There are no conflicts of interest.

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