In silico Investigations of the role of Non-Receptor Tyrosine Kinases in Enhancing Cellular Production of Extracellular Vesicles

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Background

Rheumatoid arthritis (RA) is a debilitating autoimmune disease characterised by progressive joint destruction [1], with an incident rate of 12.5 per 10000 in the UK, primarily affecting middle-aged and elderly populations[2]. Despite treatments ranging from glucocorticoids to advanced therapies like mesenchymal stem cell (MSC) transplantation, RA remains incurable, and treatments often fall short in efficacy or pose immunogenic risks[3]. Recently, extracellular vesicles (EVs) derived from mesenchymal stem cells (MSC-EVs) have been identified as key mediators of MSC immuno-modulatory function shown to reduce RA pathology[4], and are currently investigated in preclinical studies [5] [6] [7] and a phase 1 clinical trial (NCT03437759). Extracellular vesicles are phospholipid bilayer nano and micro-sized (30-1000nm diameter) containers that transport bioactive molecules [8] (**Fig. 1**). The cell-mimetic therapeutic efficacy, and low immunogenic profiles of EVs make them ideal next-generation drug delivery vectors, diagnostics, and therapies[8]. However, the main bottleneck for scaling up EV manufacture of clinically relevant levels is their low cellular yield[9]. Consequently, targeting the biogenesis mechanism of EVs, particularly non-receptor tyrosine kinases (NRTKs) that are involved in assembling the Endosomal Sorting Complex Required for Transport (ESCRT) complex essential for EV formation[10], may offer strategies to increase EV yields. Small molecule drugs (caroverine, fenoterol, forskolin) have been reported[11] to increase exosome production, but their molecular targets are not defined. The current study is an *in silico* investigation of binding interactions between those drugs and NRTKs with potential roles in EV biogenesis.

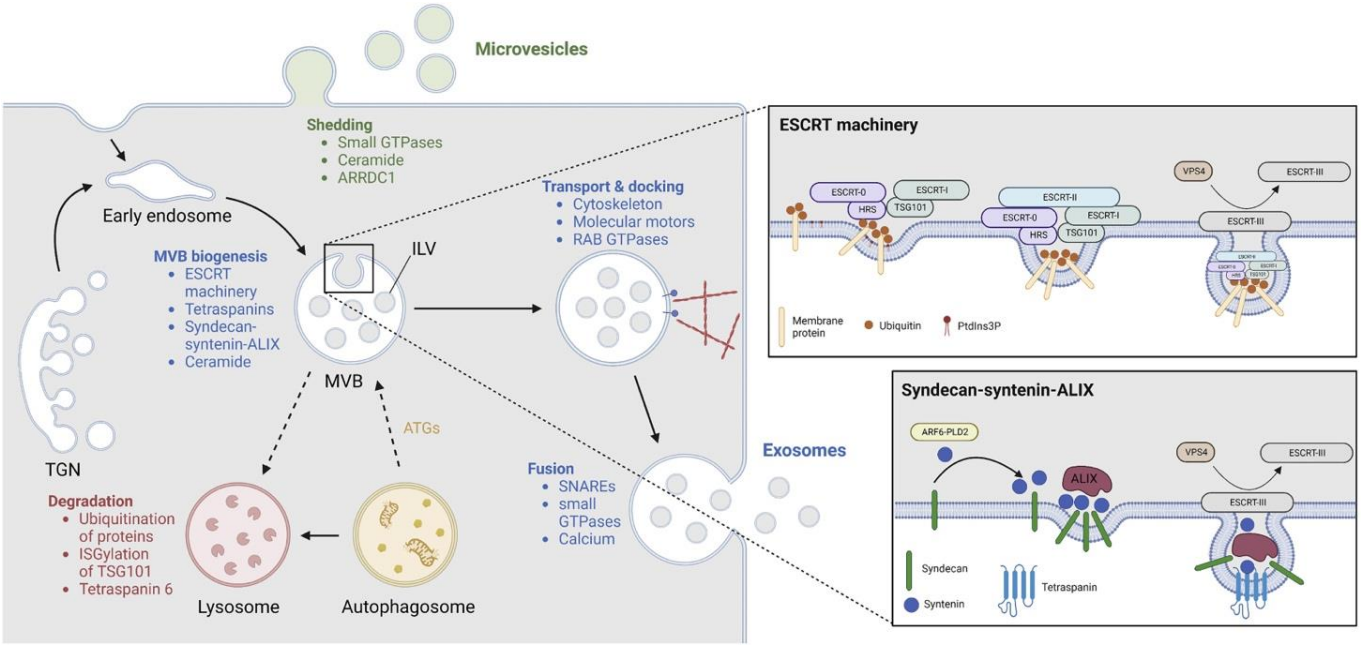

Fig 1. Extracellular vesicle biogenesis mechanism and regulators [37]. The diagram illustrates the role of the ESCRT machinery and syndecan-syntenin-ALIX complex in extracellular vesicle (EV) biogenesis. ESCRT complexes (0, I, II, III) are critical for intraluminal vesicle (ILV) formation within multivesicular bodies (MVBs), leading to exosome release. ALIX, interacting with syndecan and syntenin, supports an alternative exosome biogenesis pathway. Non-receptor tyrosine kinases (NRTKs) phosphorylate components of the ESCRT machinery and syndecan-syntenin-ALIX complex [10], potentially enhancing EV production. Additional factors, including tetraspanins, ceramide, and small GTPases, contribute to vesicle formation, transport, and docking processes, further regulating EV biogenesis and release.

Aims

The primary aim of this research project was to demonstrate a strategy for targeting the biogenesis mechanism for EVs to increase their yields. Structures of NRTKs with potential roles in EV biogenesis (BLK, YES1, FRK, SRMS) but unsolved atomic structures were approximated using a homology modelling approach. Molecular docking analyses were performed to assess whether or not candidate small molecule drugs could enhance the phosphorylation activity of the NRTKs via their binding modes, thereby stimulating EV biogenesis.

Methodology

Homology models of BLK, YES1, FRK, and SRMS were generated using ColabFold[12], SWISS-MODEL[13], AlphaFold[14], MultiFOLD[15], Phyre2[16], and RoseTTAFold[17]. Sequence and structural alignment with the template structure SRC were performed using Jalview[18] and TMalign [38] respectively. Models were visualised using Jalview and PYMOL[19]. The best models, ranked by their conformational correctness and reliability using SWISS-MODEL, were selected for further refinement with UCSF Chimera[20] and GROMACS molecular dynamics (MD) simulations[21] (ran at temperature 300 Kelvin and pressure 1 bar). Blind docking of six small molecule drugs (caroverine, fenoterol, forskolin, nitrefazole, SB215795, sitafloxacin) onto the NRTK models was performed using AutoDock Tools[22] before and after MD. Post-docking analyses were conducted using AutoDock Tools, PyMOL, and ProteinPlus[23] to study the binding poses and interactions.

Results and Discussion

1.) Homology modelling of NRTKs and structural analysis

The NRTK models (BLK, YES1, FRK, SRMS) with the most stable stoichiometric structure were built using ColabFOLD (**Fig. 2**). Models were built based on the crystal structures of highest identity and quality (**Table 1**.). All reference templates showed >30% in sequence identity, surpassing the "twilight zone" threshold[24], thus producing reliable models for further structural and functional analysis.

NRTK	NCBI Protein Sequence	Crystal Structure Template	Sequence Identity (%)	Coverage (%)	Quality (Resolution, Å)
BLK	NP_001706.2	1QCF_A	69.5	87	1.651
YES1	NP_005424.1	4K11_A	83.9	82	2.3
FRK	NP_002022.1	1QCF_A	52.3	88	1.651
SRMS	NP_543013.1	2H8H_A	42.7	90	2.2

Table 1. Sequence identity, coverage, and resolution quality of crystal structure templates for NRTKs from their NCBI protein sequences.

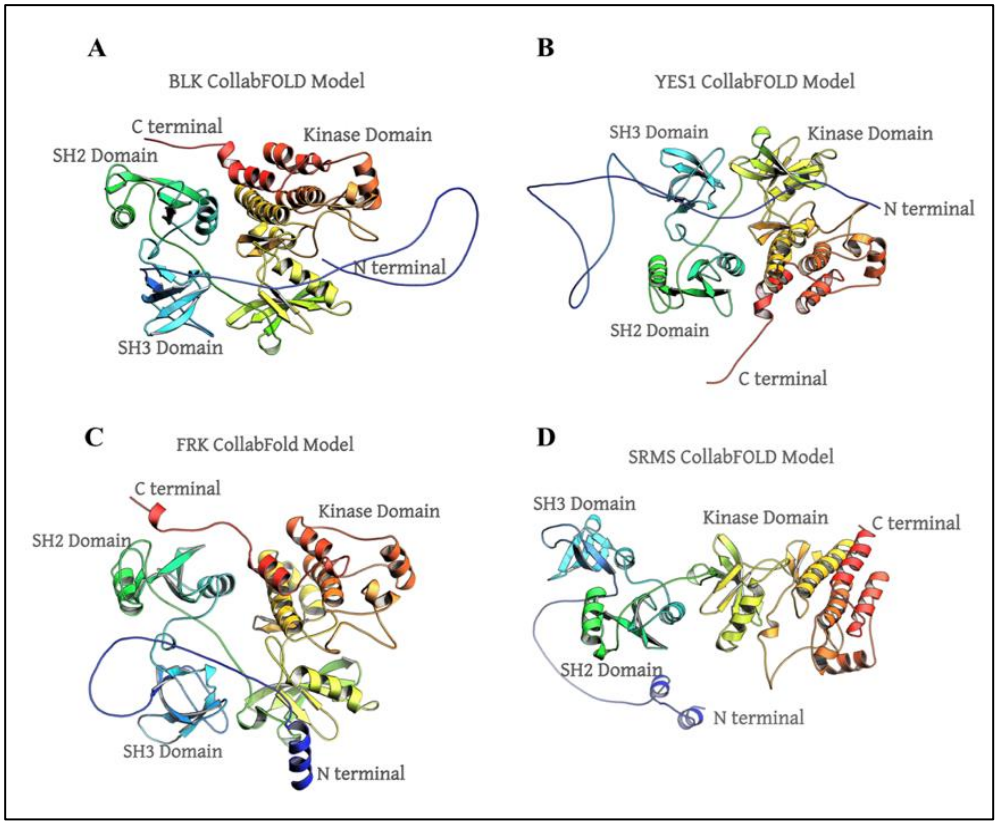

Fig 2. Homology models of human NRTKs (BLK (A), YES1 (B), FRK (C), SRMS (D)) generated using ColabFOLD showing their conserved domain architecture. The N-terminal unique domain, two adapter domains (SH3 and SH2) and C-terminal kinase

Characteristic domains of NRTK were presented in all predicted models: 1.) N terminal unique domain or intrinsically disordered region (IDR) that defines each protein's identity[25]. 2.) Src homology 3 (SH3) domain consisting of 2 antiparallel β-strands and binds proline-containing sequences[26]. 3.) Src homology 2 (SH2) domain containing a large β-sheets flanked by 2 α-helices that binds phosphotyrosine residues[27]. 4.) Kinase domain, a bilobed structure that regulates the catalytic function of NRTK[28]. A 2D schematic of the secondary structure of the models (**Fig. 3**) demonstrated consistent NRTK structural features across all four models. Model prediction quality by residue showed that the majority of low-quality regions resided in the IDRs, which lack templates due to their uniqueness (**Fig. 4**).

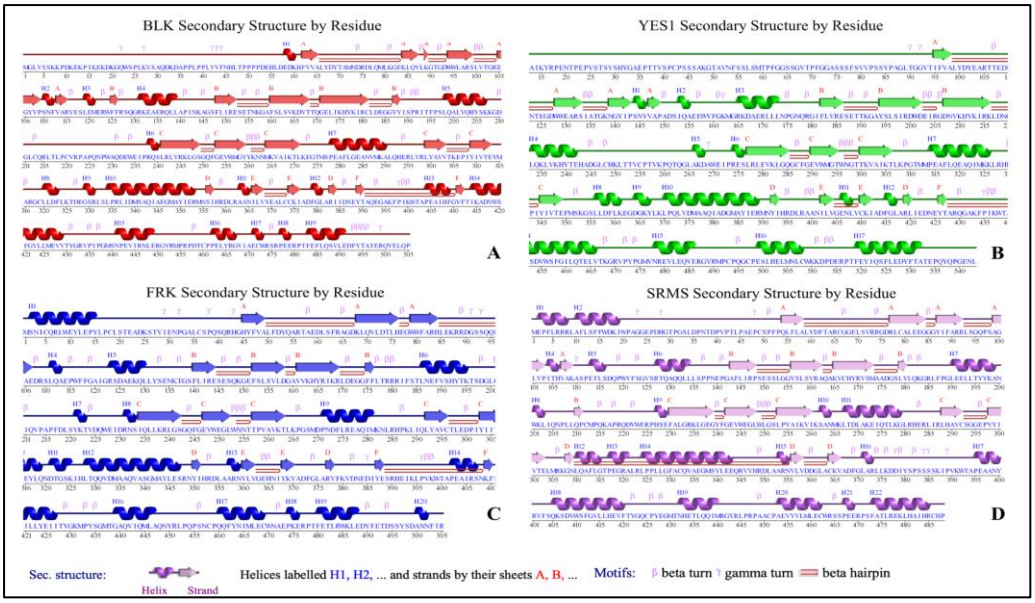

Fig 3. PDBsum[29] structural analysis of NRTK proteins (BLK (A), YES1 (B), FRK (C), and SRMS (D)). The schematic "wiring diagram" illustrates the protein's secondary structure, including strands (arrows), helices (springs), and other motifs in red (e.g., β-hairpins, γ-turns).

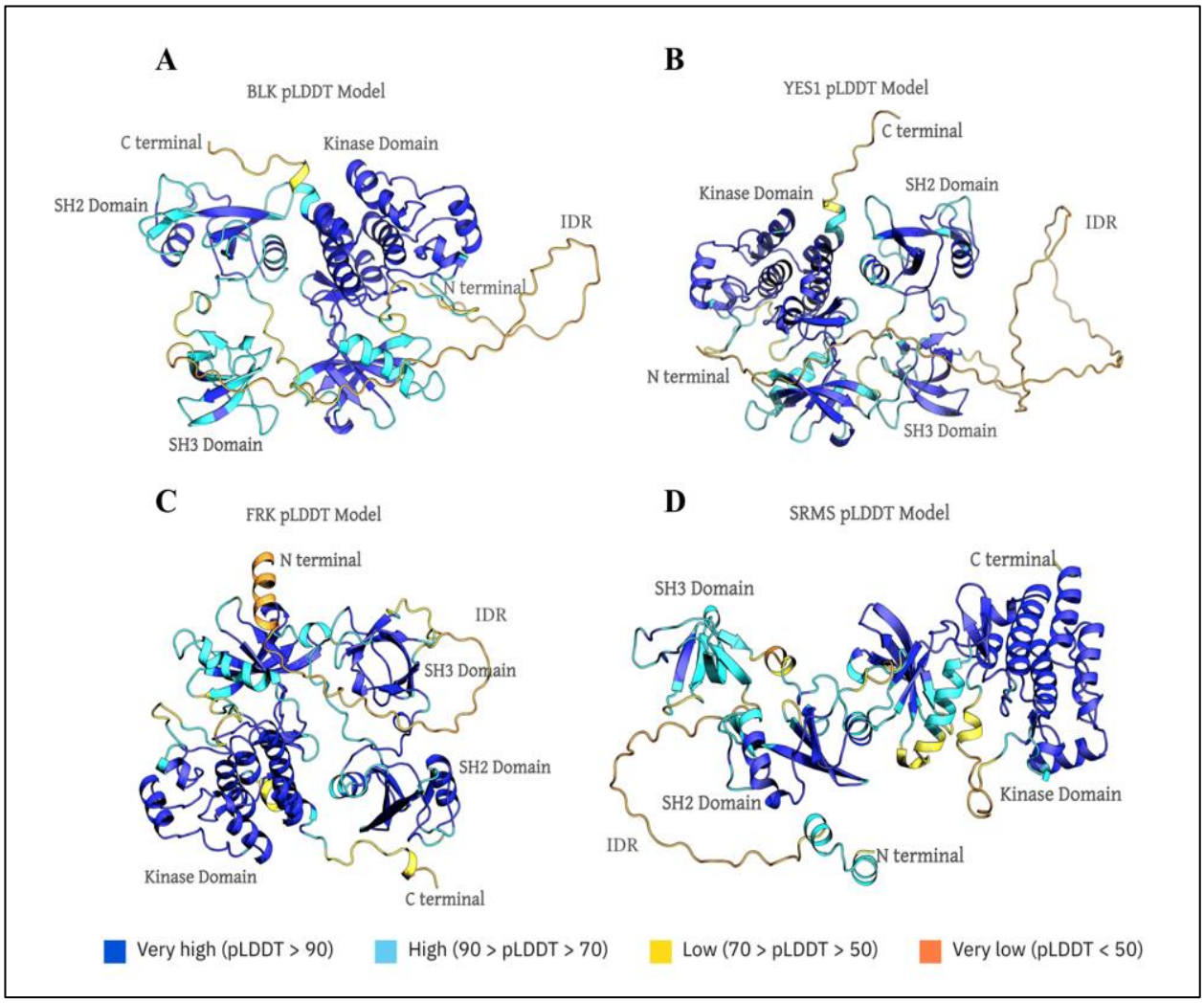

Fig 4. Prediction quality of BLK (A), YES1 (B), FRK (C), and SRMS (D) models from ColabFOLD. Structures are colour-coded by predicted Local Distance Difference Test (pLDDT) scores, indicating model confidence and structural accuracy.

2.) Sequence and Structural Evaluation of Kinase Models

Predicted models exhibited good stereochemical quality (Ramachandran favoured >90%) that improved in refined models (**Table 2**).

Protein Model	Ramachandran plot analysis				Rotamer Outlier (%)		Clash Score		MolProbity Score	
	Unrefined		Refined							
	Favoured (%)	Outlier (%)	Favoured (%)	Outlier (%)	Unrefined	Refined	Unrefined	Refined	Unrefined	Refined
BLK ColabFOLD	91.45	1.99	92.25	2.19	0.68	0.23	1.23	0.25	1.35	1.07
YES1 ColabFOLD	93.53	2.77	93.35	2.59	1.30	2.17	0.59	0.47	1.21	1.35
FRK ColabFOLD	93.24	1.39	93.24	1.39	1.12	0.90	0.62	0.49	1.18	1.11
SRMS ColabFOLD	94.86	1.03	95.06	0.41	2.20	0.73	0.52	0.65	1.29	1.06

Table 2. Structural analysis of the most structurally stable models for each NRTK protein generated from various servers. The most stable models were identified using stereochemical analysis from SwissModel and refined using Chimera. Results from both unrefined and refined states are displayed.

TM-align values of >0.5 were achieved by all models structurally aligned with the template SRC (1FMK), a well-studied Src-family kinase (SFK) member, indicating that they all share the same fold (**Fig. 5**)[30]. The root mean square deviation (RMSD) value for the NRTK, SRMS, was higher than that for the Src family kinases, FRK, BLK, and YES1.

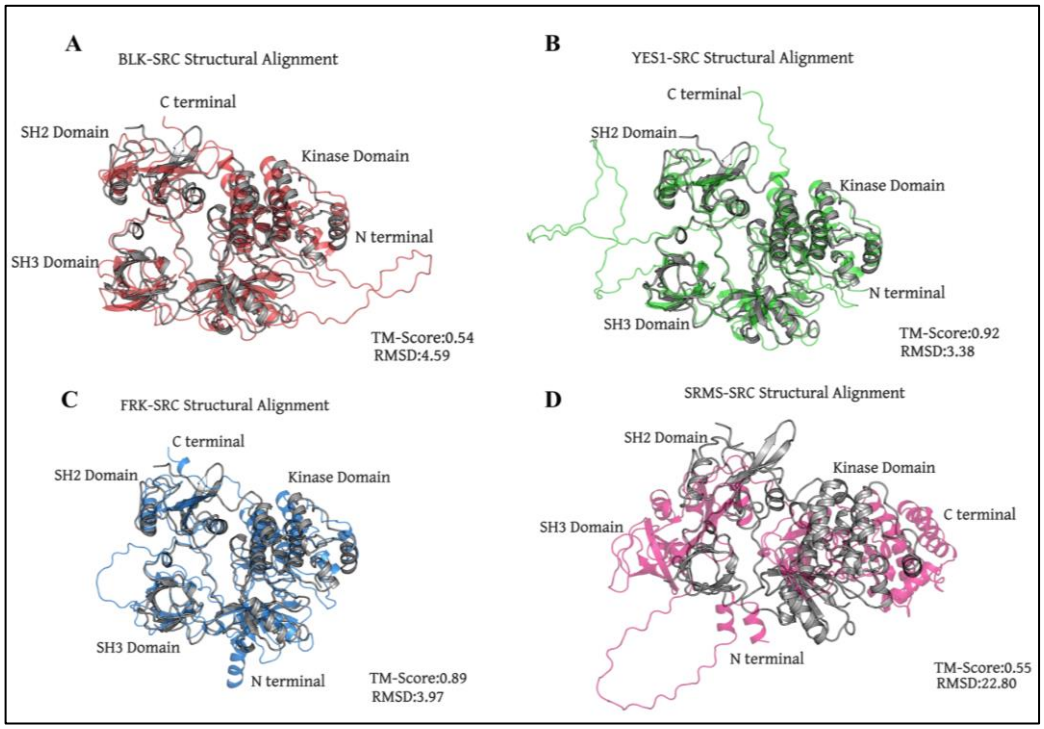

Fig 5. 3D structural alignment of NRTK models (BLK (A: red), YES1 (B: green), FRK (C: blue), SRMS (D: pink)) with the human NRTK SRC crystal structure (1FMK) in grey with TM-score and RMSD values.

The structural alignment was consistent with sequence alignment results, showing that Yes1 had the highest sequence identity (**Fig. 6 and Table 3.**) and the closest evolutionary relationship to SRC (**Fig. 7**), while SRMS exhibited the least similarity and most distant relationship to SRC.

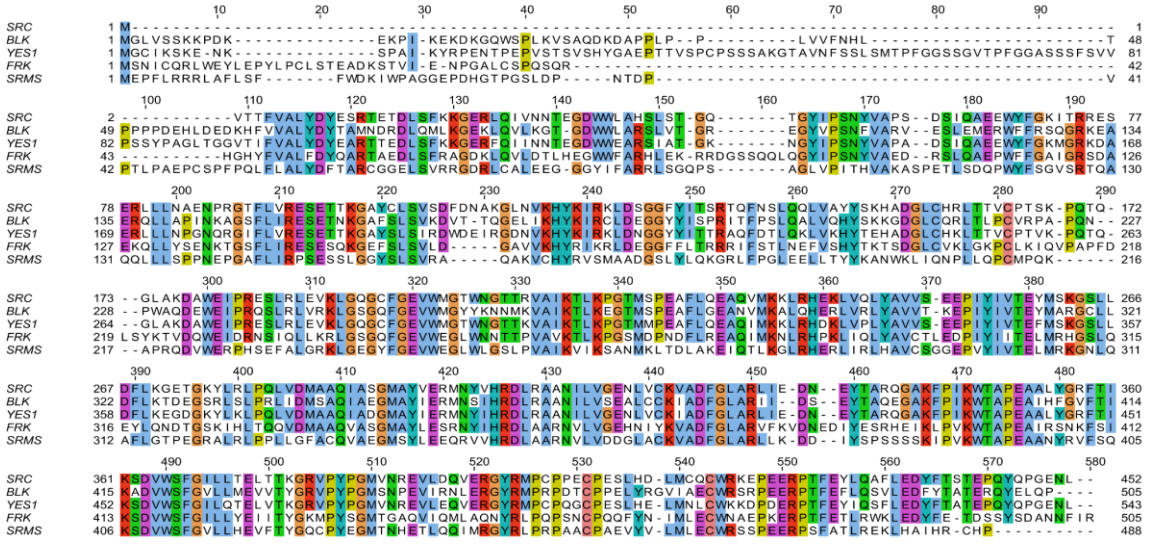

Fig 6. Sequence homology analysis of human NRTK (BLK, YES1, FRK, SRMS) compared to the template human NRTK SRC using Jalview.

Ranking	Protein	Sequence Coverage (%)	Sequence Identity (%)
1	YES1	100	70.0
2	BLK	98.7	54.4
3	FRK	98.5	45.3
4	SRMS	95.6	37.9

Table 3. Sequence identity and coverage of NRTK proteins (BLK, YES1, FRK, and SRMS) compared to the template human NRTK SRC. Proteins are ranked by sequence identity percentage compared to SRC.

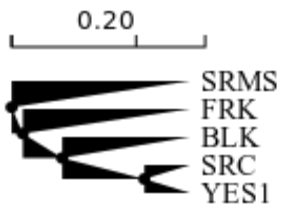

Fig 7. Phylogenetic tree of NRTKs showing evolutionary relationships.

3.) Molecular Dynamics Simulations

Molecular dynamics simulations introduced slight conformational changes in kinase models (**Fig. 8**). The simulations were run for 50 nanoseconds (ns) until the RMSD stabilised (**Fig. 9**), ensuring the results are representative of stable molecular behaviour in an ideal environment. Flexible regions highlighted by RMSF analysis are primarily IDRs, with their position confirmed by IUpred predictions[31].

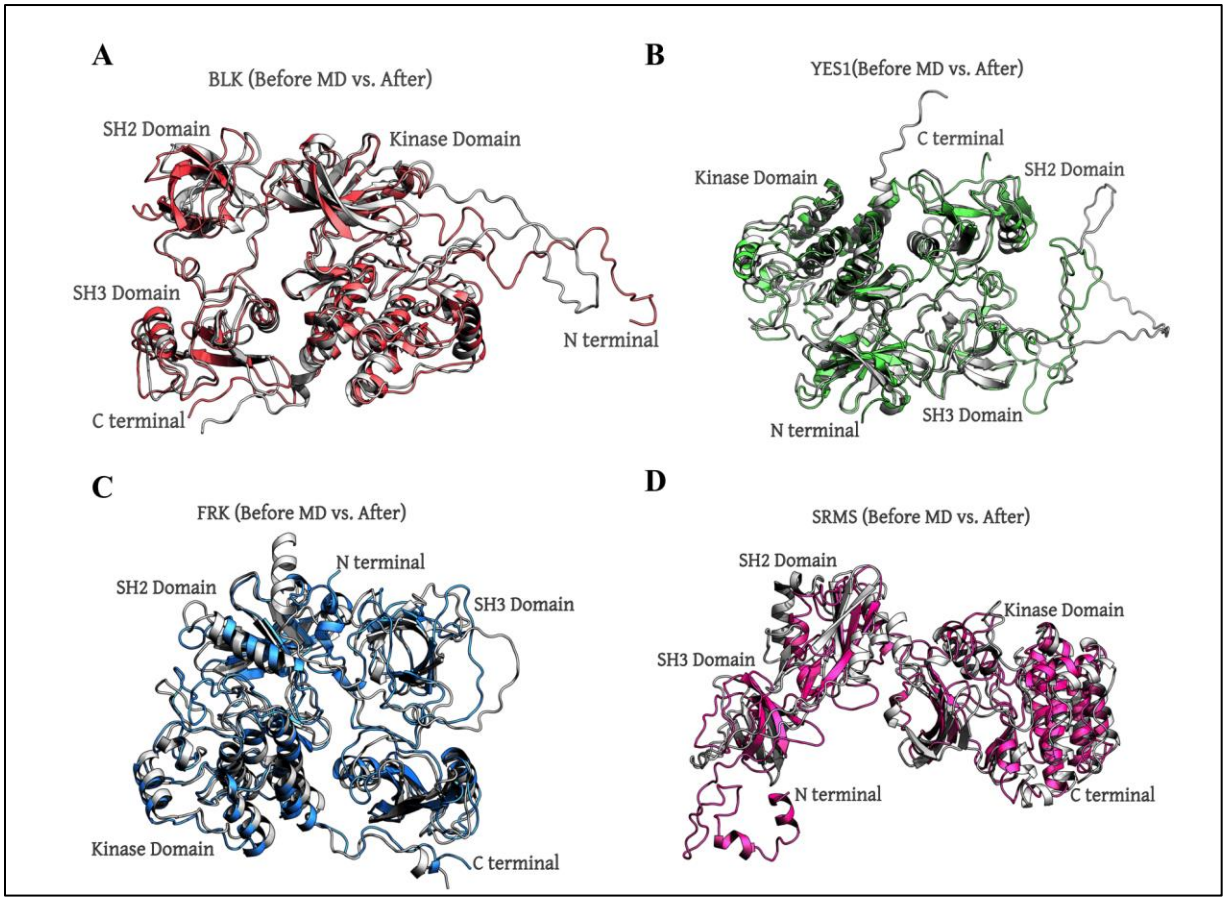

Fig 8. 3D structural alignment of NRTK models after molecular dynamics and refinement (BLK (A: red), YES1 (B: green), FRK (C: blue), SRMS (D: pink)) with the unrefined models in grey.

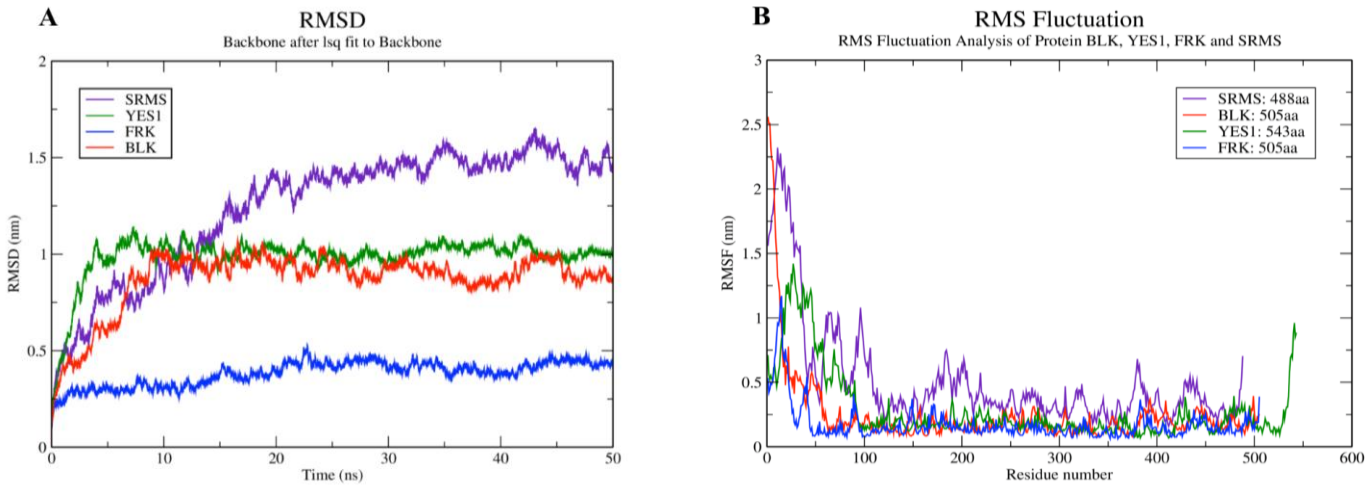

Fig 9. RMSD (A) and RMS Fluctuation (B) analysis of NRTK Models (BLK, YES1, FRK, SRMS). Panel A: RMSD of NRTK backbone over 50 ns of molecular dynamics simulation. Panel B: RMSF values of NRTK across residue numbers, indicating residue flexibility.

4.) Molecular Docking

Molecular docking analyses were performed using unrefined and MD-refined kinase models. The MD models exhibited changes in binding conformations and generally improved affinities, (**Table 4 and Fig. 10**). The binding affinity for small molecule drugs ranged from (-6.21 to -9.18 kcal/mol) for caroverine, fenoterol, forskolin and nitrefazole; and (-9.44 to -10.74 kcal/mol) for sitafloxacin and SB218795. Sitafloxacin showed the highest average affinity of -10.26 kcal/mol, followed by SB218795 and SB218795 achieved the highest binding affinity of -10.74 kcal/mol to FRK (**Fig. 11**).

No.	*Protein*	*Binding Ligand of highest affinity*		*Binding energy (Kmol/Mol)*		*Inhibition constant (Ki)*		*No of H bonds (Drug-protein)*		*Amino acid involved in interaction*	
		Before MD	*After MD*	*Before MD*	*After MD*	*Before MD*	*After MD*	*Before MD*	*After MD*	*Before MD*	*After MD*
1.	Blk	Sitafloxacin	Sitafloxacin	-10.37	-10.25	25.15 nM	30.71 nM	6	3	Arg131(A), Lys132(A), Ile174(A), Glu491(A), Tyr494(A), Thr495(A)	Thr312(A), Met315(A), Gly318(A)
2.	Yes1	Sitafloxacin	Sitafloxacin	-10.0	-10.48	46.8 nM	20.69 nM	5	5	Asp375(A), Leu526(A), Phe530(A), Thr531(A), Thr531(A)	Glu103(A), Arg105(A), Thr300(A), Tyr336(A), Phe350(A)
3.	Frk	Sitafloxacin	SB218795	-9.44	-10.56	119.99 nM	18.25 nM	4	2	Ala215(A), Ala215(A), Glu364(A), Lys369(A)	Asn103(A), Ala215(A)
4.	Srms	Sitafloxacin	Sitafloxacin	-8.43	-9.73	660.68 nM	73.84 nM	2	5	Phe52(A), Met173(A)	Asp350(A), Asp350(A), Leu375(A), Trp392(A), Thr393(A)

Table 4. This table presents the binding ligand with the highest binding energy for each NRTK protein (BLK, YES1, FRK, and SRMS) before and after molecular dynamics (MD) simulation. Inhibition constant (Ki), number of hydrogen bonds, and amino acids involved in the interaction for each NRTK protein are also displayed.

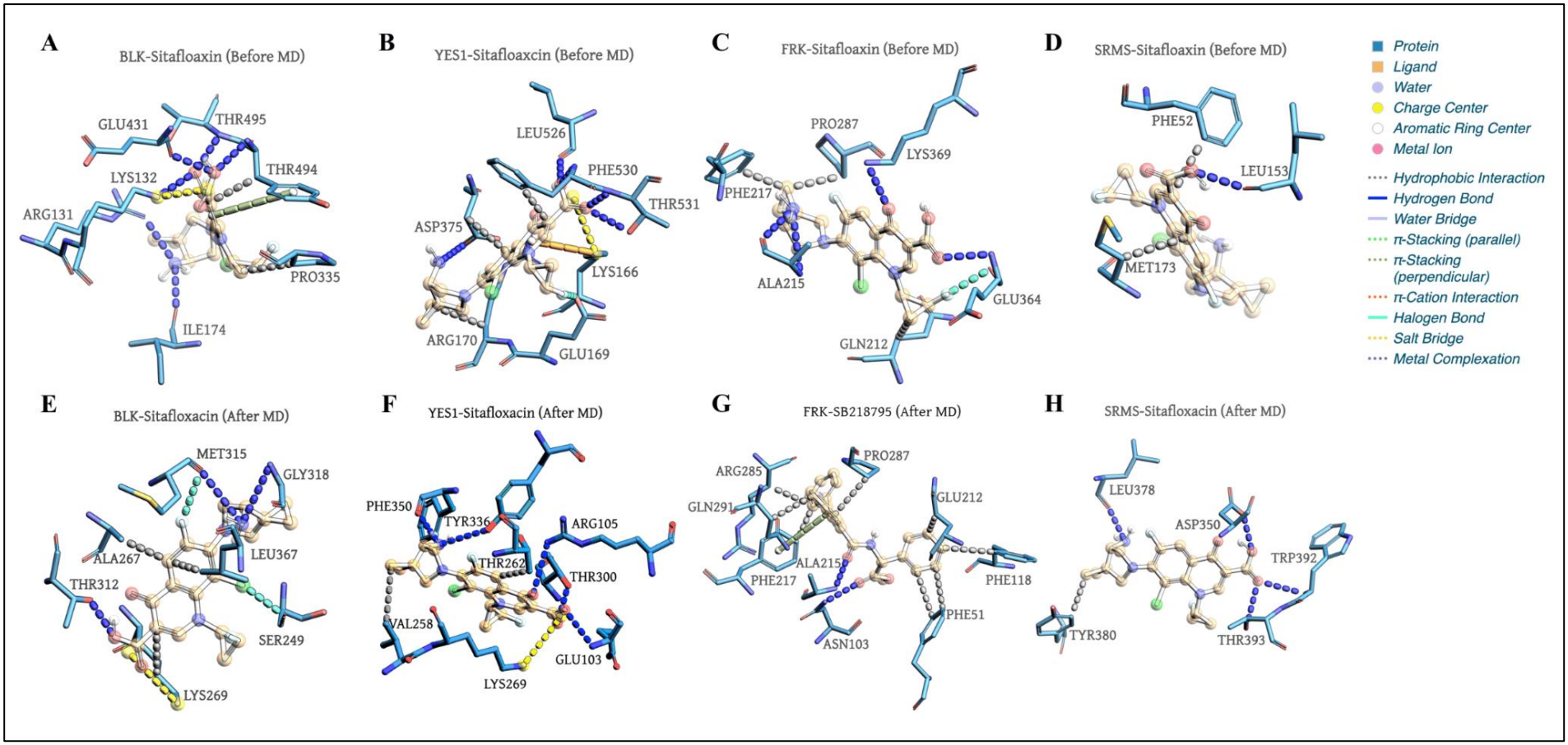

Fig 10. Binding interactions of the small molecule drug with the highest binding energy for each NRTK model (BLK (A, E), YES1 (B, F), FRK (C, G), SRMS (D, H)) before and after molecular dynamics (MD) simulation.

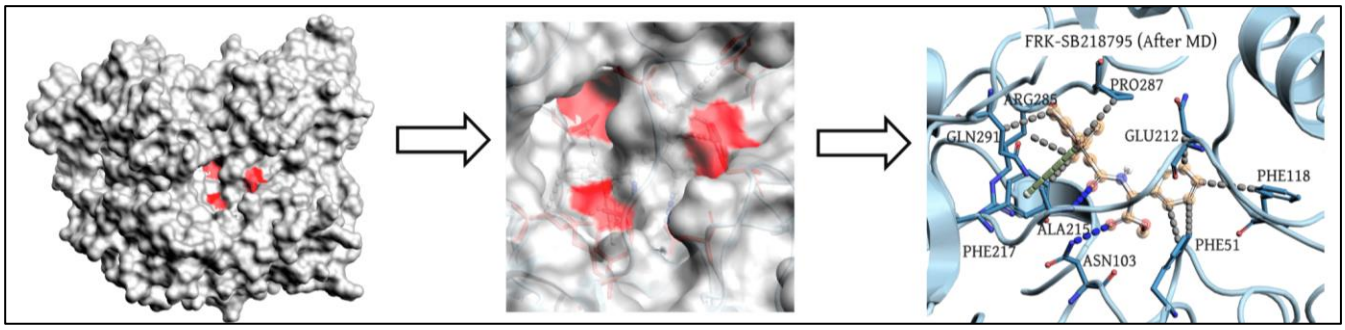

Fig 11. Position of SB218795 binding pocket in the FRK model.

Sitafloxacin and SB218795 were indicated as the most promising candidates for further investigation due to their consistent high affinity and conserved binding sites across all MD-refined models (**Table 4**). The other small molecule drugs bound with comparatively lower affinity displayed ubiquity in binding locations across all models. The interaction of SB218795 with the SH3 domain could disrupt the autoinhibitory mechanism that negatively regulates NRTK phosphorylation activity. Typically, autoinhibition is maintained when the SH3 domain binds to its proline-rich ligand in the SH2-CD linker region, leading to an inhibitory conformation that obstructs the kinase active site[32]. SB218795 may compete with the SH3 domain ligands, potentially enabling the kinase domain to adopt an open conformation and reactivate its phosphorylating function. Sitafloaxcin is predicted to target the kinase ATP binding site and could potentially act as either an inhibitor, by competing with ATP, or as an enhancer, by allosterically stabilising the active site to stimulate kinase activity.

These predictive results form a foundational basis, necessitating experimental validation to confirm the roles of SB218795 and sitafloxacin as modulators of kinase activity and investigate their ability to enhance EV production. Similar approaches have been taken previously to experimentally target proteins (NDRG1, Rab7, nSMase2) involved in the EV biogenesis using small molecule drugs (N-methyldopamine, norepinephrine, chloroquine, NH4Cl) which caused 2-3 fold increases in exosome production[33][34].

Conclusion

A potential approach to enhance EV yield may be to target NRTKs involved in EV biogenesis using small molecule drugs. This may resolve the bottleneck for EV manufacture for therapeutic applications including development of novel EV-based therapies for treating RA.

Future directions

The potential of NRTKs in enhancing EV biogenesis can be evaluated by observing EV yield in mesenchymal stem cells (MSCs) cultured with candidate small molecule drugs. NRTK mutant MSCs can be introduced as controls to validate any observed EV-stimulating effects of NRTKs. Additional small molecule drug candidates targeting NRTK signalling mechanisms to enhance EV biogenesis can be identified through High Throughput Screening (HTS). Similarly, other protein families involved in the EV biogenesis mechanism could be targeted using a broad range of techniques, such as gene editing, electrical stimulation[35], and PEGylated liposomes[36] as alternative strategies to stimulate EV cellular production.

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I am very grateful to the Biochemical Society for granting me the opportunity to gain hands-on experience in computational pharmaceutical research, which has broadened my understanding of molecular pharmacology. My sincere thanks go to Dr. Bernice Wright and the Biochemical Engineering department at University College London (UCL) for their invaluable supervision and support during my 6-week stay. I am specifically thankful to Dr. Cheng Zhang (Research Fellow) for overseeing my work and teaching me the applications of Molecular Dynamics Simulation.

Throughout this studentship, I have become more confident in my abilities applying bioinformatics tools in the context of biopharmaceutical drugs discovery and evaluation. These experiences have affirmed my commitment to pursuing a research career in molecular pharmacology, particularly in understanding the mechanisms of drug-induced cell signalling and toxicity. The skills I have developed during the Biochemical Society summer studentship will be invaluable as we extend this project into the wet lab for my final year dissertation at UCL, also as I apply for PhD studies to further explore new therapeutic targets and their cellular mechanisms for rare diseases, contributing to the field of pharmacology research.

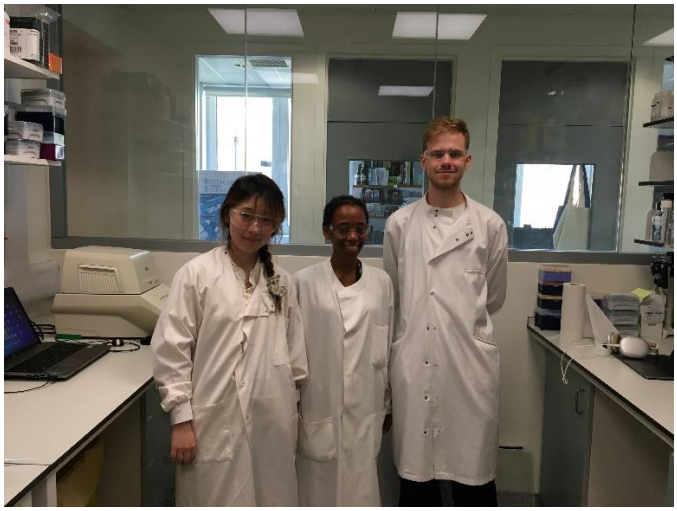

Image: From left to right – Yoki Wang (student), Bernice Wright (supervisor), and Vasilii Blinov (student colleague) in the Analytical Lab (Bernard Katz building) in the Department of Biochemical Engineering, UCL.

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