Poxviridae Protein kinases as targets for control of LSDV , Monkeypox like outbreaks

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Abstract

Lumpy skin disease virus has infected cows across the world resulting in the death or destruction of at least a million cows. Poxviridae family includes 88 such viruses that cause deadly diseases like smallpox, monkeypox and lumpy skin disease. Pox virus induced infections have been known to be sporadic and zoonotic. We identified two novel protein kinases in poxviridae genomes. Multiple sequence alignment and phylogenetic analysis of these pox viral protein kinases revealed the sequence conservation and evolutionary relationships of these viral protein kinases. AI driven methods revealed 3D structures and active site of the two protein kinases. The viral kinases also showed remarkable structural conservation. Virtual screening of pox virus kinases identified LSTK and LYK as druggable targets and 2 FDA approved kinase inhibitors (lapatinib and pazopanib) were recognized as potential inhibitors of LSTK and LYK. Molecular dynamics simulations identified these inhibitors to be competitive inhibitors of the new kinases. These findings provide valuable insights that can be leveraged in the development of antiviral therapeutics for pox virus infections LSDV and Monkeypox.

Poxviridae Protein kinases as targets for control of *LSDV* , Monkeypox like outbreaks

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Abstract:

Lumpy skin disease virus has infected cows across the world resulting in the death or destruction of at least a million cows. Poxviridae family includes 88 such viruses that cause deadly diseases like smallpox, monkeypox and lumpy skin disease. Pox virus induced infections have been known to be sporadic and zoonotic. We identified two novel protein kinases in poxviridae genomes. Multiple sequence alignment and phylogenetic analysis of these pox viral protein kinases revealed the sequence conservation and evolutionary relationships of these viral protein kinases. AI driven methods revealed 3D structures and active site of the two protein kinases. The viral kinases also showed remarkable structural conservation. Virtual screening of pox virus kinases identified LSTK and LYK as druggable targets and 2 FDA approved kinase inhibitors (lapatinib and pazopanib) were recognized as potential inhibitors of LSTK and LYK. Molecular dynamics simulations identified these inhibitors to be competitive inhibitors of the new kinases. These findings provide valuable insights that can be leveraged in the development of antiviral therapeutics for pox virus infections LSDV and Monkeypox.

Abbreviations: *LSDV* – Lumpy skin disease virus; LSTK –*LSDV* serine threonine kinase; LYK – *LSDV* tyrosine kinase; MSTK- Monkeypox serine threonine kinase; LYK – *LSDV* tyrosine kinase; MLKL – Mixed lineage kinase domain-like.

1. Introduction

Lumpy skin disease (LSD) is caused by a virus that belongs to the genus *Capripoxvirus* within the family *Poxviridae* . Lumpy Skin Disease virus (LSDV) primarily infects cows [1] and also affect other species such as water buffaloes, sheep, goats[2]. Lumpy skin disease virus has infected millions of cows across the world and the zoonotic nature of these viruses threatens other closely related animals. Monkeypox is one such member of *Poxviridae* family (Subfamily: *Chordopoxvirinae* , Genus *Capripoxvirus*) that has shocked the entire planet during COVID by infecting humans who are immunocompromised. Many of the pox virus induced infections are sporadic and zoonotic in nature[3]. Lumpy skin disease is characterized by the formation of nodules or lumps on the skin and mucous membranes, fever and reduced milk production [4,5]. Pox viruses such as *Molluscum contagiosum* can cause human skin infections[6] as well.

Poxviridae viruses are double stranded DNA viruses that contain typically 100-150 genes[7] that appear to have been captured primarily from hosts by genetic recombination events during evolution [8]. LSDV genome has 156 putative genes and three of these genes encode viral protein kinases, that may phosphorylate specific proteins to control various cell signalling processes. These kinases might play a role in the LSDV's replication, pathogenesis and other cellular functions like translation initiation[9] and nuclear egress, evasion of host immune response as seen from other viruses [10]. Protein Serine/threonine [10] and tyrosine kinases[11] have been known to be present in viral genomes and protein phosphorylation has also been found an important mechanism that regulates viral replication [12]. The discovery of a vaccinia virus kinase (VPK2) has shown the importance of protein phosphorylation in poxviral biogenesis [13]. Targeting LSDV and other conserved family protein kinases could be an essential step in containing the infection, preventing the global spread of the virus and to stop economic burden arising due to cattle death.

In the present work, we have employed biochemical-informatics approaches for the identification of LSDV viral protein kinases, predict their structures and model their active sites, conservation followed by virtual screening for inhibitors. Two interesting LSDV kinases; a serine/threonine kinase (LSTK) and a tyrosine kinase (LYK) were identified with homologs and high degree of conservation across various viruses. Prediction of a structure has taken a big leap forward from the major experimental approaches to *in silico*methods[14-16]. Modern structure prediction tools using deep learning and artificial intelligence-based approaches demarcate the beginning of a new era in protein structural bioinformatics [17]. A myriad of programs incorporate information from amino acid sequences to predict the distance and torsion distribution of the protein by employing deep learning, machine learning based computational algorithms by effectively combining homology modeling, threading and *ab initio* strategies[18,19]. Among these, ALPHAFOLD2[20] and ESMfold [21] have been used to predict the protein structure models approximated to high accuracy. Protein kinases have been the most druggable targets[22]. Since, the kinases in most organisms have well conserved active sites and catalytic domains, we adopted*bioinformatics* approaches to repurpose FDA approved human kinase

inhibitors to LSDV and monkeypox kinases. The utilization of existing drugs for the treatment of LSD through repurposing strategies may yield promising outcomes.

2. Materials and Methods

2.1. Multiple sequence alignment of LSDV Serine/Threonine kinase and Tyrosine kinases

Putative protein kinases predicted from *LSDV* genome analysis[7] were screened in SMART[23], CDART[24], CDD [25] and pfam[26] to confirm their protein kinase domains and families. Sequences of a serine/threonine protein kinase (LSTK) and a tyrosine kinase (LYK) from *LSDV* and other poxviridae were obtained from NCBI. BLASTp was performed for both sequences in NCBI and the related sequences were examined for multiple sequence alignment. The multiple sequence alignment of sequences obtained from protein clusters from NCBI for both LSTK and LYK were aligned using ClustalW[27].

2.2. Phylogenetic analysis of LSDV Kinases: The IQ-TREE program[28], was employed to construct phylogenetic tree based on maximum likelihood. Best-fit substitution model was selected using ModelFinder algorithm[29], based on the Bayesian information criterion (BIC), with ultrafast bootstrapping (1000 iterations) as described in detailed in Supplementary methods. The tree was visualized using the FigTree software (v1.4.4)[30].

2.3. Structure prediction for LSTK and LYK and structural comparison: The sequences of LSTK (NCBI Gene ID- 921586; Accession: NP_150459) and LYK (Gene ID- 22595678; Accession: AAN02711.1) were used to model 3D structure of the kinases. Structures were predicted by comparing two AI based programs, AlphaFold2 (AF2) [20]and ESMFold (EF) [31], [21]. The quality and accuracy of the predicted models were evaluated using Ramachandran plot, using Gaussian KDE (kernel density estimation) to plot the density of favoured torsion angles (φ and ψ) in the protein structure. The structures of the human MLKL (Mixed Lineage Kinase Domain-Like Protein) and *Monkeypox* STK were predicted using AlphaFold2 for structural comparisons of *LSDV* kinases were performed using Chimera-X[32]. The degree of similarity between the proteins' structures was assessed by determining their RMSD (Root Mean Square Deviation) and structure alignment (TM-Template Modelling) scores by UCSF Chimera[32] and TM-score[33]. Structures obtained from AlphaFold2 and ESMFold were compared by their RMSD, pLDDT[34], perplexity[21], and pTM scores.

2.4 Ligand preparation for virtual screening: A total of 88 approved human kinase inhibitors were obtained from the DrugBank database [35]. The SMILES to 3D-structures generation was done using Open Babel[36]. Ligprep[37]was used to prepare the structures of the inhibitors by assigning bond order and bond angles and by minimizing the structures using the OPLS-2005 force field. The Epik[38]feature is used to ensure the correct protonation state of the ligand and to generate tautomers of the compounds at a target pH of 7.4 $\pm$1.0. A maximum of 5 stereoisomers were created for each chiral molecule[39].

2.5 Receptor preparation and grid generation: The target proteins (LSTK and LYK) were prepared by addition of missing hydrogens, partial charges and side chains using Protein Preparation Wizard module (Schrödinger suite 2020-3). Energy minimization was carried out using default constraint of 0.3 Å RMSD and OPLS-3 force field. The Sitemap tool [40] was used to identify the active site region of the Kinases. Receptor grid is generated using the Receptor Grid Generation Wizard in the Glide Grid module (Schrödinger suite 2020-3).

2.6. Virtual screening - Drug Repurposing: The predicted structures of LSTK and LYK were screened against a library of FDA-approved kinase inhibitors by High-Throughput Virtual Screening (HTVS) using the Schrödinger Maestro module [41]. HTVS and Standard Precision (SP) docking was used to assess the effectiveness of ligand binding towards target protein. PyMol and Maestro tools were used for molecular visualization[42].

2.7. Molecular simulations: The classical molecular dynamics (MD) simulations were carried out on selected inhibitors, using GROMACS 2022[43], to evaluate the substrate and inhibitor binding interactions of LSTK and LYK. The protein was parameterized with CHARMM-36force field provided in GROMACS[44]. Ligand topologies and coordinate files were generated for the CHARMM-36 force field using the SwissParam tool[45].

Two types of protein-ligand complexes for LSTK and LYK were designed for the simulation, one with bound ATP and the other with docked inhibitor at the active site in presence of Magnesium ions. The protein-ligand complexes underwent energy minimization using the steepest-descent algorithm[46] and the Verlet cut-off scheme[47]. An ensemble equilibration with a restricted constant number of particles, volume, and temperature /canonical ensemble (NVT) and an ensemble equilibration with a constant pressure, and temperature (NPT) ensemble were then performed for a total time of 1ns under Particle Mesh Ewald (PME) electrostatics and 3D periodic boundary conditions[48]. Berendsen thermostat[49] was used in both the NVT and NPT runs. For the NPT run an additional Berendsen barostat was used. Finally, a production MD run of 10ns was carried out using a modified Berendsen thermostat and Parinello-Rahman barostat[50] before proceeding further with ligand binding energy calculations and active site modelling.

2.8. Principal component analysis

The FDA approved veterinary drugs for different diseases were selected for the comparative analysis against human kinase inhibitor drug dataset (88 compounds). 16 Chemical and molecular descriptors (molecular weight (M.W), heavy atoms, aromatic heavy atoms, Fraction of CSP^3, rotatable bonds, Hydrogen bond acceptors, H-bond donors, Molecular refractivity (MR), Topological polar surface area (TPSA), Bioavailability, lead likeness, Synthetic accessibility and lipophilicity factors such as iLogP, XLogP, WLogP and MLogP were calculated using SwissADME [51], and ChemDraw[52]. The PCA analysis was performed by classical mean-centering and the normalization using R open source program to reduce 18-dimensional vectors of each compound to a 2D vector.

Results and Discussion

3.1. Conservation of LSDV protein kinases: Around 350 viral genomes have been identified to encode S/T/Y protein kinases[53]. Viruses with double stranded DNA and single stranded RNA as genetic material encode kinases in their genomes.*Poxviridae* family of double stranded DNA viruses have 2 primitive protein kinases embedded in their genomes. In Lumpy skin disease virus, we identified a serine/threonine kinase (LSTK) and a tyrosine kinase (LYK) from their sequences. Eukaryotic protein kinases typically have 7 α-helices and 5 β-strands and 2 long loops representing catalytic site containing HRD domain and activation site that has the well-known DFG motif [54]. A comprehensive sequence and structure-based analysis to find related kinases among viruses enabled us to identify the conserved residues or motifs in kinase catalytic domain across*Poxviridae* . We observed no related kinases in eukaryotic genomes, that resemble LSTK, whereas LYK had a distant relative amongst vertebrates called mixed lineage kinase domain-like protein (MLKL) that is critical for necroptosis[55]. We note that these novel kinases from *Poxviridae* may have functional assignments due to their replication dependency for cellular machinery, in higher organisms. They may also have similarity to viral oncogenes in function in regulating cell signalling, human cancers and diseases. The viral protein kinases are different from eukaryotic and prokaryotic protein kinases [56] in having only kinase domain.

LSTK has shown a high degree of conservation and presented clear structural motifs and sequence motifs revealing the importance of the conserved sequences (**Figure 1A, 1B**). LYK clustered in 2 different groups of kinases based on their sequence conservation as overall LYK from all animals has not shown great level of conservation (**Figure 2AI, 2BI**). These 2 different groups of viruses having different sequences and seem to have categorically enriched certain sequences in confinement to infect land animals (**Figure 2AI, 2BI**) or birds (**Figure 2AII, 2BII**) primarily. Although, LYK from both groups differed significantly in sequence similarity yet revealed structurally similarity as well that may be an index of functional or substrate specificity. Variations reflected true viral nature of modified sequences, to adapt to different host tissue and cellular milieu and to bind to specific sequences when infecting a specific host. Short sequence motifs were highly conserved amongst STKs of *poxviridae* (**Figure 1A**) whereas LYK showed high level of variations (**Figure 2A**). Motifs that were highly conserved in*LSDV* kinases were present in both α-helices, β-strands as well as loops (Figure 1B and 2B). Short Linear Motifs (SLiMs) were predominant, but in a catalytic active site we see major kinase domain like long sequence conservation as well. While LSTK presented 19 α-helices (18 in monkeypox kinase) and 11 β-sheets (**Figure 1B**), LYK whose homolog was absent in *monkeypox*

showed 13-14 α-helices and 7-8 β-sheets (**Figure 2B**). Each of the kinases showed unique motifs that aid in structure conservation and that might be necessary for protein function. A unique YVVKFVYE (**Figure 1AI,1BI**) at β4 gave an impression of FYVE domains, that bind to phospholipids[56]. Domain / motif prediction software tools (Interpro[57], Scansite[58], pfam[59], Cofactor[60]and Schrodinger), anticipated active site of LSTK to span residues 80-359, while the ATP binding site (predicted by Schrodinger and ATPbind[61]) is expected to be located within residues 95-346. Similarly, for LYK, the predicted active site ranges from residues 14-188, with the ATP binding site also predicted to be within residues 95-346. These regions exhibit a high degree of conservation, with minimal variations observed (**Figure 1C, 2C**). Prokaryotic proteins were believed to be enriched with rare amino acids as opposed to modern living entities[62].

We constructed maximum likelihood phylogenetic trees using protein sequences from 26 different viruses belonging to 7 genera for LSTK and 22 different viruses from 7 genera for LYK. The phylogenetic analysis of LSTK and LYK proteins revealed a remarkable level of conservation among the related viruses with a high degree of similarity. The evolutionary distance between the viral kinases was found to be very small, indicating common evolutionary lineage (**Figure 1C**). The phylogenetic analysis of viral kinases revealed conserved structure and sequence for both LSTK and LYK proteins across the studied genera. The LSTK of Lumpy skin disease virus showed close relationship with Sheeppox and Goatpox virus (**Figure 1C**). While LYK of *LSDV* showed similarities with Myxoma, Deerpox, Sheeppox, and Goatpox virus with a common ancestor (**Figure 2C**). This suggests that viral kinases have undergone minimal diversification, indicating their critical role and functional significance in viral replication and pathogenesis.

The phylogenetic analysis of LSTK and LYK also revealed several notable amino acid substitutions across the protein sequences and varying degrees of conservation and divergence in the sequences. Amino acid frequency analysis revealed enrichment of lysine, leucine and asparagine in both LSTK and LYK (**Figure 1D, 2D**) but no selective enrichment of tryptophan, cysteine, tyrosine, phenylalanine, which were generally found to be abundant in the prebiotic environment. Hence it can be concluded that there is possibility that these kinases have been acquired by gene capture from higher organisms or happened due to current evolutionary and environmental conditions. In LSTK, amino acids like isoleucine, leucine, lysine, and phenylalanine, exhibited a higher frequency of substitution (> 0.09) (**Figure 1D**). Similarly, in LYK, isoleucine, leucine, and lysine showed a higher frequency (> 0.09) (**Figure 2D**). These amino acids displayed a relatively higher rate of substitution, suggesting their potential functional or structural adaptability to host[63]. On the other hand, in LSTK, amino acids such as cysteine, tryptophan, and methionine had lower substitution values, with a frequency below 0.02 indicating their importance in structural integrity. In LYK, cysteine, tryptophan, histidine, alanine, glutamine, and methionine also exhibited a lower frequency (<0.02). These amino acids exhibited a relatively lower rate of substitution, indicating a higher degree of conservation in their respective positions within the protein sequences.

3.2. AI based modeling of 3D structures of LSTK and LYK:*LSDV* has several variants that have been sequenced by independent efforts [64-66]. Efforts are ongoing to identify the complete genome sequence of all variants of monkeypox[67] although whole genome sequencing of hMPXV1 has been completed [68]. Bioinformatics analysis of these sequences have identified putative protein kinase sequences in these genomes [7,69]. We hypothesized that if we could predict their structures, we could repurpose FDA approved kinase inhibitors on the potential kinase sequences to combat *LSDV* and monkeypox. Since, there are no experimentally derived structures of the *LSDV*kinases in the protein data bank, few were challenged to employ the 2 most recent AI based protein prediction algorithms.AF2 and ESMfold2 employ AI algorithms based on template-free modelling[20]. LSTK and LYK whose structures were predicted, were also compared with two similar proteins: *Monkeypox* MSTK and human MLKL homologs of the *LSDV* kinases (**Figure 3A-D**). For each of these four proteins, the AF2 algorithm generated top-ranked models. Both kinases are monomeric and have only one domain (protein kinase domain). In order for the predictions to be practically valuable, it is necessary to have a confidence measure that is well-calibrated and resolved at the sequence level. The AF2 model based predicted local distance difference test (pLDDT) that ranges from 0 to 100, where higher scores indicate greater confidence in the predicted structure[20,70] enabled us to confidently compare and screen the predicted structures. Ramachandran plots were used to validate predicted structures.

In the current study, all the top-ranked protein models had a pLDDT score more than 85, indicating that the predicted structures are reliable. Various metrics were utilized to assess the quality of the structure, among which pLDDT stands out as a better index of quality of predicted protein structure in approximation to its native structure[33]. The pLDDT scores of the LSTK and LYK structures demonstrated commendable confidence values (**Figure 3A**), indicating a noteworthy level of accuracy in the prediction of the protein's main chain. The structure details were compared and analysed to gather information about number of α-helices and β-sheets, while, the LYK showed 14 helices and 8 sheets in the foreseen structure. To further validate the generated protein structure quality, the SAVES (v 6.0) server (https://saves.mbi.ucla.edu/) was utilized [71]. Ramachandran plot indicated a significant proportion of residues falling in right quadrants (**Figure 3B**). The low percentage of residues in the generously allowed regions is indicative of good structural quality, although their presence suggests the existence of structural irregularities or flexibility in some areas of the protein (**Figure 3B**). In the LSTK structure, only 0.5% (2 out of 447 residues) were found in the disallowed region. In contrast, no residues were located in the disallowed region in the LYK structure, which is another positive indicator of good protein structure quality. According to the ERRAT server [72], the overall quality factor was determined to be 90.3 for LSTK and 89.7 for LYK. AF2 also utilizes a pairwise error prediction technique to generate pTM/predicted template modeling scores, wherein the contribution of each residue is weighted based on the probability of its experimental resolution. The assessment results revealed that the pTM scores of the predicted LSTK and LYK structures were 0.85 and 0.88, respectively, indicating a remarkable level of accuracy in protein structure prediction.

In addition to the use of the AF2 method **(Figure 3A)** , the ESMFold (Evolutionary Scale Modeling) deep learning-based method was also employed for the prediction of protein structures[21] (**Figure 3C**). The findings suggested that the predicted structures displayed high accuracy. The results of both AF2 and ESMFold methods demonstrated significant predictive ability for the structures of LSTK and LYK proteins, with several mathematical and statistical validations. The combination of these multiple metrics provides compelling evidence to support the notion that the predicted LSTK and LYK models possesses a high level of quality, thereby making it suitable for further studies **(Figure 3C)** . Ramachandran validation confirmed the accuracy model**(Figure 3D)** .

3.3. Prediction of the 3D structures of MSTK and hMLKL: The results of the sequence homology analysis indicated that the*LSDV* kinases, LSTK and LYK, exhibit significant similarity with*Monkeypox* Ser/Thr kinase and human MLKL proteins, respectively. AF2 and ESMFold were utilized to predict the structures of these two proteins, facilitating their subsequent comparative analysis**(Figure 3A-3D)** . The reasons to compare the *LSDV* kinases with their homologs were because; i) Comparing structures may give us a chance to understand their functions, ii) designing inhibitors can come in handy to target the kinases (Monkeypox for instance) for other similar pox outbreaks if required. iii) knowing what MLKL binds to and regulates in its signaling may give us an approximate idea of LYK mechanism as well when the virus infects the host. The aforementioned validation measures and matrices were computed for the predicted structures, including pLDDT scores for both AF2 and ESMFold models. Additionally, both AF2 and ESMFold predicted models for MSTK displayed similar matrices as their homologs.

Overall AF2 and ESMFold produced high-quality 3D structures for all four proteins, with relatively minor variations in their predictions that can be attributed to differences in their respective algorithmic architectures for protein structure prediction. Since AF2 demonstrated greater accuracy by the measure of the index of the computed matrices for both, LSTK and LYK, we have chosen AF2-predicted models for further analyses [31].

3.4. Structural comparison (LSTK vs MSTK and MLKL vs LYK): We compared structural similarities for LSTK/LYK with their nearest homologues, by superimposition of the AF2 predicted LSTK model with MSTK and LYK with hMLKL proteins and observed significant structural similarities and differences **(Figure 4).** The LSTK super imposed with MSTK exhibited a moderate structural similarity, whereas, the LYK displayed major structural difference with predicted and PDB based structure of hMLKL (**Table 1**). Though the LSTK and MSTK (**Figure 4A**); LYK and hMLKL have good sequence homology in sequences

(**Figure 4B**), their structural alignments (RMSD scores) reveal structural differences (**Table 2**). RMSD can reveal structural deviations even if two structures are almost identical with slight variations like minor differences in a flexible terminus or a loop [31]. MSTK and LSTK presented similar structural features but for the flexible loop (**Figure 4A**). The primary structural difference between LSTK and MSTK is the presence of an additional α-helix in LSTK, resulting in a total of 19 helices in LSTK compared to 18 in MSTK and the structure of LYK differs from that of hMLKL in having one additional helix and sheet. We also compared hMLKL that has been predicted by AI and also the MLKL whose structure has been solved by x-ray crystallography and deposited in PDB for a gain of confidence on our superposition studies (**Figure 4C**). These findings may point to the functional similarities as well as diversity on offer due to enzymatic flexibility, adaptability as an aspect of specificity found in closely related proteins.

3.5. Virtual screening / Drug Repurposing: Protein kinases in general have immense potential as antiviral drug targets[73,74]. LSTK and LYK were subjected to virtual screening against a total of 88 FDA approved human kinase inhibitors. With the structural prediction followed by active site prediction we subjected LSTK (**Figure 5A-5B**) and LYK (**Figure 6A-6B**), to virtual screening. We also examined the druggability index (D-score) calculated by Sitemap tool [40] of both LSTK and LYK, and the scores (1.032 and 1.087) point out to their capacity to bind small molecules. We have used molecular dynamics to measure the likelihood of inhibitors to bind to the active sites of the two kinases. Majority of the inhibitors exhibited comparable binding affinities towards LSTK and LYK. Erdafitinib exhibited promising binding affinity with a docking score of -8.39 Kcal/mol followed by Lapatinib (-7.66 Kcal/mol) and Baricitinib (-7.52 Kcal/mol) towards LSTK (**Figure 5C-E**). The three ligands Erdafitinib, Lapatinib and Baricitinib (growth factor receptor antagonists) interacted with LSTK via three (Lys112, Ala259 and Asp346), three (Lys112, Gly99 and Asn315) and two (Gln80 and Ala259) hydrogen bonds, respectively. LSTK-inhibitor complexes demonstrated the presence of hydrophobic interactions from neighbouring residues contributing to the stability of the complexes. All 88 kinase drugs were found to interact with the active site of LSTK at varying affinities, and the top 15 inhibitors showed binding affinities/ docking scores, ranging -8.39 to -6.65 Kcal/mol (**Figure 5F-5G**)

The molecular docking studies of LYK also showed promising candidates; with top three molecules Pazopanib, Ponatinib (tyrosine kinase inhibitors) and selumetinib (MEK1/2 inhibitor) exhibited promising binding with LYK (docking scores of -9.24, -7.01 and -6.73 Kcal/mol) (**Figure 6C-6E**). Hydrogen bonds are involved in the stabilization of interaction between the top three inhibitors with LYK. While, Pazopanib forms four hydrogen bonds (Asn29, Pro30, and Tyr104) that facilitate its interaction. Selumetinib, interacts with LYK through three hydrogen bonds (Asn18, Lys90, and Ile103) and Ponatinib forms a single hydrogen bond (Asn29) with LYK. Hydrophobic interactions have also played the significant role in the stabilization of LYK-ligand complexes. Similar to LSTK, the active site of LYK was found to interact with varying affinities to all 88 kinase compounds, and the top 15 inhibitors exhibited the binding scores ranging -9.24 to -5.11 Kcal/mol (**Figure 6F-6G**). No prior computational studies have been conducted on *LSDV* /LYK kinases. These studies can be used to test the activity and inhibition of these kinases *in vitro* .

3. 6. MD simulations and Binding energy: To assess the dynamic stability of the protein-ligand complexes, MD simulations were conducted on the LSTK and LYK complexes with the hit compounds. Protein bound to ATP-Mg2+ and the protein- inhibitor complexes were used virtual screening. The best inhibitor from the binding energy calculation was subjected to bind to the ATP-binding site to proceed with molecular dynamics simulations. An all-atom 10ns MD simulation was conducted on the ATP-Mg^{2+}-LSTK (**Figure 7A**), Lapatinib-LSTK (**Figure 7B**), ATP-Mg^{2+}-LYK (**Figure 7E**) and Pazopanib-LYK (**Figure 7F**), complexes. Lapatinib, showed increased binding energy two times to that of the ATP-protein complex showing that it can displace ATP as a competitive inhibitor. LYK binding to pazopanib also displaced ATP from the protein complex. The amino acids Asp314, Asp346, and Ser96 located in the ligand binding site of LSTK interact with both ATP and lapatinib (**Table 3**). All three amino acids form interactions with the phosphate groups of the ATP molecule (**Figure 7A**). Specifically, Ser96 and Asp346 form hydrogen bonds with the oxygen atoms of the beta phosphate, while Asp314 interacts with the oxygens on the phosphate groups of the ATP using a salt-bridge mode of interaction. The inhibitor lapatinib interacts with the same

three amino acids in the same regions as ATP, forming hydrogen bonds and utilizing the key ATP-binding sites (**Figure 7B)** . This suggests a possible competitive inhibition mechanism for lapatinib (**Figure 7C**). The GROMACS calculation confirms this observation, as the binding energy value of the lapatinib-LSTK (-810.956) complex is much higher than that of the ATP-LSTK pair (-366.012) (**Table 4**). All the calculated binding energies are a combination of Lennard Jones and Coulombic interaction energy functions that describe the effect of polar and non-polar interactions between the binding site amino acids and the ligand. For the LYK, both the pazopanib and ATP share some, but not all, common residues. Specifically, the ATP molecule interacts with Asn29, Asn62, Arg94, Tyr104, and Lys102 via salt-bridge interactions (**Figure 7E**). The inhibitor pazopanib forms hydrogen bonds with Asn29, Asn62, and Tyr27, out of which Asn29 and Asn62 also interact with ATP (**Figure 7F**). Both ligands bind to different tyrosine residues in the same binding pocket (**Figure 7G**). The calculated binding energy values suggest that pazopanib (-473.983 KJ/mol) exhibits greater stability upon binding to the kinase pocket in comparison to its ATP (-434.901 KJ/mol) counterpart (**Table 2**). This observation allows for the possibility of another instance of competitive inhibition.

Competitive binding of the Inhibitor and protein complexes: To evaluate the stability of the inhibitors bound to LSTK and LYK, the inhibitors, lapatinib-LSTK (2.685±0.36 Å) and pazopanib-LYK (2.078±0.37 Å), both exhibited low RMSD values (**Figure 7D & 7H**) indicating their stability. Notably, the pazopanib bound to LYK showed a lower RMSD than the native ATP-Mg2+ ligand. In contrast, the lapatinib bound to LSTK showed a slightly higher RMSD than ATP-Mg2+ (ATP-LSTK = 2.030 ± 0.47 Å). A comparison of the binding energies of the complexes revealed that the pazopanib-protein complex (E = -473.983±19.09 KJ/mol) was more stable than the ATP-protein complex (E = -434.901±14.56 KJ/mol) in the case of LYK. Lapatinib-protein complex (E =-810.956±12.20 KJ/mol) was highly stable relative to the ATP-protein complex (E = -366.012±14.08 KJ/mol) in the case of LSTK (**Table 4**). Notably, the magnitude difference in the binding free energy values between the ATP and lapatinib in the case of LSTK was substantial. These observations clearly demonstrate that the screened inhibitors more effectively bind to the protein active site than the conventional ATP-Mg^{2+} ligand molecule. Further, the findings of the present investigation deduced that these hits (lapatinib and pazopanib) hold the promising prospect to be a candidate lead compounds against*LSDV* kinases in Lumpy skin disease and potentially monkeypox as well.

3.7. Principal component analysis: The structural features/physiochemical properties of small molecules are the key molecular descriptors that determine the molecule's pharmacokinetic and pharmacodynamic behaviour [75] and are critical to drug design, including solubility, stability, and bioavailability, which are important considerations in the early steps of drug design[76]. These molecular representations are used to assess the structural diversity/similarity of small molecules[77]. By computing the descriptors, we assessed the distribution of FDA-kinase inhibitors in the chemical space of FDA-Cattle drugs as well as human and cattle anti-viral drugs. We performed a comprehensive analysis of molecular descriptors of FDA approved drug molecules (Kinase inhibitors, cattle drugs, human antiviral and cattle antiviral) followed by their clustering into different groups to identify similar molecules sharing physicochemical descriptor space with other drugs. We performed PCA analysis and compared structural similarity/diversity on the set of 16 physicochemical descriptors (**Supplementary Table S1**) for a total of 492 drugs including kinase inhibitors (88), cattle drugs (319), human antiviral (74) and cattle antiviral (11). We computed a total of ten principal components (PCs) and observed that the first three PCs account for a substantial portion of the total variance in the data. Hence, the first three PCs were utilized to generate PC1 vs PC2 vs PC3 plots and to compare the molecular representation between various drugs based on their properties (**Figure 8**). The three principal components account for 90.983% of the variance in the complete dataset (PC1-57.83%, PC2-23.75% and PC3-9.39% respectively) (**Figure 8A-8C**). In the distinct chemical spaces occupied by the kinase inhibitors, the analyzed data showed considerable overlap between Kinase inhibitors with both cattle FDA approved and cattle antiviral drugs (**Figure 8D**) implying that they all possess similar drug like properties. Based on the virtual screening results the top three kinase inhibitors for both LSTK (Erdafitinib, Lapatinib and Baricitinib) and LYK (Pazopanib, Selumetinib and Ponatinib) were compared with cattle antiviral drugs. The six kinase inhibitors were found to occupy a common chemical space with cattle antivirals. From the

results we have identified the top three cattle antiviral drugs such as ganciclovir, acyclovir and vidarabine, that showed overlap with kinase inhibitors (**Figure 8E**). The spatial molecular property distributions of kinase inhibitors are comparable to those of FDA-approved cattle drugs and cattle antiviral drugs. We surmise that the newer LSTK, LYK binding inhibitors do fall in the chemical space intended for anti-virals used to treat cattle and they can be further tested to control *LSDV* and Monkeypox infections.

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Figure legends

Figure 1: Multiple sequence alignment (MSA) of serine/threonine protein kinase (LSTK) from (AI, AII) *Poxviridae* . Sequences have been divided into two groups for the ease of identification of motifs. Sequence logos displayed on top of aligned sequences. Evolutionary conservation of amino acid residues was highlighted for all the aligned blocks of the MSA. The number of α-helices (blue) and β-strands (yellow) were displayed and highlighted with different colors in the sequences. Short Linear Motifs (SLiMs) that are predominant and conserved in both α-helices and β-strands of the (BI) *LSDV* and (BII)*Monkeypox.* (C) Phylogram showing LSTK homologs in*Poxviridae* inferred by IQ-TREE. (D) Amino acid substitution frequency of LSTK across *Poxviridae.*

Figure 2: Multiple sequence alignment (MSA) of tyrosine kinase (LYK) from (A1, AII) *Poxviridae* . Sequences have been divided into two groups for the ease of identification of motifs. Sequence logos displayed on top of aligned sequences. The sequences of (BI) *LSDV*and (BII) *Fowlpox* were visually represented, with α-helices highlighted in blue and β-strands in yellow. (C) The phylogram of LYK in*LSDV* and other closely related *Poxviridae* was constructed using IQ-TREE. Different colors were used to represent each genus within the *Poxviridae* family. (D) The frequency of amino acid substitutions in the LYK protein sequences among the diverse members of the *Poxviridae* family.

Figure 3: AI based prediction and assessment of protein structures using two different learning models. (A) AlphaFold2 predicted structures for LSTK, LYK, MSTK and hMLKL. (B) Ramachandran plots for the predicted LSTK, LYK, MSTK and hMLKL structures. (C) ESMFold predicted structures for LSTK, LYK, MSTK and hMLKL. (D) Ramachandran plots for the ESMFold predicted LSTK, LYK, MSTK and hMLKL structures. The model confidence for each protein was assessed with pLDDT and pTM scores, which are represented below respective structures. The blue color region in each protein structure indicates the high model confidence.

Figure 4

Comparative structural analysis of LSTK and LYK with other related serine/threonine kinases. (A) Superimposition of predicted protein structures of *Monkeypox* serine/threonine kinase (MSTK) with LSTK. (B) Superimposition of predicted LYK structure with predicted hMLKL. (C) Superimposition of predicted LYK structure with predicted hMLKL along with PDB derived hMLKL structure.

Figure 5

Virtual screening of the FDA-approved kinase library targeting LSTK. (A) The predicted LSTK structure (shown as a line representation) along with the depiction of its active site. (B) Surface representation of the predicted binding site of LSTK. (C,D,E) Surface and 2D representations of the ligand-protein complexes with the top three inhibitors. (F) A common binding site shared by all the kinase inhibitors. (G) Docking scores of the top fifteen inhibitors towards LSTK.

Figure 6

In-silico screening of the FDA-approved kinase inhibitors against LYK. (A) The predicted structure of LYK, represented as a line model, along with its active site. ((B) The binding site of LYK was predicted using sitemap tool and visualized as a surface representation. The molecular interactions of top three inhibitors of LYK were depicted in three different formats: (C, D, E) Surface and 2D illustration of the ligand-protein

complexes. (F) A common binding site shared by all the kinase inhibitors. (G) Docking scores of the top fifteen inhibitors towards LYK.

Figure 7

MD simulations for the LSTK and LYK proteins with ATP, Mg+2, and inhibitor complexes. (A) The binding poses of ATP and Mg^{+2} and (B) lapatinib, with LSTK protein represented in surface conformation along with the zoomed view in a circle. (C) The cartoon representation of LSTK binding with lapatinib, ATP and Mg^{+2} in the same active site. (D) RMSD plot of the LSTK-ATP and LSTK-Lapatinib complexes over a simulation time of 10 ns. The binding pose conformations of (E) ATP and Mg^{+2}, (F) pazopanib with the LYK protein, represented in a surface conformation and visualized with a circular zoomed view. (G) The cartoon representation of LYK binding with pazopanib, ATP and Mg^{+2} in the same binding site. (H) RMSD plot of the LYK-ATP and LYK- pazopanib complexes over a simulation time of 10 ns.

Figure 8

Principal component analysis (PCA) using structural and physiochemical descriptors of the 88 human kinase FDA approved inhibitors compared with human anti-viral (74), cattle anti-viral (11) and other general cattle (319) drugs. A set of 16 molecular descriptors were used to generate the PCA plots. (A) PCA plot of PC1 versus PC2; (B) PC2 vs PC3; (C) PC1 vs PC3; (D) Three dimensional presentation of PC1 vs PC2 vs PC3. (E) The comparison of top three inhibitors obtained from virtual screening results of LSTK and LYK with cattle anti-viral drugs. The top three inhibitors (FDA approved human kinase inhibitors), represented by green square dots, were found to share common descriptors with the cattle anti-viral drugs, indicated by red triangle dots.

*Supporting Figure S1***:**

Molecular binding of pazopanib and lapatinib against receptor tyrosine kinases. (A) *In silico* binding interactions of pazopanib with Vascular endothelial growth factor receptor 2 (VEGFR2) in different mammals. (B) *In silico* binding interactions of lapatinib with Erb-B2 Receptor Tyrosine Kinase 4 (ERBB4) across various mammals. The cartoon representation of ligand-protein complexes is displayed, with the corresponding binding free energies (ΔG) mentioned below each complex.

Supporting Table S1 : The predicted molecular descriptors used for PCA analysis in detailed.

Supporting Table S2: The predicted structural and physiochemical descriptors of FDA approved kinase inhibitors, Human anti-viral drugs, Cattle anti-viral and Veterinary drugs.

Supporting Table S3: The contributed percentage of each principal component in a PCA plot represents the proportion of variance explained by each component.

Supplementary Methods : Complete detailed methods for each section have been described.

Tables

Table 1 : Structure prediction index:

Protein	**Sequence Identity**	**Sequence Similarity**	**Alphafold2 α-Helix**	**Alphafold2 β sheets**	**Alphafold2 PLDDT**	**Alphafold2 PTM**	**ESMFold α-Helices**	**ESMFold β sheets**	**ESMFold PLDDT**	**ESMFold PTM**
LSTK	73 %	85%	19	11	88.8	0.857	22	10	63.475	0.67
MSTK	73 %	85%	18	11	88.4	0.863	18	11	66.981	0.737
LYK	30%	55%	14	8	86.9	0.881	12	8	85.311	0.89
hMLKL	30%	55%	13	7	86.1	0.859	11	8	83.281	0.876

Table 2 : Sequence structure alignment index:

Superimposed proteins	**RMSD**	**Sequence alignment score**	**Tm score**
MSTK + LSTK	0.676 A?	1874.4	0.5515
hMLKL + LYK	0.987 A?	567.8	0.2394
hMLKL(Pdb) + LYK	0.953 A?	545.4	0.1099

Table 3 : Amino acids present in the binding pocket of LSTK and LYK. A list of all the amino acids present in the binding pocket of kinase domain that hold the ligand in its position. Residues in the same binding pocket are highlighted with a common colour (For LSTK, the color green was used to represent it, while the color red was used for the LYK).

Protein	*Ligand*	*Amino acids in the ATP binding site that show interactions*
LSTK	ATP-Mg	I95,S96,T97,G98,Y100,G101,V103,K105,V110,K11
	Lapatinib	Q80,F85,Y86,P87,I95,S96,T97,G98,G99,Y100,V10
LYK	ATP-Mg	Y27,I28,N29,R32,F61,N62,R94,S97,K102,Y104.
	pazopanib	E17,N18,Q20,I22,Y27,I28,N29,P30,I31,R32,F61,N

Table 4 : The binding energies of each LSTK and LYK in complex with ATP and inhibitor. The total interaction energy is a summation of the Lennard-Jones interaction energy (LJ) and the Coulombic interaction energy (Coul). SR stands for short-range interactions which are the key interactions determining the local stability of the ligand in the binding pocket.

Protein-Ligand complex	Energy (KJ/mol)	Energy (KJ/mol)
	Average	Err. Estimate
LJ-SR-LSTK-ATP	-148.18	5.4
Coul-SR-LSTK-ATP	-217.832	13
Total-Energy-LSTK-ATP	-366.012	14.0769315
LJ-SR-LSTK- lapatinib	-196.575	2.2
Coul-SR-LSTK- lapatinib	-614.381	12
Total-Energy-LSTK- lapatinib	-810.956	12.2
LJ-SR-LYK-ATP	-190.515	4
Coul-SR- LYK -ATP	-244.386	14
Total-Energy- LYK -ATP	-434.901	14.5602198
LJ-SR- LYK – pazopanib	-218.201	1.9
Coul-SR- LYK – pazopanib	-255.782	19
Total-Energy-LYK- pazopanib	-473.983	19.0947637

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