Cheminformatics Project

Ya-Ting Yang

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Drug Repositioning Strategy for Novel Therapeutic Indications

A) Motivation - The new therapeutic indication and the protein target (s) you want to hit.

B) Target information - Any relevant protein structure(s) and/or existing medications that act on this target, and any drawbacks they may have. Ligands, controls, considerations. All should be clearly outlined. Anything pertinent from clinicaltrials.gov?

C) Compound selection - What library(ies) of approved drugs will you use? Are you going to use them all? How did you filter your selection? Property analysis.

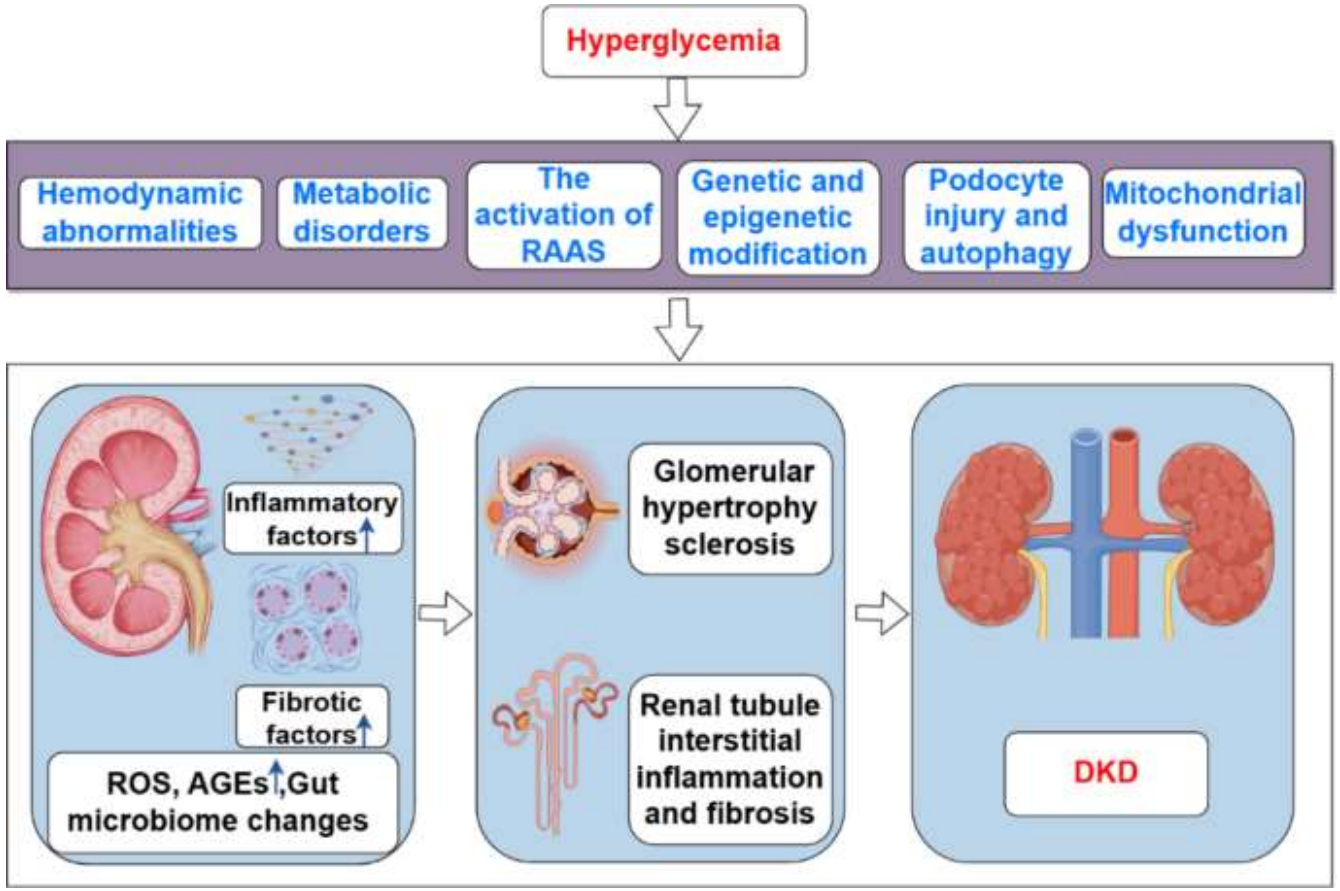
D) Computational model building and application - Use methods in the course at your disposal: virtual screening, machine learning, model building, cheminformatics to pick which compound(s) you think have the highest chance of working. What feature(s) of the selected compounds make them particularly attractive? Or what could be the challenges?

E) Budget - How many compound(s) will you purchase (estimated cost?), and how will you test them? What other considerations come into play?

F) Outlook - Timeline estimates, estimate future hurdles.

A) Motivation - The new therapeutic indication and the protein target (s) you want to hit.

Diabetic Kidney Disease

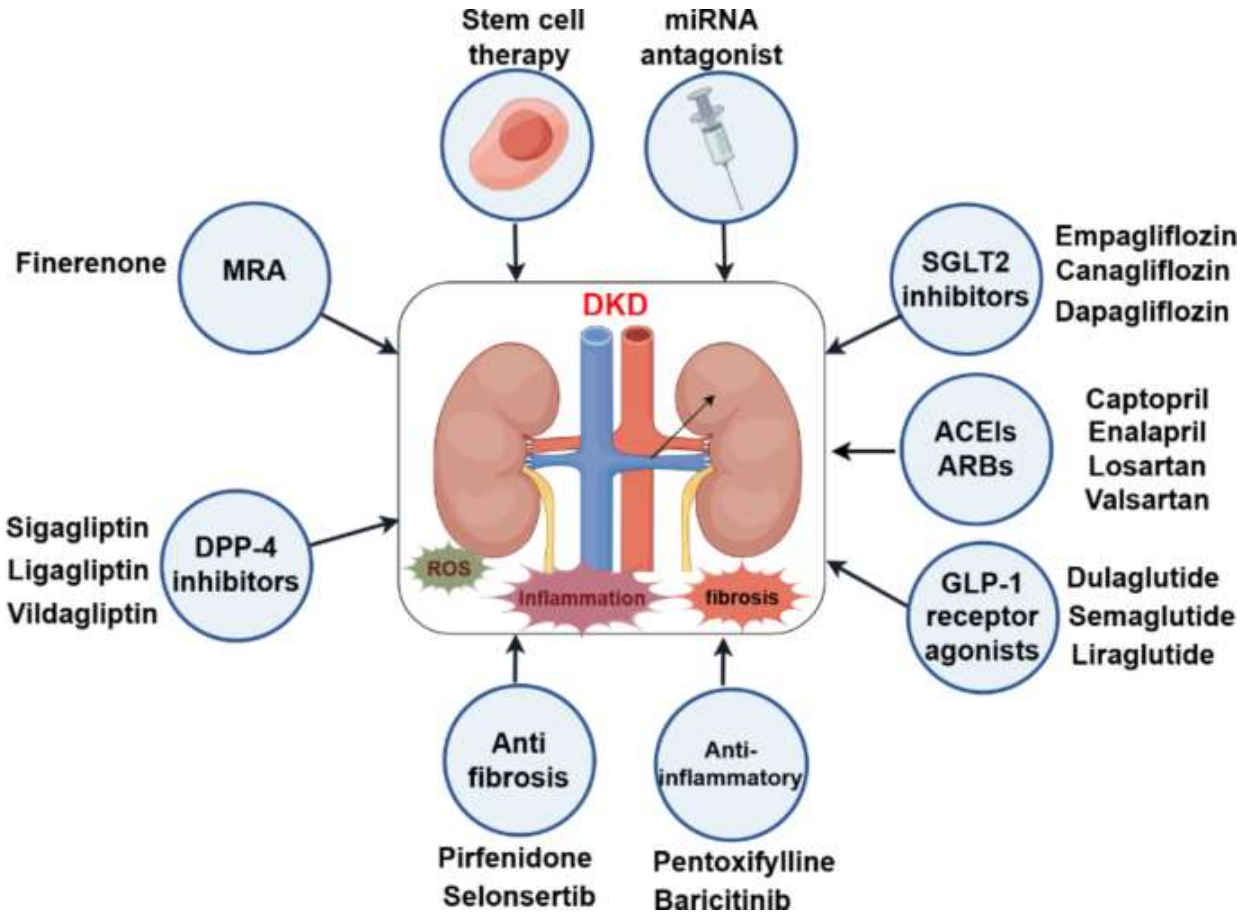


1. Persistent hyperglycemia exposes the kidney to an inflammatory environment, driving mitochondrial dysfunction and generating excessive reactive oxygen species (ROS).
2. Increased oxidative stress and disrupted nitric oxide synthesis exacerbate endothelial dysfunction, leading to increased vascular permeability and thickening of the basement membrane.
3. The progressive structural damage to the **glomeruli and renal tubules** results in a decline in the glomerular filtration rate (eGFR).
4. The progression of DKD is often accompanied by worsening proteinuria and a continuous decline in eGFR, which are important clinical indicators for assessing disease status.
5. Kidney injury can affect the overall metabolic balance, further triggering cardiovascular disease, neuropathy, anemia, and other complications.

(Thomas MC, 2015; Vladimir Petrovic, 2025)

Diabetic Kidney Disease

Tubulointerstitial fibrosis is a key driver of DKD progression.



Current Clinical Medications

Ex. SGLT2 inhibitors (e.g., Empagliflozin):Reduce glucose reabsorption, with renoprotective effects.

Innovative Therapeutic Exploration

Stem cell therapy: Promote renal tissue repair

However, the current medications for DKD are not for renal anti-fibrosis development.

Antifibrosis

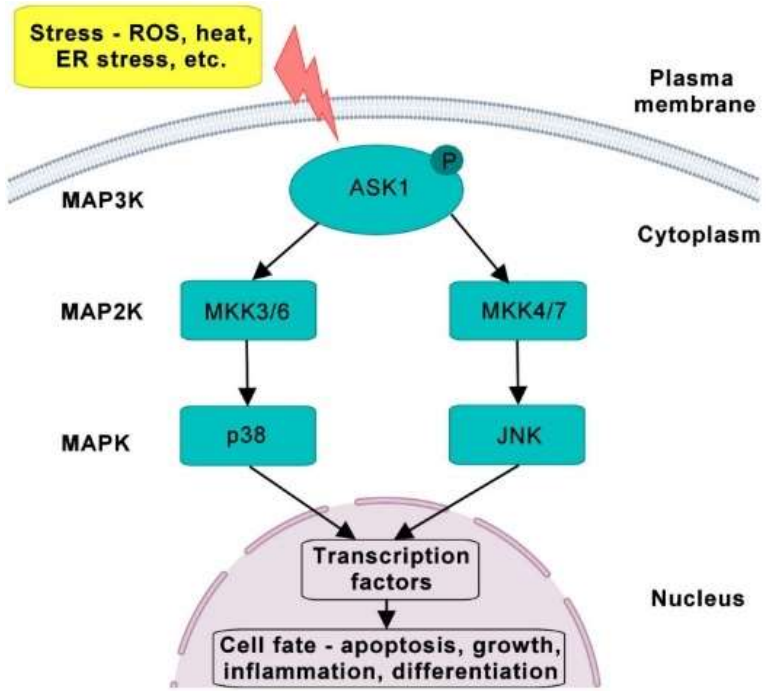
Pirfenidone -> Idiopathic Pulmonary Fibrosis (IPF)

Selonsertib -> DKD Phase II study

(Zhao, 2025)

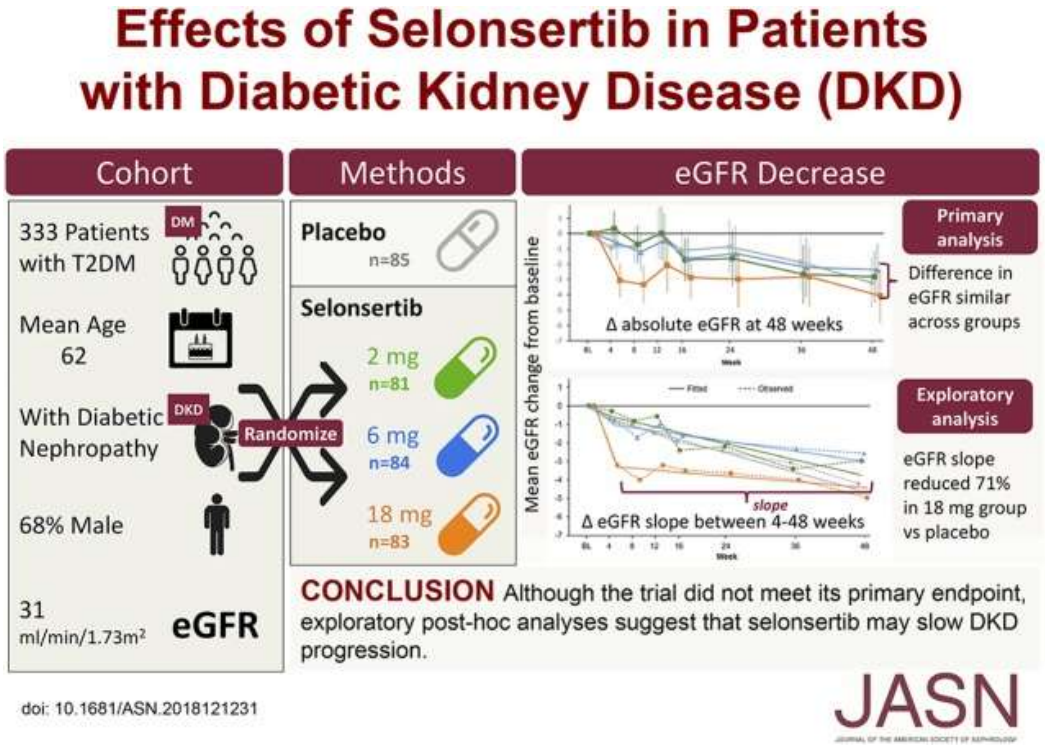
A) Motivation - The new therapeutic indication and the protein target (s) you want to hit.

ASK1 is an important node in DKD



The Role of ASK1 in Diabetes and DKD

- Increased β-cell apoptosis and insulin resistance
- Associated organ damage (heart, kidney, retina, brain)
- JNK and p38 can suppress insulin signaling via the TNFα pathway



Selonsertib (NCT02177786)

- Specific for inhibiting ASK1
- No significant improvement in eGFR within 1 year

Evaluation Challenges

- DKD progression is slow, long-term observation of eGFR changes is required
- Clinical trial design needs longer follow-up and larger sample size to observe potential protective effects

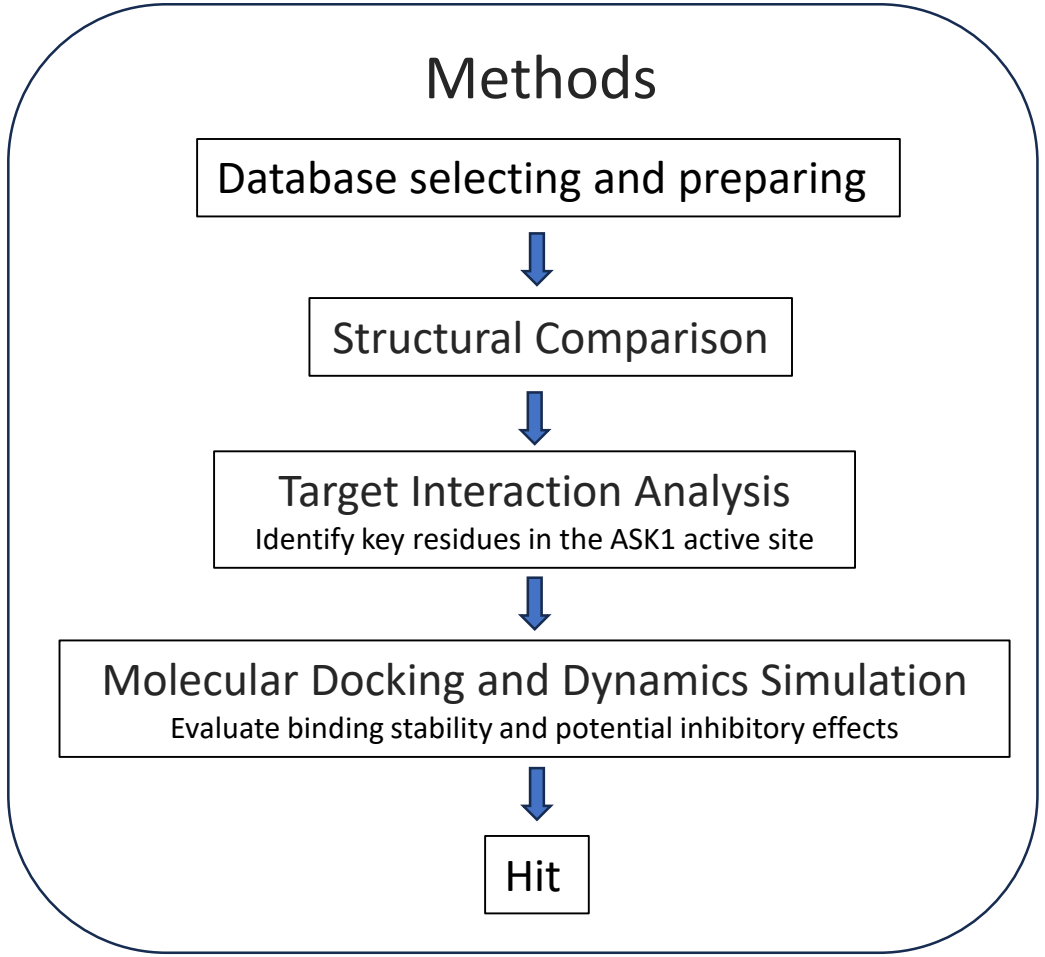
(Obsilova V, 2021; Lin JH 2015; Chertow GM 2019)

A) Motivation - The new therapeutic indication and the protein target (s) you want to hit.

Targeting ASK1 as a Novel Anti-fibrotic Approach for DKD

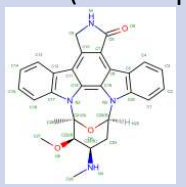
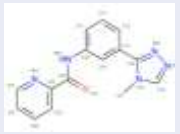
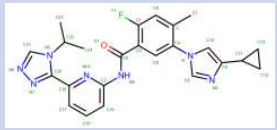
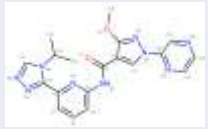
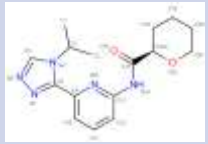
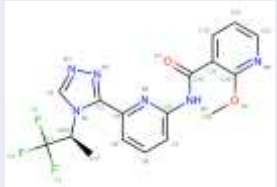
The Potential and Current Status of ASK1

- ASK1 is a key kinase closely related to tubulointerstitial fibrosis in the kidney. Although selonsertib failed in Phase 2 and 3 trials for DKD/NASH
- The accumulated pharmacological and safety data provide a foundation for future drug design
- The academic and industrial communities are actively exploring more optimized molecules and new inhibition mechanisms



Research Objectives Utilize structure-guided virtual screening and drug repurposing strategies to identify potential ASK1 inhibitor compounds from approved drugs

B) Target information

PDB ID	Resolution	Ligand (2D Diagram)	Note
2CLQ	2.3 Å	STU (Staurosporine) 	Broad-spectrum kinase inhibitors, used as reference for the kinase pocket structure
6E2M	2.25 Å	KK7 	An experimental ASK1 inhibitor developed by Gilead Sciences, which has not yet entered clinical trial stage.
6OYT	2.82 Å	Selonsertib (GS-4997) 	Developed by Gilead Sciences, for the treatment of non-alcoholic steatohepatitis (NASH) and diabetic kidney disease (DKD), terminated at Phase 3 and Phase 2 clinical stages.
6VRE	2.29 Å	RFG 	An experimental compound developed by Gilead Sciences, a structural analogue of Selonsertib.
6XIH	2.65 Å	V3S 	Experimental ASK1 inhibitor
7MU6	2.17 Å	A1AWT 	Experimental compound

Target: PDB 6OYT, ligand: Selonsertib (GS-4997), salmon

Pocket residues ➜ L686–V694 (LGKGTYGIV) + K709
Feature Evaluation ➜ Hydrogen bonding sites, hydrophobic patches

Control: PDB 2CLQ, ligand: Staurosporine (STU), yellow

Broad-spectrum kinase inhibitors, able to bind to ASK1, PKC, CDK, etc.
Potent inhibition ➜ Used as a binding benchmark
Poor selectivity, high toxicity ➜ Only for comparison, not as clinical candidates

- Select PDB 6OYT as the potential comparative ligand, using the PDB 2CLQ ligand Staurosporine (STU) as the control molecule.
- Observe the reference points L686-V694 (LGKGTYGIV) and K709 in the ATP binding site.
- Analyze the size of the binding pocket, the number of hydrogen bonds, the hydrophobic regions, and establish drug screening criteria.

C) Compound selection

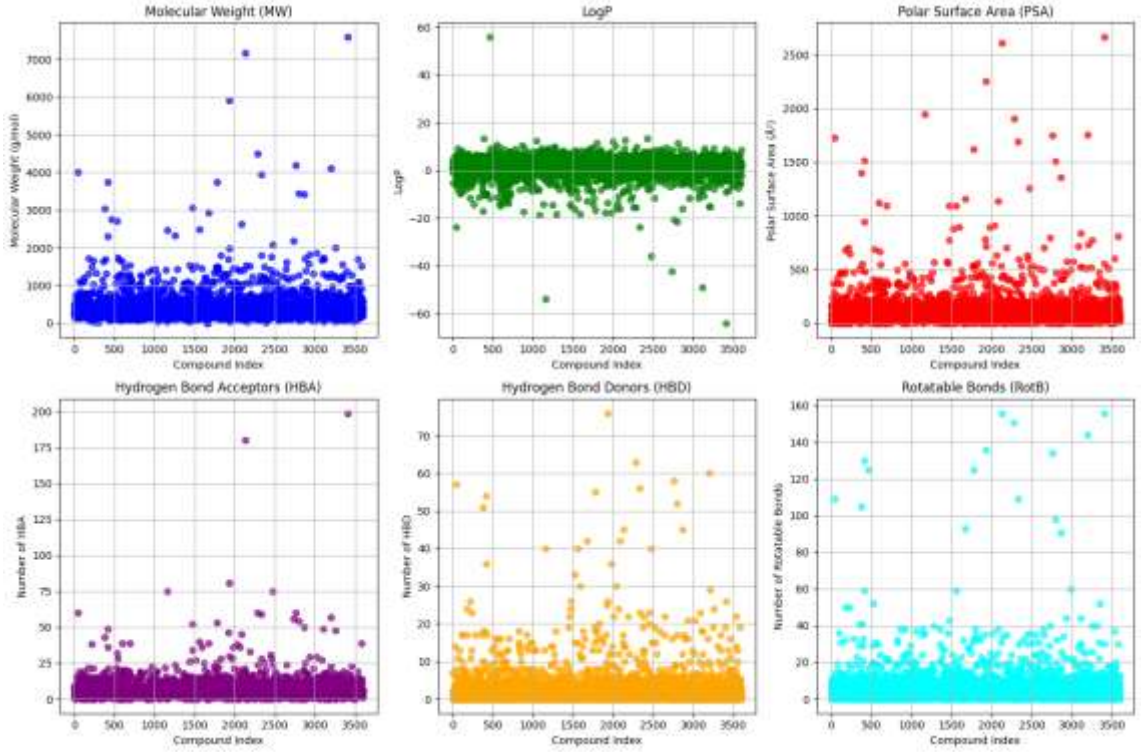
Source of Drugs and Filtering Process Database Source: ChEMBL **FDA-approved** small molecules (Total 4,392 samples)

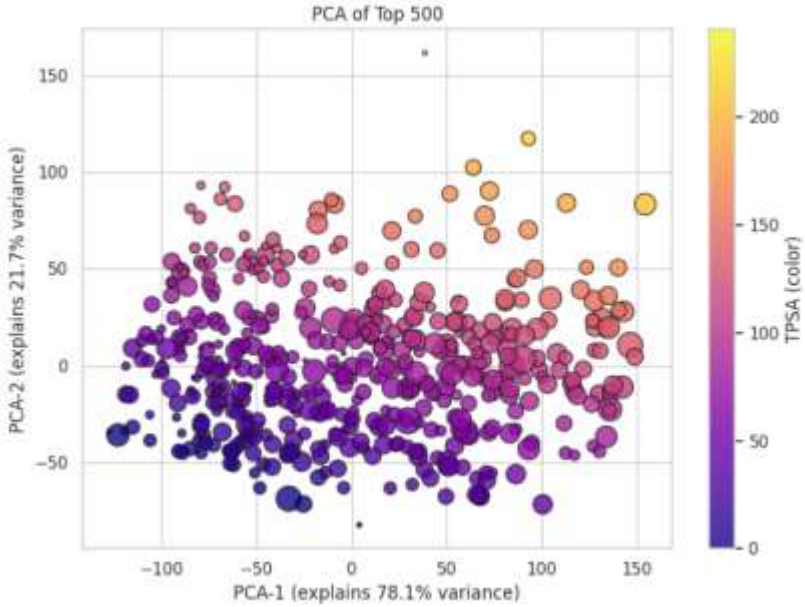
PAINS filter, Desalt, Canonicalize → 3,593 entries

Lipinski's Rule of Five (MW 250–500, LogP -5~5, HBD ≤ 5) → 1,736 entries

Set reference standards based on Selonsertib properties (MW: 445.49)

Property analysis





Molecular Weight (MW): 0-1000 g/mol

Polar Surface Area (PSA): 0-500 Å^2

Hydrogen Bond Acceptors (HBA): 0-25

LogP: -10 and 10

Rotatable Bonds (RotB): 0-20

Hydrogen Bond Donors (HBD): 0-10

Performed PCA analysis on the **top 500** candidate compounds

PCA-1 (78.1%): Dominated by molecular weight & polarity

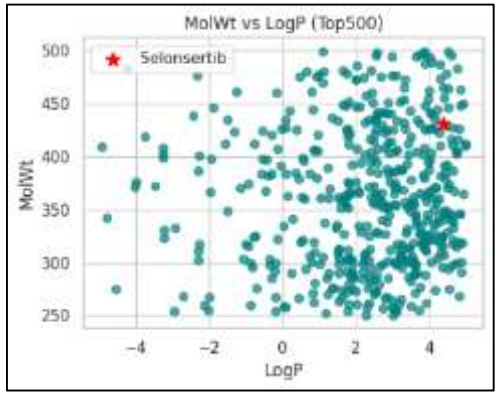
PCA-2 (21.7%): Mainly Rotatable bonds and TPSA

Most molecules are concentrated in the middle range of properties, favorable for oral absorption. A small portion of high TPSA or high flexibility molecules have potential polar pocket adaptability, worth further analysis.

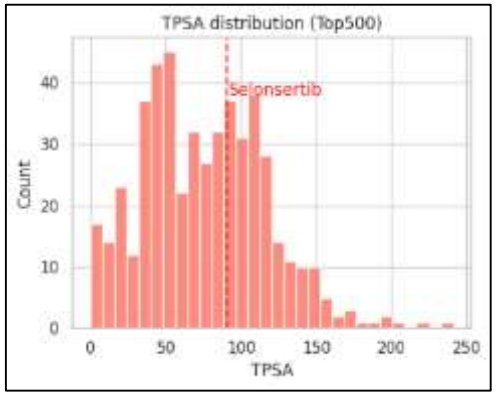
C) Compound selection

Top 500 FDA Approved Small Molecules Distribution

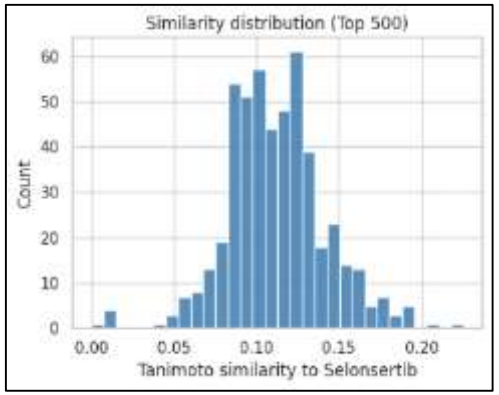
Verifying whether the candidate drugs possess basic drug-like properties



Most drugs are concentrated in a reasonable oral drug range (MolWt around 300-450, LogP between 0-4), with Selonsertib represented by the red star, located within the main dense area, indicating that other candidate compounds also possess similar drug-like properties.



Most drugs have a TPSA (Total Polar Surface Area) between 40-120, which meets the criteria for good absorption. Selonsertib has a TPSA of around 90, falling in the middle range, indicating that the selected drugs are mostly of moderate polarity, suitable for penetrating cell membranes.

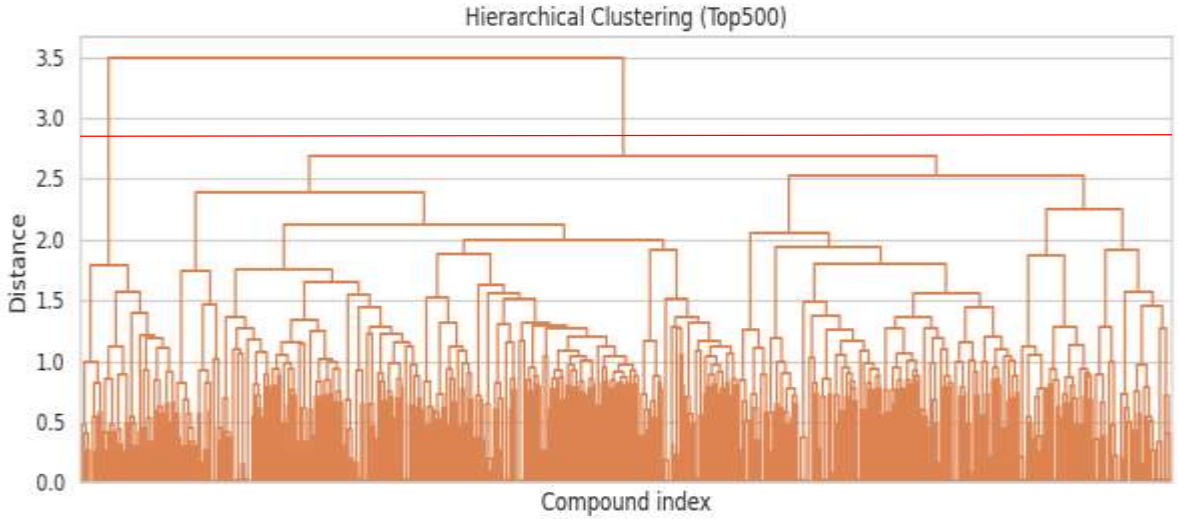


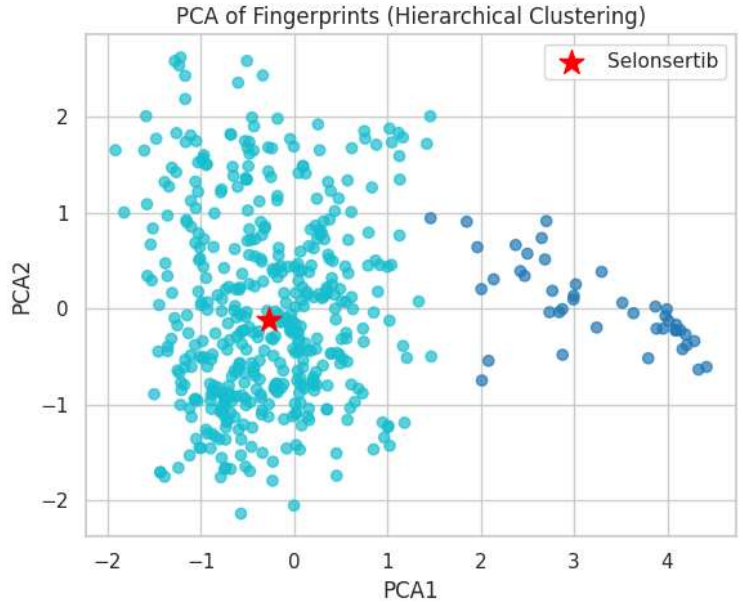
The Tanimoto similarity to Selonsertib for the top 500 compounds mostly falls in the range of 0.08-0.15, indicating that although these compounds have different structures, they retain some common features, and there is a chance they may possess similar activities.

The number of molecules with Tanimoto similarity to Selonsertib ≥ 0.5: 0 / 500

C) Compound selection

Top 500 FDA Approved Small Molecules Structural Diversity



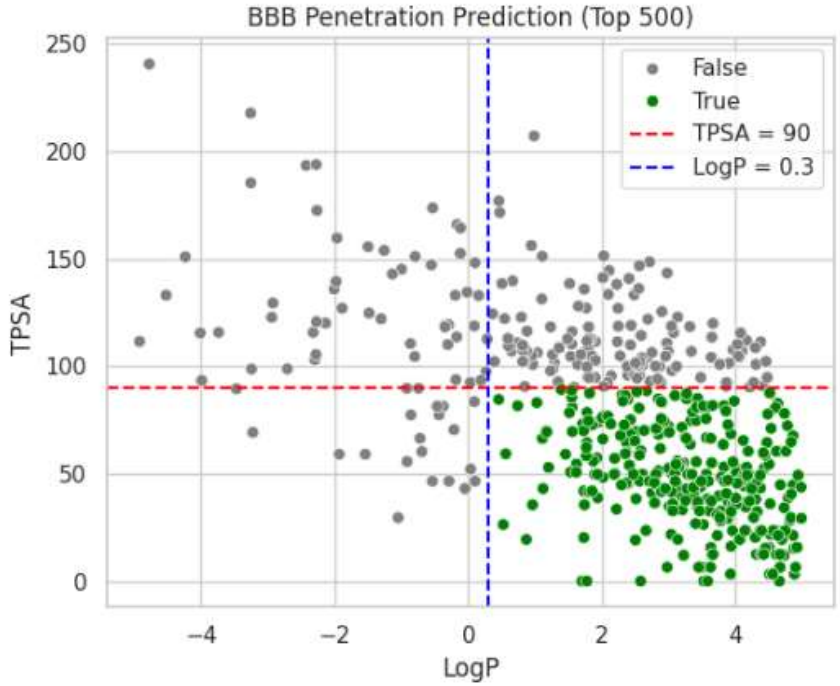


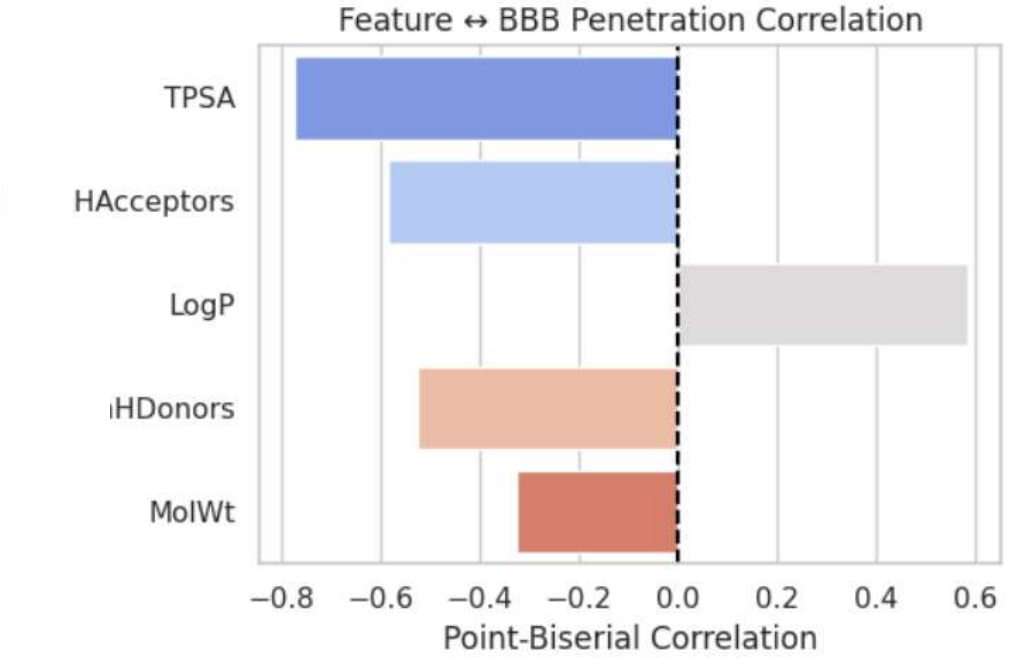
- Using the molecular fingerprints of each compound, their structural similarity was calculated.
- A dendrogram was constructed to show how similar molecules aggregate in a hierarchical manner.
- The top 500 molecules were primarily divided into 5 groups, indicating that these drugs cover a variety of different structural scaffolds.

- The high-dimensional fingerprint information was transformed into a 2D space (PCA1 and PCA2), visualizing the distribution of molecular structures.
- A total of 2 clusters, showing that groups has a distinct distribution in the chemical space.
- Selonsertib (red star) falls in a specific region, which can be compared with other groups to identify structurally novel but potentially high-performing molecules.

C) Compound selection

Blood-Brain Barrier (BBB) Penetration Prediction Analysis





- TPSA < 90: Indicates lower polarity, which is favorable for penetration
- LogP > 0.3: Indicates a certain degree of lipophilicity, which is beneficial for cell membrane permeability
- Green dots (True) predicted to penetrate the BBB. Gray dots (False) predicted to not penetrate the BBB. Most penetrating compounds fall in the bottom right quadrant → "Low polarity, high lipophilicity"

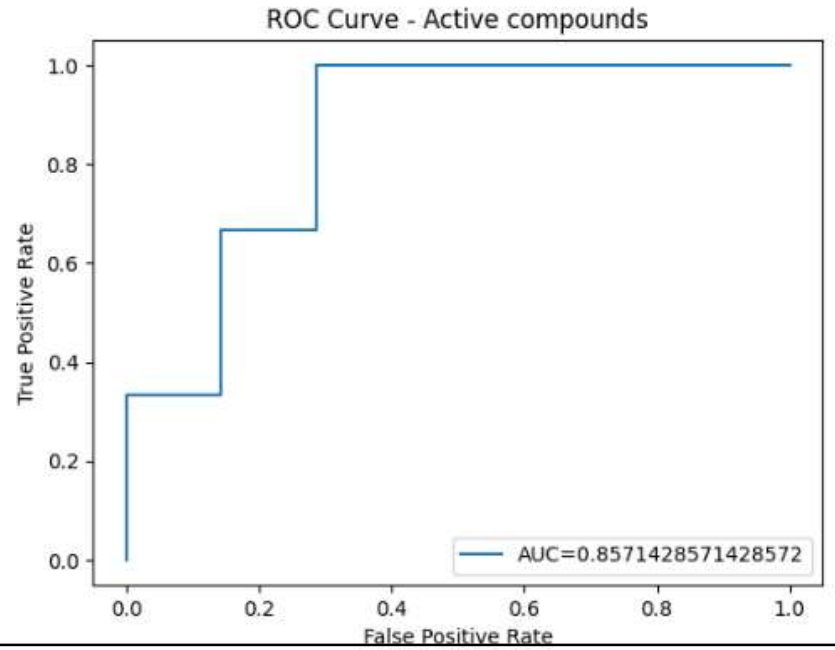
- The x-axis represents the Point-Biserial correlation, and the higher the absolute value, the more discriminative the feature is.
- TPSA (-0.8): Higher polarity → Lower penetration ability
- Number of hydrogen bond acceptors (-0.6)
- LogP (+0.47): Higher lipophilicity → Higher probability of penetration

Method Validation (docking, ligand preparation)

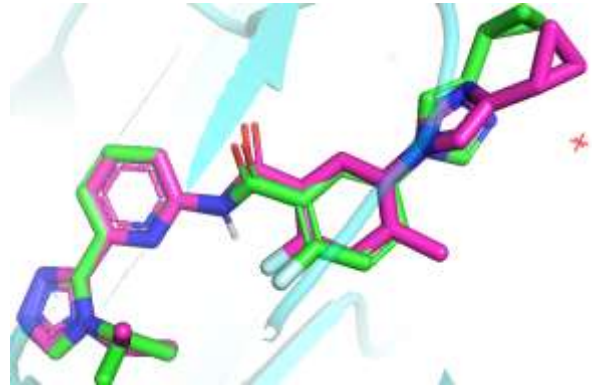
	Steps

Assemble a benchmark set	• ChEMBL ASK1 records
	  • pChEMBL > 8 = 117 (actives)
	  • pChEMBL ≤ 8 = 629 (inactives)
	• Random pick 7 ZINC decoys / 3 pos for similarity test

Dock into 6OYT ATP pocket	• Reference residues L686-V694 & K709 • Scoring = minimized Affinity (kcal mol^{-1})
Evaluate	• ROC curve → **AUC 0.857**



	ID	Active	Sim	minimizedAffinity
0	Molecule_0	True	1.000000	-9.92415
6	Molecule_6	False	0.112676	-9.72801
2	Molecule_2	True	0.582609	-9.61569
4	Molecule_4	False	0.105634	-9.48467
1	Molecule_1	True	0.701923	-9.21337
5	Molecule_5	False	0.124183	-9.07940
7	Molecule_7	False	0.116438	-8.97435
3	Molecule_3	False	0.122449	-8.81358
8	Molecule_8	False	0.095890	-8.65004
9	Molecule_9	False	0.131387	-8.14936



Ligand pose validation. Overlay of original (green) and docked (pink) ligands in ASK1 (cyan) confirms accuracy of docking protocol.

The result of docking validation

- ROC AUC = 0.857 demonstrates strong discriminatory power between known ASK1 actives (pChEMBL > 8) and decoys.
- This confirms that the binding pocket and scoring scheme are suitable for the virtual screening of 500 FDA-approved drugs.

ASK1: https://www.ebi.ac.uk/chembl/explore/target/CHEMBL5285

D) Computational model building and application

Preliminary Virtual Screening

Focused on **Top 500** FDA-approved filtered drugs after Lipinski + PAINS + canonicalization filters.

↓

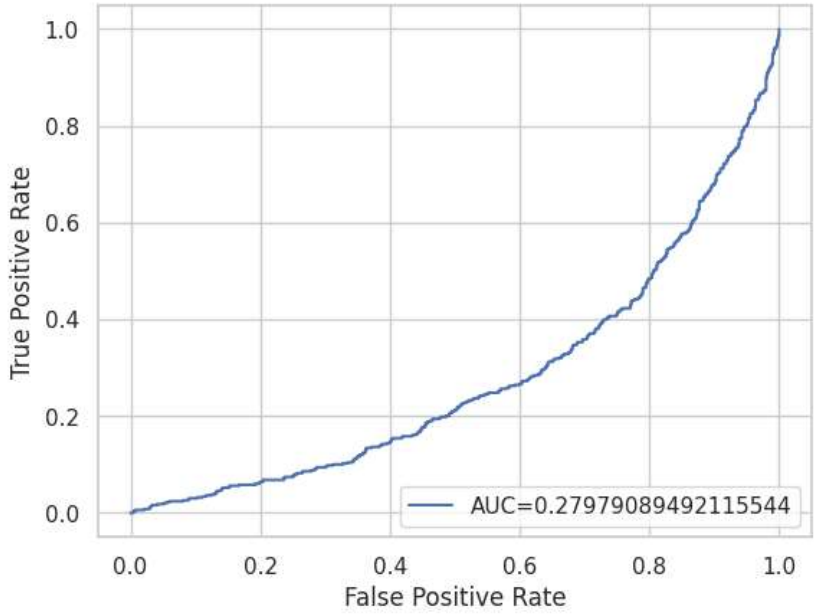
✓ 500 positives (Selonsertib-like)
✓ 1000 random decoys from ZINC

↓

Control molecules:
✓ Selonsertib (Molecule_0)
✓ STU (Molecule_1500) as positive control

↓

Docking tool: **Smina**
Scoring metric: minimizedAffinity (kcal/mol)



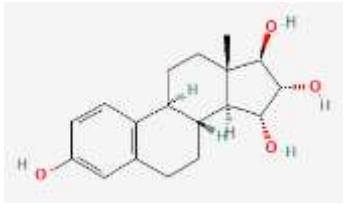
ID	Active	smiles	Sim	minimizedAffinity
Molecule_276	1	C[C@]12CC[C@@H]3c4ccc(O)cc4CC[C@H]3[C@@H]1C[C@...	0.106383	-11.21652
Molecule_396	1	Cl.NC1=NCC2c3ccccc3Cc3ccccc3N12	0.088889	-11.00645
Molecule_39	1	CC1CN(c2cc3c(cc2F)c(=O)c(C(=O)O)cn3-c2ccc(F)cc...	0.155340	-10.88220
Molecule_0	1	C1=CC(=C(C=C1N2C=C(N=C2)C3CC3)C(=O)NC4=CC=CC(=...	1.000000	-9.62043
Molecule_1500	1	C[C@@]12[C@@H]([C@@H](C[C@@H](O1)N3C4=CC=CC=C4...	NaN	-8.40030

Selonsertib (NJV, GS-4997) smiles:
C1=CC(=C(C=C1N2C=C(N=C2)C3CC3)C(=O)NC4=CC=CC(=N4)C5=NN=CN5C(C)C)F
STU smile: C[C@@]12[C@@H]([C@@H](C[C@@H](O1)N3C4=CC=CC=C4C5=C6C(=C7C8=CC=CC=C8N2C7=C53)CNC6=O)NC)OC

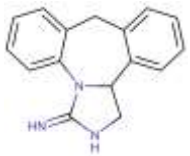
Preliminary Virtual Screening

The best 3 candidates

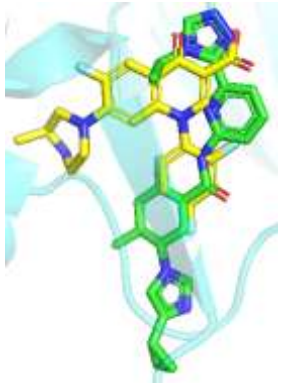
Candidate 1 (Morlecule_276)
Estriol



Candidate 2 (Molecule_396)
Epinastine hydrochloride

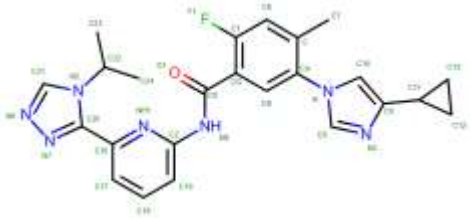


Candidate 3 (Molecule_39)
Temafloxacin hydrochloride



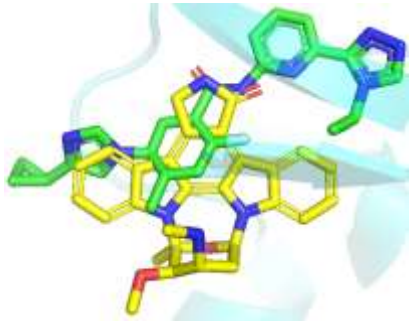
(Yellow: candidates, green: Selonsertib)

Selonsertib



(Yellow: Selonsertib,
green: Selonsertib)

Control



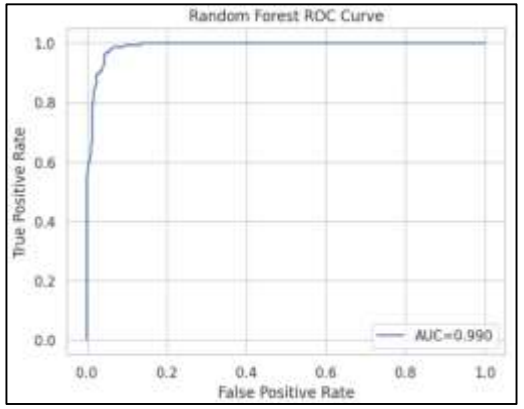
(Yellow: STU
green: Selonsertib)

The result of the preliminary virtual screening

- Docking is technically valid (good controls)
- However, the AUC value is only around 0.28, indicating that the docking scores have limited ability to distinguish active molecules.
- To improve the predictive performance and credibility of the candidate molecules, I will proceed with training a machine learning model.

Machine Learning - Enhanced Virtual Screening Pipeline

	Action	Key Numbers / Notes
1. Docking pre-filter	• Dock all filtered FDA drugs with Smina • Keep molecules with minimized Affinity < –7 kcal mol^{-1}	**650** candidates from 1500 molecules
2. Random Forest model	• Descriptors: MolWt, LogP, TPSA, H-bond counts + docking score • Data split 70 / 30 (500 actives / 1 000 inactives) • 5-fold stratified CV	**AUC 0.99** **Accuracy 95.1 %** **F1 score 0.93**

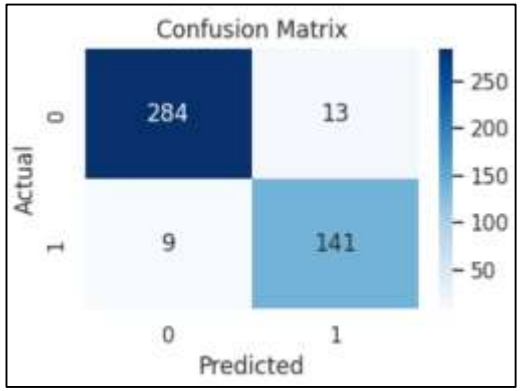



```
Accuracy : 0.951
F1-score : 0.928
Classification report:
              precision    recall  f1-score   support

           0       0.97      0.96      0.96       297
           1       0.92      0.94      0.93       150

    accuracy                           0.95       447
   macro avg       0.94      0.95      0.95       447
weighted avg       0.95      0.95      0.95       447
```

The ROC curve has an AUC of 0.99, indicating that the model can effectively distinguish active and inactive molecules.




```
Feature Importances
MolLogP              0.444928
MolWt                0.337916
TPSA                 0.112596
minimizedAffinity    0.059131
NumHAcceptors        0.027561
NumHDonors           0.017868
```

Confusion Matrix

True Negatives 284, True Positives 141
False Positives 13, False Negatives 9
→ Very low false negatives, the exclusion of inactive molecules is particularly reliable.

Feature Importances

It can be seen that the model mainly relies on overall physicochemical features, with MolLop accounting for the largest portion at 44.4%, while the docking score is only an auxiliary basis. This suggests that **hydrophobicity** plays a critical role in ASK1-related inhibition, possibly by improving membrane permeability or enhancing binding affinity in the hydrophobic kinase pocket.

Machine Learning - Enhanced Virtual Screening Pipeline

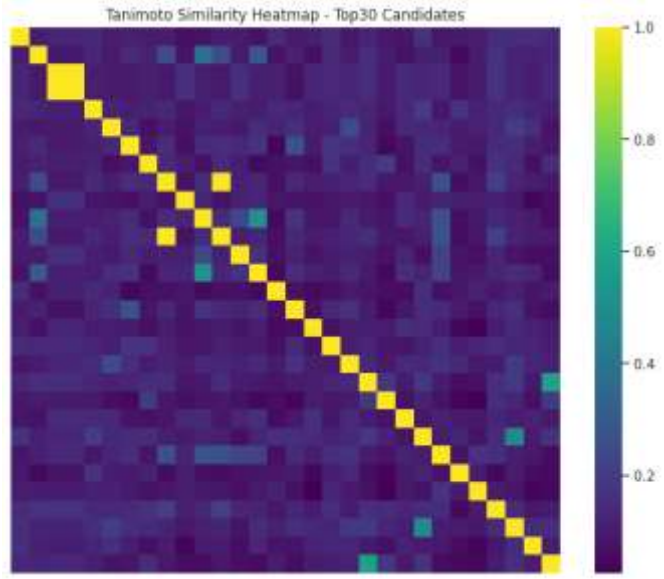
RF predicted probability + binding affinity for dual ranking

Top 30 candidates

(binding affinity < -7 kcal/mol + highest RF predicted probability)

Top Candidates Sorted by minimizedAffinity:

	ID	minimizedAffinity	RF_proba	Active
3	Molecule_3	-9.67462	1.0	True
328	Molecule_329	-9.53918	1.0	True
366	Molecule_367	-9.45183	1.0	True
359	Molecule_360	-9.10800	1.0	True
1	Molecule_1	-8.83861	1.0	True
45	Molecule_45	-8.73701	1.0	True
286	Molecule_287	-8.72563	1.0	True
334	Molecule_335	-8.53303	1.0	True
316	Molecule_317	-8.52158	1.0	True
333	Molecule_334	-8.48741	1.0	True
473	Molecule_475	-8.31893	1.0	True
321	Molecule_322	-8.29707	1.0	True
479	Molecule_481	-8.19705	1.0	True
290	Molecule_291	-8.15260	1.0	True
50	Molecule_50	-8.07942	1.0	True
295	Molecule_296	-7.96911	1.0	True
357	Molecule_358	-7.92506	1.0	True



- To avoid the candidate molecules being too structurally similar, Tanimoto fingerprint similarity analysis was performed.
- The average similarity (Avg Similarity) is 0.122.
- While the maximum similarity (Max) reached 1.000 (indicating one pair of structures are completely identical), the overall structures still exhibit a high degree of diversity.

D) Computational model building and application

BBB Penetration Prediction Analysis

Although DKD does not require entry into the central nervous system (CNS), there are still the following reasons for evaluation

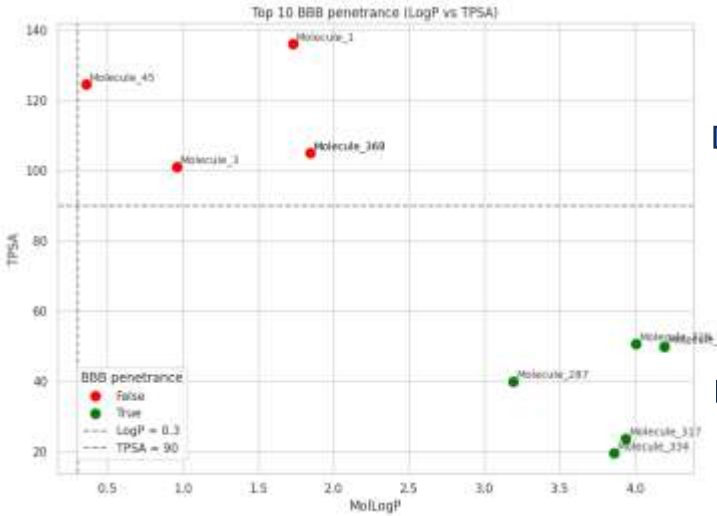
- To avoid potential central side effect risks
- To provide drug repurposing potential (such as for oxidative stress-related neurological diseases)

Evaluation only for the Top 10 candidate drugs

- LogP > 0.3: Higher lipophilicity, which aids in crossing the blood-brain barrier
- TPSA < 90 Å²: Lower polarity, which facilitates passage through the brain capillary endothelial cells

Results

- Preliminary findings suggest that some candidate molecules may possess BBB permeability, providing clues for central action potential.



Top 10 Compounds that do not pass the BBB penetration criteria

ID	minimizedAffinity	RF_proba	Active	MolLogP	TPSA	BBB_Penetration	Smiles
Molecule_3	-9.67462	1.0	True	0.9627	100.87	False	C[C@H]1CO[C@@H]2Cn3cc(C(=O)NCc4ccc(F)cc4F)c(=O)c(O)c3C(=O)N12
Molecule_367	-9.45183	1.0	True	1.8468	104.89	False	CC[C@@]1(O)C(=O)OCc2c1cc1n(c2=O)Cc2cc3c(CN(C)C)c(O)ccc3nc2-1
Molecule_360	-9.10800	1.0	True	1.8468	104.89	False	CC[C@@]1(O)C(=O)OCc2c1cc1n(c2=O)Cc2cc3c(CN(C)C)c(O)ccc3nc2-1
Molecule_1	-8.83861	1.0	True	1.7324	135.95	False	CNC(=O)c1nnc(NC(=O)C2CC2)cc1Nc1cccc(-c2ncn(C)n2)c1OC
Molecule_45	-8.73701	1.0	True	0.3606	124.44	False	CC(C)C[C@H](NC(=O)[C@H](Cc1ccccc1)NC(=O)c1cnccn1)B(O)O

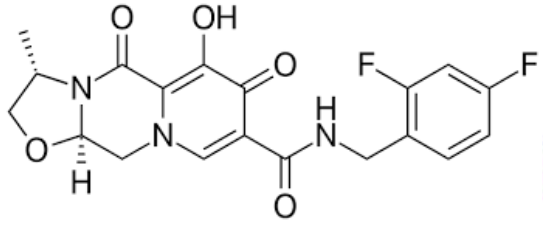
Top 10 compounds that meet the BBB penetration criteria

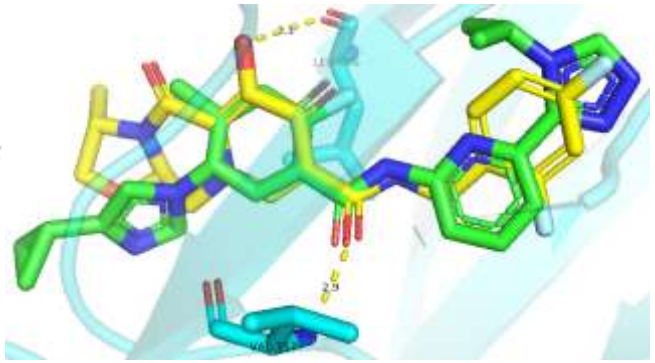
ID	minimizedAffinity	RF_proba	Active	MolLogP	TPSA	BBB_Penetration	Smiles
Molecule_329	-9.53918	1.0	True	4.00778	50.50	True	N#Cc1ccc2c(c1)N(CCCN1CCC(O)CC1)c1ccccc1S2
Molecule_287	-8.72563	1.0	True	3.19380	39.72	True	CCOc1ccccc1O[C@@H](c1ccccc1)[C@@H]1CNCCO1
Molecule_335	-8.53303	1.0	True	4.19580	49.69	True	C[C@]12CC[C@@H]3c4ccc(OC5CCCC5)cc4CC[C@H]3[C@@H]1C[C@@H](O)[C@@H]2O
Molecule_317	-8.52158	1.0	True	3.94040	23.47	True	O[C@@](CCN1CCCC1)(c1ccccc1)C1CCCCC1
Molecule_334	-8.48741	1.0	True	3.86260	19.37	True	CN(C)c1ccc2nc3ccc(N(C)C)cc3[s+]c2c1

Top 4 Best candidate drugs

For DKD candidate drugs, I selected those that do not pass the BBB barrier.

Candidate 1, Molecule_3, Cabotegravir





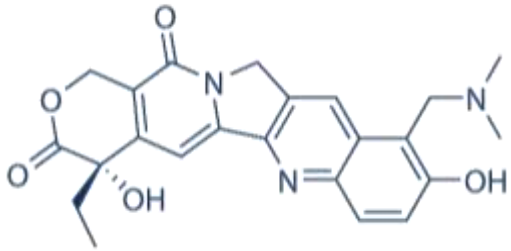
Current Use & Pharmacology

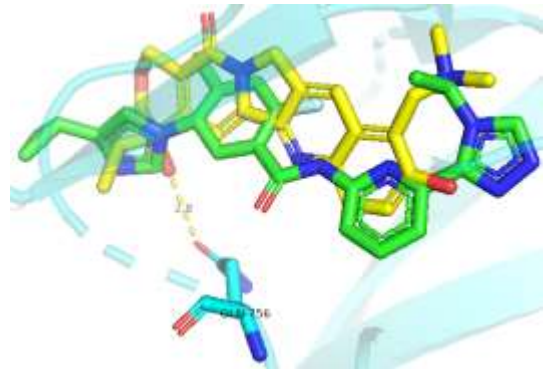
- Approved for HIV-1 treatment and pre-exposure prophylaxis (PrEP).
- Potent integrase strand transfer inhibitor (INSTI), preventing viral DNA integration.
- Long half-life (~40 days), low CNS side effects, suitable for monthly or bi-monthly IM injection.

Interaction with ASK1

- No known direct link to ASK1 signaling; antiviral mechanism is independent of oxidative stress or MAPK pathways.
- Docking shows tight binding to ASK1 ATP site (Leu686, Val757) potential mechanism-switch repositioning.

Candidate 2, Molecule_367, Topotecan





Current Applications and Pharmacology

- Small cell lung cancer, ovarian cancer, cervical cancer; administered intravenously or orally.
- Topoisomerase I inhibitor, causing DNA breaks and cell apoptosis.
- Bone marrow suppression is the dose-limiting toxicity; short half-life requires consecutive daily dosing.

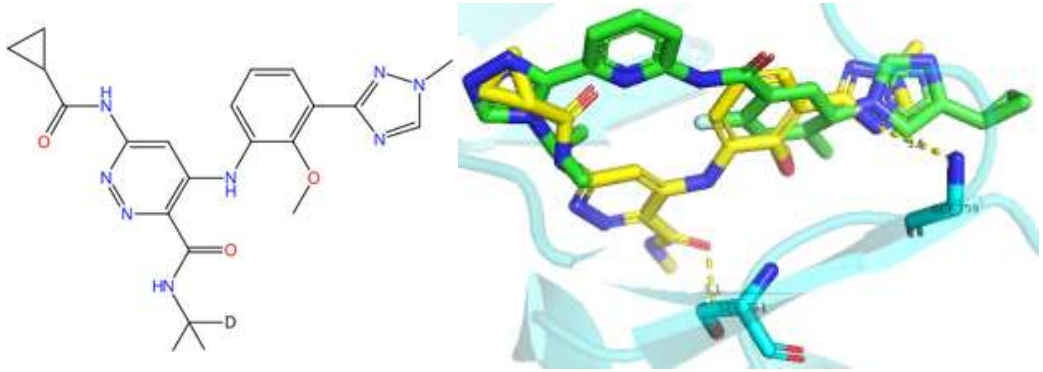
Interaction with ASK1

- No direct reports, but Topotecan can increase ROS and promote DNA damage; theoretically, it may activate ASK1 → p38/JNK.
- Docking shows potential binding in the L686/V757/Gln756 region, inhibiting ASK1 also reduces self-induced ROS.

Top 4 Best candidate drugs

For DKD candidate drugs, I selected those that do not pass the BBB barrier.

Candidate 3, Molecule_1, Deucravacitinib



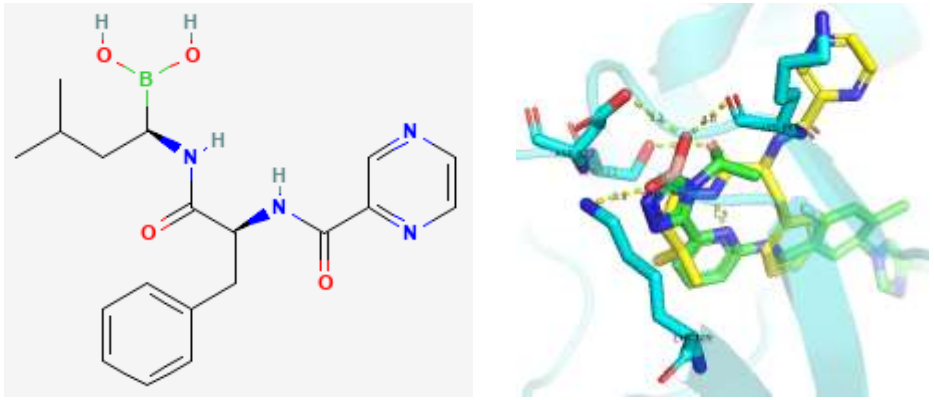
Current Applications and Pharmacology

- Moderate to severe plaque psoriasis (oral, once daily).
- First-in-class TYK2 allosteric inhibitor, with specificity higher than other JAKs.
- Reduces IL-23/12 signaling, with relatively low side effects (infections, dyslipidemia).

Interaction with ASK1

- TYK2-STAT overlaps with inflammation and IFN pathways; ASK1 is also part of the MAPK family, potential complementary anti-inflammatory effects.
- Docking shows binding capability with Gly759 and Ser761, with stable conformation.

Candidate 4, Molecule_45, Bortezomib



Current Applications and Pharmacology

- Multiple myeloma, mantle cell lymphoma; administered subcutaneously or intravenously.
- Reversible inhibition of the 26S proteasome β5 subunit, leading to the accumulation of misfolded proteins and inducing ER stress.

Interaction with ASK1

- ER stress can activate the ASK1-JNK pathway; low doses may potentially "shut down the loop" to combat fibrosis.
- Docking successfully binds to Lys688/Asp822/Lys709/Ser821, with multiple hydrogen bonds and hydrophobic stacking.

Budget

I plan to purchase four candidate compounds (Cabotegravir, Topotecan, Deucravacitinib, and Bortezomib), along with a positive control (Selonsertib, an ASK1 inhibitor). Each compound will be procured in 25 mg with an estimated total cost of $4720.79.

Candidates	minimizedAffinity	Similarity	Supplier	Qty	Price
Selonsertib (Control)	-9.62043	1.000000	Sigma	25mg	$135
Cabotegravir	-9.67462	0.094017	MedChemExpress	25mg	$168.59
Topotecan	-9.45183	0.094828	Cayman	25mg	$797.5
Deucravacitinib	-8.83861	0.225490	SelleckChem	25mg	$1470
Bortezomib	-8.73701	0.151515	MedChemExpress	25mg	$2149.7

Total: $4720.79

To validate their potential in DKD treatment

1. Conduct cell-based assays using **human renal tubular cells (HK-2)** to assess ASK1-related signaling (e.g., p-JNK, ROS) and cytotoxicity.
2. Promising candidates will then be tested in a pilot animal study, using a **DKD mouse model** to evaluate efficacy on renal function and fibrosis.

E) Budget

Experimental Design

Assay (In intro)	Purpose	Evaluations
Cell viability (MTT)	Rule out cytotoxicity or non-specific inhibition	CC50, relative viability
ASK1 kinase assay	Confirm whether compound inhibits ASK1 enzymatic activity	IC50, % inhibition
Western blot	Evaluate downstream signaling (p-JNK, p-p38)	The changes in phosphorylation levels
Immunohistochemistry	Assess protein localization and expression in cells (e.g., fibronectin, TGF-β, ASK1)	Staining intensity and localization
ROS assay	Test if compound reduces intracellular ROS	Fluorescence intensity, ROS level

Compounds with significant results in cell assays will be tested in animal experiments.

Assay (In vivo)	Purpose	Evaluations
db/db diabetic mouse model	Mimic DKD pathological condition	Body weight, blood glucose, proteinuria
Renal function analysis	Therapeutic effect on kidney function	Urinary protein, serum creatinine, eGFR
Histology	Assess glomerulosclerosis and fibrosis	H&E, Masson, PAS staining
Immunohistochemistry	Evaluate ASK1 pathway markers (e.g., p-JNK, p-p38, fibronectin, collagen IV) in kidney tissue	Staining intensity, localization in glomeruli or tubules
Western blot	Confirm ASK1 downstream signaling (p-JNK, p-p38)	p-JNK/p-p38 levels

F) Outlook - Timeline estimates, estimate future hurdles.

Timeline estimates

month

- 1 — Docking → Hit List
- 1 — Compound Purchased & QC
- 3 — In vitro Studies:
  MTT, ASK1 kinase assay
  WB, IHC, ROS assay
- 3 — In vivo studies:
  Pathological condition, renal function analysis
  Histology, IHC, WB
- 1-2 — Provisional patent
- 4-6 — Apply for Phase 1 clinical seed funding

Clinical trial

Future hurdles

Kinase Selectivity

✖ ASK1 shares homology with other MAP3Ks; off-target effects may cause toxicity.

☑ Structure scan and optimization.

Cytotoxicity Risk

✖ High toxicity may block progression to animal testing.

☑ Dose-response profiling; viability screen with CC_{50} > 80%.

In Vivo Variability

✖ db/db show no significant outcomes in DKD.

☑ Change to STZ-induced mice.

Budget Constraints

✖ High cost of in vivo studies.

☑ Break project into funding phases; apply for seed funds; negotiate core facility discounts.

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