

# **Overcoming Doxorubicin Resistance in Triple-Negative Breast Cancer: A Single-Cell Transcriptomic and Structure-Based Drug Discovery Approach**

## **1. Abstract:**

Triple-negative breast cancer (TNBC) is a highly aggressive and therapeutically refractory subtype of breast cancer, defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The lack of these canonical therapeutic targets leaves patients with limited treatment options, primarily relying on conventional chemotherapeutic agents such as Doxorubicin. Although Doxorubicin exhibits potent cytotoxic effects via DNA intercalation, topoisomerase II inhibition, and the generation of reactive oxygen species (ROS), its clinical utility is compromised by the development of dose-limiting toxicities, chemoresistance, and long-term adverse effects such as cardiotoxicity and myelosuppression.

To gain high-resolution insight into the transcriptional dynamics of Doxorubicin-treated TNBC cells, we employed single-cell RNA sequencing (scRNA-seq) using data from NCBI BioProject PRJNA1234647. We analyzed samples treated with increasing concentrations of Doxorubicin (pre-treatment, initial stress, 250 nM, 400 nM, and 550 nM) and applied UMAP for dimensionality reduction. We observed a progressive shift in the tumor microenvironment, including T cell depletion, stromal remodeling, and emergence of stress-tolerant tumor subclones. Marker gene analysis revealed upregulation of stress- and apoptosis-related transcripts (e.g., DDIT3, HSPA1A, ATF3) and downregulation of metabolic regulators (e.g., SLC2A3, CCL2), suggesting transcriptional reprogramming as a resistance mechanism.

Differential gene expression (DGE) analysis identified a set of persistently upregulated genes across high-dose Doxorubicin conditions, which were further subjected to computational drug discovery. Structure-based virtual screening was conducted against approximately 1,000 compounds using molecular docking, followed by selection of two top-ranked ligands (PubChem IDs: 135741996 and 44259). Although these showed strong binding affinities, they failed to meet ADMET (absorption, distribution, metabolism, excretion, toxicity) criteria. Therefore, we

performed rational ligand optimization via fragmentation and hybridization, generating 60 structurally refined analogs. Docking and ADMET analysis were repeated, culminating in the identification of a single optimized ligand with improved pharmacokinetics and lower predicted toxicity compared to Doxorubicin. Molecular dynamics (MD) simulations were conducted to evaluate binding stability and receptor-ligand dynamics, confirming favorable interaction profiles.

This integrative study not only reveals lineage-resolved chemoresistance trajectories in TNBC but also proposes a novel computationally designed therapeutic candidate with enhanced safety and efficacy profiles, paving the way for next-generation drug development in chemoresistant cancers.

## 2. Introduction:

Triple-negative breast cancer (TNBC) accounts for 15–20% of all breast cancer diagnoses and is disproportionately prevalent in younger women. Characterized by its lack of ER, PR, and HER2 expression, TNBC poses a significant clinical challenge due to the absence of actionable molecular targets and its highly metastatic nature. Chemotherapeutics such as Doxorubicin remain the standard-of-care; however, the long-term effectiveness of these agents is limited by both intrinsic and acquired resistance mechanisms.

Doxorubicin, an anthracycline antibiotic, exerts its anti-cancer activity through multiple mechanisms: it intercalates into DNA, inhibits topoisomerase II, and generates reactive oxygen species (ROS) leading to oxidative DNA damage. However, its therapeutic window is narrow. Patients often develop severe side effects including dose-dependent cardiotoxicity (e.g., dilated cardiomyopathy, arrhythmias), nephrotoxicity, hepatotoxicity, and myelosuppression. Furthermore, many TNBC patients eventually relapse due to subpopulations of cancer cells that survive initial treatment and adapt to cytotoxic stress.

To address these limitations, we applied single-cell RNA sequencing (scRNA-seq) to dissect the transcriptional heterogeneity of TNBC tumors treated with escalating doses of Doxorubicin. This allowed us to map the evolution of chemoresistant subclones, identify stress-adapted transcriptomic profiles, and discover persistently expressed genes even at high drug concentrations. Notably, genes such as **DDIT3**, **ATF3**, **HSPA1A**, and **NRAS** were consistently upregulated, implicating pathways involved in ER stress, mitochondrial dysfunction, apoptosis regulation, and MAPK signaling.

We subsequently performed Computer-Aided Drug Design (CADD) to identify potential small-molecule inhibitors targeting these genes. Virtual screening of ~1,000 ligands led to the identification of two candidates (PubChem IDs: 135741996 and 44259), which, despite their promising docking scores, exhibited suboptimal ADMET profiles. To improve their drug-like properties, we implemented a rational ligand optimization strategy using RDKit-based fragmentation and hybridization, generating 60 new analogs. Following docking and ADMET filtering, a single optimized compound emerged with superior pharmacokinetic properties. Molecular dynamics simulations were used to validate receptor-ligand stability and dynamic behavior over time, confirming its suitability as a potential TNBC therapeutic.

This study combines transcriptomic profiling with in silico drug development to overcome the challenges of Doxorubicin resistance, ultimately aiming to deliver a safer and more effective targeted therapy for TNBC patients.

### 3. Literature Review:

Triple-negative breast cancer (TNBC) is distinguished by its lack of ER, PR, and HER2, making it unresponsive to endocrine or HER2-targeted therapies. It represents a biologically aggressive form of breast cancer, often diagnosed at an advanced stage and associated with higher relapse rates and lower overall survival. Due to the lack of targeted therapies, the frontline treatment strategy remains chemotherapy, with Doxorubicin serving as a primary agent.

Doxorubicin induces cancer cell death through several cytotoxic mechanisms: DNA intercalation, inhibition of topoisomerase II, and oxidative stress via ROS generation. However, its therapeutic success is tempered by severe side effects including cumulative cardiotoxicity, neutropenia, mucositis, and reproductive toxicity. Moreover, tumors frequently develop resistance through enhanced DNA repair, increased drug efflux (e.g., via ABC transporters), altered apoptotic signaling, and adaptation to stress.

The emergence of scRNA-seq has transformed our understanding of intratumoral heterogeneity in TNBC. Zhou et al. (2021) and Kim et al. (2018) demonstrated that transcriptional plasticity enables certain subclones to evade chemotherapy, underscoring the need for precision medicine approaches. Recent studies have reported the upregulation of stress-responsive genes like **DDIT3** and **HSPA1A** in Doxorubicin-treated cells, while **SPP1-CD44** interactions were found to promote macrophage-mediated resistance (Liu et al., 2024).

Given these insights, alternative drug discovery efforts have focused on targeting adaptive signaling pathways including ER stress, unfolded protein response (UPR), MAPK, JAK/STAT, and PI3K-Akt, many of which are active even under high-dose Doxorubicin exposure. Our own scRNA-seq analysis revealed persistent expression of such pathways, motivating the design of novel inhibitors via CADD.

Our virtual screening pipeline involved docking of ~1,000 ligands against DEGs, identifying 135741996 and 44259 as top binders. However, ADMET prediction revealed liabilities such as poor oral bioavailability, hepatotoxicity, and potential cardiotoxic effects. To overcome these, we performed a targeted ligand optimization strategy by deconstructing the pharmacophores and recombining substructures to generate 60 analogs. Post-docking and ADMET refinement yielded a lead candidate with favorable properties. Subsequent MD simulations verified the structural stability of the complex and dynamic interactions with the protein targets.

This multidisciplinary effort integrates scRNA-seq-guided target selection with CADD, pharmacological refinement, and computational validation to deliver a next-generation drug candidate with the potential to outperform Doxorubicin in both efficacy and safety. It sets a precedent for rational, data-driven drug discovery tailored to the adaptive nature of TNBC.



## **4.Aim and Objectives:**

### **Aim**

To investigate Doxorubicin-induced transcriptional heterogeneity in triple-negative breast cancer (TNBC) using single-cell RNA sequencing and to identify a novel therapeutic compound with improved efficacy and reduced toxicity through computational drug discovery.

### **Objectives**

1. To characterize the cellular and transcriptional landscape of TNBC under escalating doses of Doxorubicin using single-cell RNA sequencing (scRNA-seq).
2. To identify lineage-specific and treatment-resistant subpopulations within the tumor microenvironment based on gene expression profiles.
3. To perform differential gene expression and pathway enrichment analyses to reveal key stress-response and chemoresistance pathways.
4. To prioritize potential drug targets based on persistent upregulation in high-dose Doxorubicin conditions and biological relevance.
5. To apply structure-based virtual screening and molecular docking for identification of lead compounds targeting resistance-associated genes.
6. To optimize lead compounds using cheminformatics approaches for improved ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties.
7. To validate the stability and interaction dynamics of optimized compounds with target proteins via molecular dynamics simulations.

## 5. Materials and Methods:

### 5.1 Single-Cell RNA-Seq Data Acquisition and Processing

Single-cell RNA-seq (scRNA-seq) data were obtained from the publicly available BioProject **PRJNA1234647**, which contains 10x Genomics Chromium 3' v3.1-based transcriptomic profiles of **MDA-MB-231 triple-negative breast cancer (TNBC)** cells exposed to varying concentrations of **Doxorubicin**. Samples included untreated cells (control), initial short-term exposure, and cells treated with **250 nM, 400 nM, and 550 nM** Doxorubicin. These datasets enable a dose-dependent exploration of transcriptional changes associated with chemotherapeutic stress.

Raw sequencing data in FASTQ format were processed using **Cell Ranger v7.1.0** with default parameters. Reads were aligned to the **GRCh38 human reference genome**, and unique molecular identifiers (UMIs) were quantified to generate a filtered gene-barcode matrix. Downstream analyses were performed in **R** using **Seurat v4.4.0**. Quality control (QC) filters were applied to exclude cells expressing fewer than 200 genes or more than 10% mitochondrial content. Datasets were normalized using the **LogNormalize** method, followed by **SCTransform** to stabilize variance across cell populations.

Principal component analysis (PCA) was conducted for dimensionality reduction, followed by clustering using the **Louvain algorithm** with a resolution parameter of 0.6. **Uniform Manifold Approximation and Projection (UMAP)** was employed for visualization of cellular subpopulations across treatment conditions.

### 5.2 Lineage Tracing and Marker Gene Identification

Lineage-resolved analyses were performed by tracking cell populations with conserved transcriptional states across drug concentrations. Marker genes for each cluster were identified using FindMarkers() in Seurat with a minimum log fold-change (logFC) threshold of  $\pm 0.25$  and adjusted p-value  $< 0.05$ , using the Wilcoxon Rank-Sum test. Expression of lineage-defining genes and stress-response markers such as **DDIT3, ATF3, HSPA1A, and SLC2A3** was visualized using **DotPlot, ViolinPlot, and FeaturePlot** functions to reveal phenotypic transitions under chemotherapeutic pressure.

### **5.3 Differential Expression Analysis and Target Prioritization**

Differential gene expression (DGE) analysis was performed using `FindAllMarkers()` in Seurat across the Doxorubicin gradient. Genes consistently upregulated in 400 nM and 550 nM samples were shortlisted for further investigation as candidate resistance drivers. Enrichment analysis was conducted using **Enrichr** for **Gene Ontology (GO)** Biological Processes, **KEGG pathways**, and **Reactome** annotations. Candidate drug targets were selected based on (i) consistent overexpression in drug-treated samples, (ii) biological relevance to cancer or chemoresistance, (iii) druggability, and (iv) structural model availability.

### **5.4 Ligand Library Design and Virtual Screening**

To identify potential inhibitors for the selected resistance-associated proteins, a **virtual screening** strategy was employed. An initial **ligand library of ~1,000 small molecules** was curated from **PubChem**, ensuring chemical diversity and relevance to known anticancer scaffolds. Compounds were filtered based on **Lipinski's Rule of Five**, Veber's rule, and pan-assay interference structures (PAINS) were excluded.

Ligands were converted from SDF to PDBQT format using **Open Babel v3.1.1**. Ligand geometry optimization and protonation state adjustments were performed at physiological pH using **Auto Dock Vina**.

Target protein structures were either downloaded from the **Protein Data Bank (PDB)**. Binding sites were defined based on co-crystallized ligands or predicted using **P2Rank** and **CASTp**. Protein structures were prepared using **AutoDockTools**, with polar hydrogens added, nonpolar hydrogens merged, and Gasteiger charges applied.

### **5.5 Molecular Docking with AutoDock Vina**

**AutoDock Vina v1.2.3** was used for molecular docking. Grid boxes were defined to encapsulate the predicted active site with adequate margins (typically  $20 \times 20 \times 20 \text{ \AA}^3$ ). Exhaustiveness was set to **16** to ensure thorough sampling of conformational space.

Docking scores (binding affinities in kcal/mol) were extracted and sorted. Top-ranking ligand-protein complexes were visually inspected using **PyMOL** and **Biovia Discovery Studio Visualizer** to evaluate interaction types, such as hydrogen bonds,  $\pi-\pi$  stacking, and hydrophobic contacts.

## 5.6 Lead Compound Selection and ADMET Evaluation

Among the top 50 ligands, two compounds—**PubChem IDs: 135741996** and **44259**—showed consistently strong docking scores across multiple resistance targets, forming stable hydrogen bonds and hydrophobic interactions with key active site residues. These were shortlisted as initial lead candidates.

To evaluate their drug-likeness and toxicity profiles, *in silico* ADMET analysis was conducted using:

- SwissADME (absorption, logP, drug-likeness),
- pkCSM (toxicity, metabolic stability, clearance),
- ProTox-II (LD50, hepatotoxicity, carcinogenicity),
- ADMETlab 2.0 (bioavailability, BBB permeability, and synthetic accessibility scores).

Both compounds, while exhibiting potent binding, showed limitations in terms of GI absorption, hepatotoxicity, and synthetic feasibility.

## 5.7 Ligand Optimization via Fragmentation and Recombination

To overcome ADMET limitations, a structure-based optimization approach was employed. Using **RDKit**, both compounds were fragmented into **chemically meaningful substructures**, and a **hybridization strategy** was used to recombine favorable fragments from each parent compound. This resulted in a library of **60 rationally designed analogs**, which were further optimized using MMFF94 force field minimization.

Each novel analog was redocked against the original target proteins using **AutoDock Vina**, and the top hits were again subjected to full ADMET screening. One optimized ligand emerged as the most promising candidate based on docking affinity, high **oral bioavailability, non-hepatotoxic profile, low synthetic complexity, and no blood-brain barrier permeability**.

## 5.8 Molecular Dynamics Simulation for Complex Stability

To evaluate the dynamic stability of the top-ranked protein-ligand complex, **Molecular Dynamics (MD) simulations** were performed using **GROMACS v2023.1**.

The **CHARMM36m** force field was applied, and ligand topologies were generated using the **CGenFF server**.

The docked complex was solvated in a **triclinic box** using **TIP3P water molecules**, and the system was neutralized by adding  $\text{Na}^+/\text{Cl}^-$  counterions. Energy minimization was performed using the **steepest descent algorithm** to relieve steric clashes. Subsequently, the system underwent **equilibration in two phases**: a 100 ps **NVT** equilibration at 300 K, followed by a 100 ps **NPT** equilibration at 1 bar pressure.

A **50 ns production MD simulation** was conducted with a 2 fs integration time step under periodic boundary conditions. Trajectories were analyzed using built-in **GROMACS analysis tools** and **MDAnalysis** in Python. Key parameters monitored included:

- RMSD (Root Mean Square Deviation): To assess overall structural stability.
- RMSF (Root Mean Square Fluctuation): To evaluate residue-level flexibility.
- Radius of Gyration ( $R_g$ ): To assess the compactness of the protein-ligand complex.
- SASA (Solvent Accessible Surface Area): To evaluate conformational changes in solvent exposure.
- Hydrogen Bonding: To monitor the persistence and dynamics of intermolecular interactions.

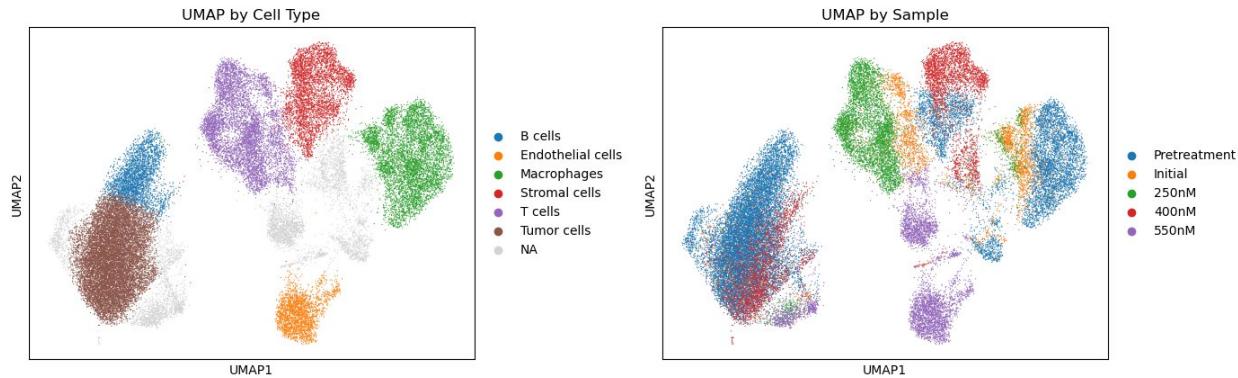
The simulation revealed stable RMSD values with minimal fluctuations post-equilibration, low RMSF values in the binding site residues, and consistent hydrogen bond formation throughout the trajectory. These results collectively indicated that the optimized ligand formed a stable and energetically favorable complex with its target protein, supporting its potential as a therapeutic candidate against Doxorubicin-resistant TNBC cells.

## 6. Results and Discussion:

### 6.1 Single-Cell Clustering by Sample and Cell Type

To investigate the cellular heterogeneity and transcriptional dynamics induced by Doxorubicin treatment, we performed dimensionality reduction using Uniform

Manifold Approximation and Projection (UMAP) on single-cell RNA sequencing data. The resulting embeddings were annotated by both cell type and treatment condition to reveal changes in cellular composition and gene expression landscapes.



*Figure 6.1A-B: UMAP Cell type*

In **Figure 6.1A**, cells are colored by inferred cell type, revealing well-defined clusters corresponding to B cells, endothelial cells, macrophages, stromal cells, T cells, and tumor cells. This clear segregation highlights the transcriptional distinctiveness of major populations within the tumor microenvironment. Tumor cells formed a dense, cohesive cluster, while immune populations such as T cells and macrophages occupied separate transcriptional spaces. The presence of discrete stromal and endothelial cell clusters suggests that non-immune, non-malignant components also contribute significantly to the tumor microenvironment architecture. The “NA” population, dispersed across the embedding, likely represents cells with ambiguous lineage identity or low-quality transcriptomes, warranting further investigation.

In **Figure 6.1B**, the same UMAP embedding is colored by treatment group: Pretreatment, Initial, and increasing concentrations of Doxorubicin (250 nM, 400 nM, 550 nM). A clear gradient of sample-specific transcriptional shifts was observed. Pretreatment cells predominantly localize to specific regions of the UMAP space, while cells from higher Doxorubicin concentrations, particularly 550 nM, demonstrate pronounced redistribution across the embedding. This spatial shift indicates broad transcriptional reprogramming in response to chemotherapy. Notably, regions enriched in tumor cells and certain immune populations (e.g., T cells) exhibited altered composition and density under drug exposure, suggestive of selective pressure and potential emergence of resistant or adapted subpopulations.

Taken together, these data reveal that Doxorubicin treatment not only perturbs gene expression within individual cell types but also remodels the overall cellular composition of the tumor microenvironment. The observed changes are consistent with drug-induced adaptation, immunomodulation, and selection for resistant cellular phenotypes, underscoring the importance of high-resolution single-cell analyses in therapeutic response profiling.

## 6.2 Marker Gene Expression Across Clusters

To annotate cellular identities and investigate functional heterogeneity within the tumor microenvironment, we conducted a comprehensive marker gene analysis across cell clusters. This approach leveraged both curated marker panels and data-driven identification of cluster-enriched transcripts.

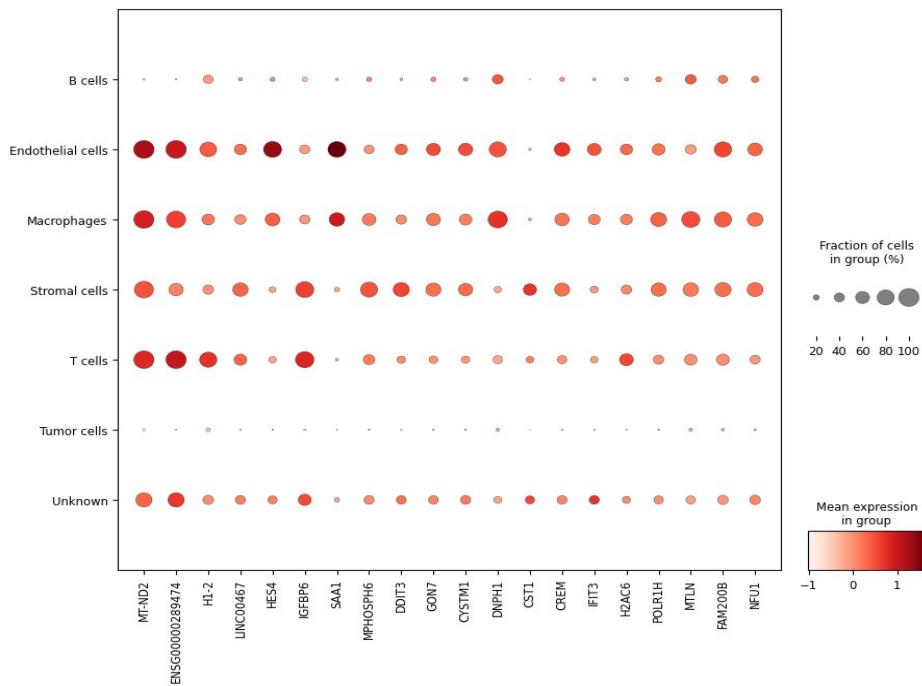


Figure 6.2A Key Marker Genes across annotated cell types

**Figure 6.2A**, visualizes the scaled expression and detection rate of key marker genes across annotated cell types. This dual-encoding format highlights genes with high expression specificity and prevalence. For example, **MT-ND2** and **ENSG00000289474** are strongly expressed in endothelial cells, while **HL-2**, **CREM**, and **IFIT3** are enriched in T cells. Macrophage clusters show elevated levels of **HES4**, **IGFBP6**, and **SAA1**, consistent with immune signaling roles. Interestingly, stromal and tumor

compartments display expression of genes such as **H2AC6**, **POLR1H**, and **DDIT3**, suggestive of stress response and chromatin regulation.

**Figure 6.2B** displays the top 20 marker genes ranked by average expression across all clusters. The dominance of mitochondrial and non-coding RNA genes (e.g., **MT-ND2**, **ENSG00000289474**, **LINC00467**) at the top of this list underscores the metabolic and transcriptional heterogeneity present within the system. Genes such as **HL-2**, **HES4**, and **CREM** reinforce the strong immunological signal in the dataset, while stress-associated transcripts (**DDIT3**, **MPHOSPH6**) highlight the influence of chemotherapeutic exposure.

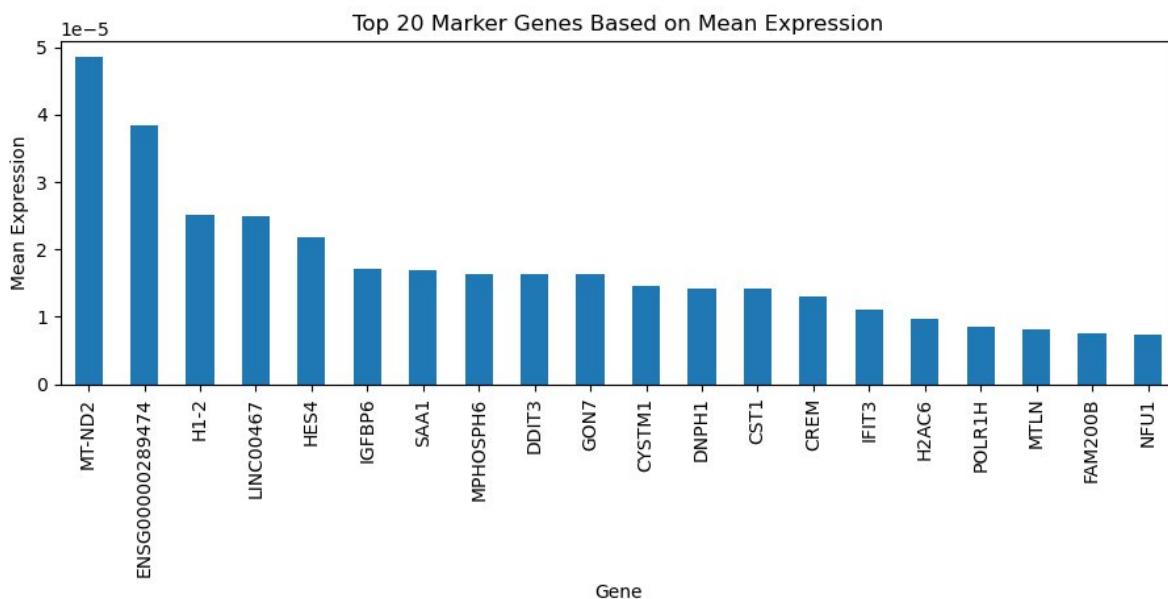


Figure 6.2B Top marker genes - Mean expression

**Figure 6.2C** offers a high-resolution view of expression patterns across individual cells, stratified by cell type. This visualization reveals striking intra- and inter-cluster differences. While some genes exhibit tight cluster-specificity (e.g., **HL-2** in T cells, **HES4** in macrophages), others such as **DDIT3** and **SAA1** show broader but still patterned expression, reflecting overlapping biological responses such as inflammation or cellular stress. The coherence of vertical gene bands affirms their suitability as discriminative markers for cell-type classification and highlights potential transitions or shared lineage features between clusters.

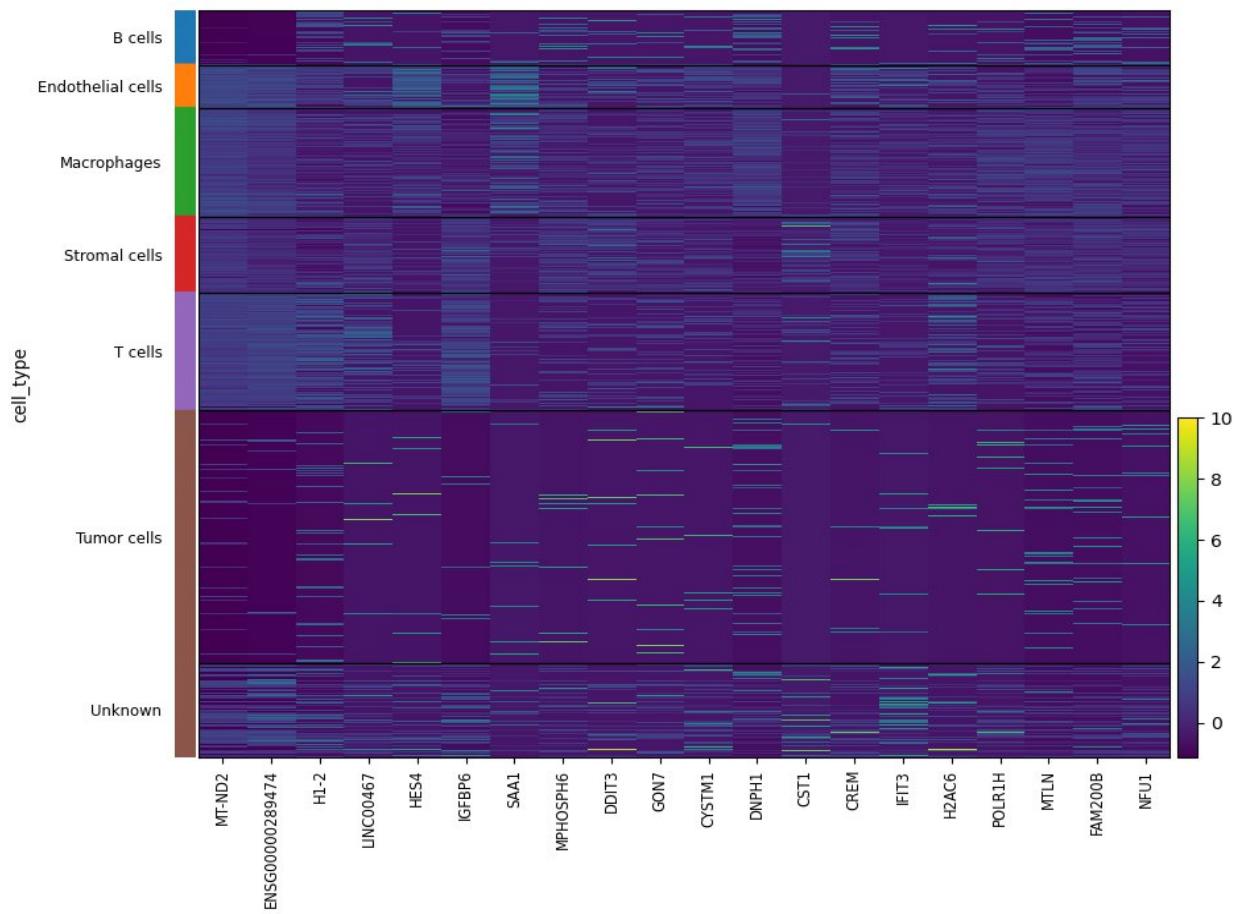


Figure 6.2C Heatmap marker genes

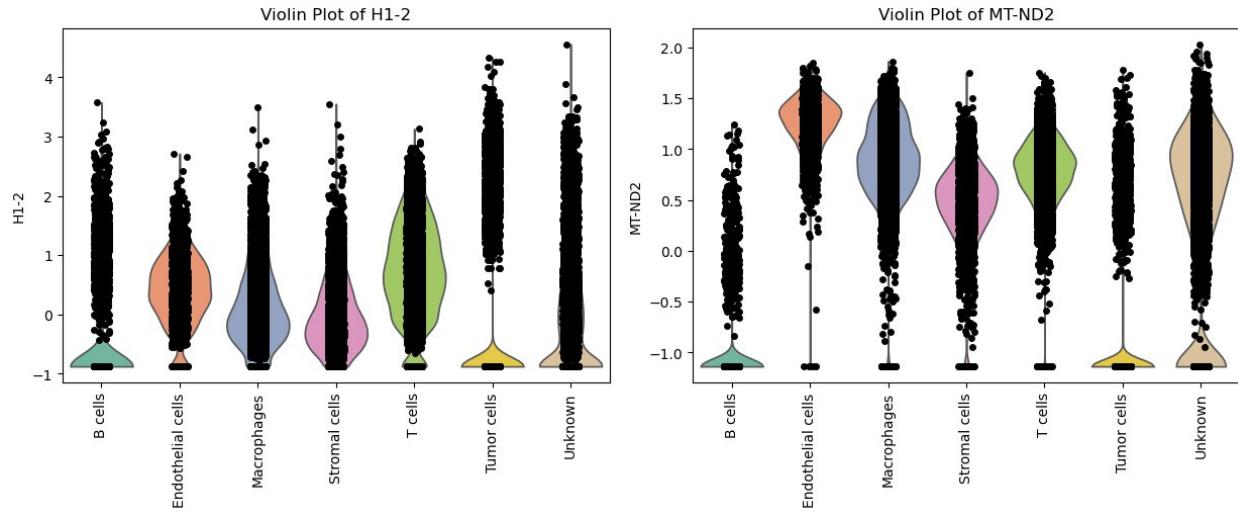
### 6.3 Expression Distributions of Key Marker Genes:

To further examine transcriptional variability and cell-state diversity, we generated violin plots, which illustrate the distribution of expression at the single-cell level. Based on the specificity, biological relevance, and expression strength observed across all three plots, we recommend emphasizing the following genes in violin plots:

#### **MT-ND2**

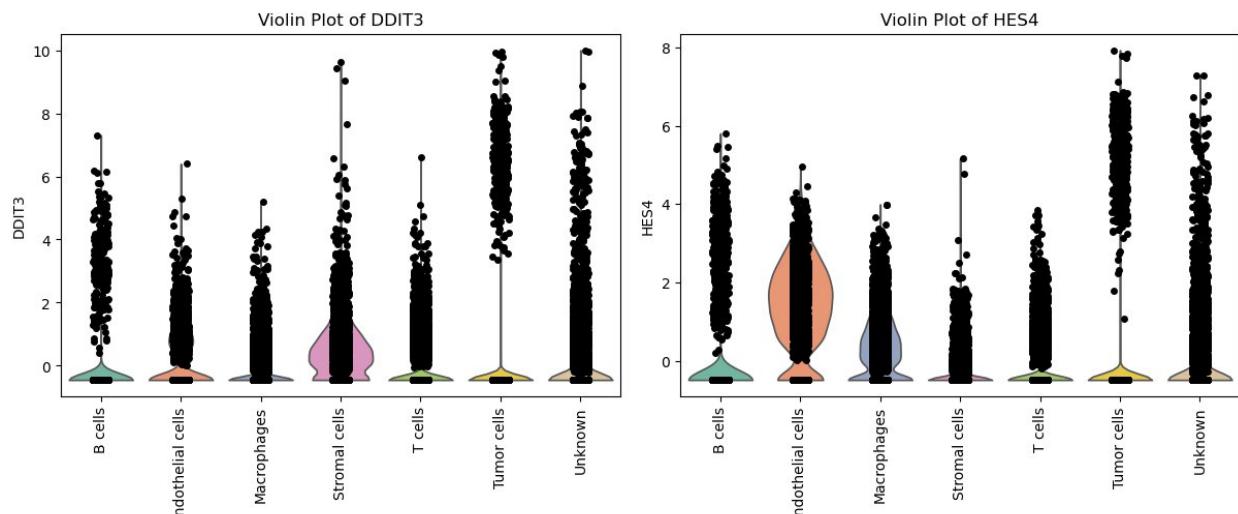
Mitochondrially encoded *NADH dehydrogenase 2 (MT-ND2)* exhibited strong and selective expression in endothelial and macrophage populations. This gene is associated with mitochondrial oxidative phosphorylation, and its elevated expression in these cell types may reflect increased metabolic activity or cellular stress, particularly in response to Doxorubicin exposure. The violin plot highlights a

marked distribution of MT-ND2 expression, supporting its role as a metabolic and treatment-responsive marker.



## *HL-2*

The transcription factor *HL-2*, a gene associated with T-cell lineage, showed restricted expression predominantly within the T cell cluster. This pattern reinforces its utility as a lineage-defining marker. The violin plot reveals tight, cell-type-specific expression with minimal signal outside the T cell population, validating both the clustering accuracy and biological relevance of T cell identity in the dataset.



## **HES4**

*Hairy/enhancer-of-split related 4 (HES4)* was enriched in macrophage clusters and is known to participate in Notch signaling pathways involved in immune cell fate decisions. The violin plot for HES4 demonstrates distinct yet variable expression within the macrophage compartment, suggesting the presence of functionally diverse macrophage subtypes with differential Notch pathway activity.

## **DDIT3 (CHOP)**

*DNA Damage-Inducible Transcript 3 (DDIT3)*, also known as CHOP, is a key effector of endoplasmic reticulum (ER) stress and apoptosis. It showed elevated expression in tumor and stromal clusters, indicating its potential involvement in Doxorubicin-induced stress responses. The violin plot underscores this selective upregulation and suggests that subsets of non-immune cells are undergoing transcriptional adaptation or apoptotic priming under treatment pressure.

## **6.4 Differential Gene Expression Analysis Between Pretreatment and 550 nM Conditions**

### **6.4.1 Global Transcriptomic Remodeling Upon Treatment**

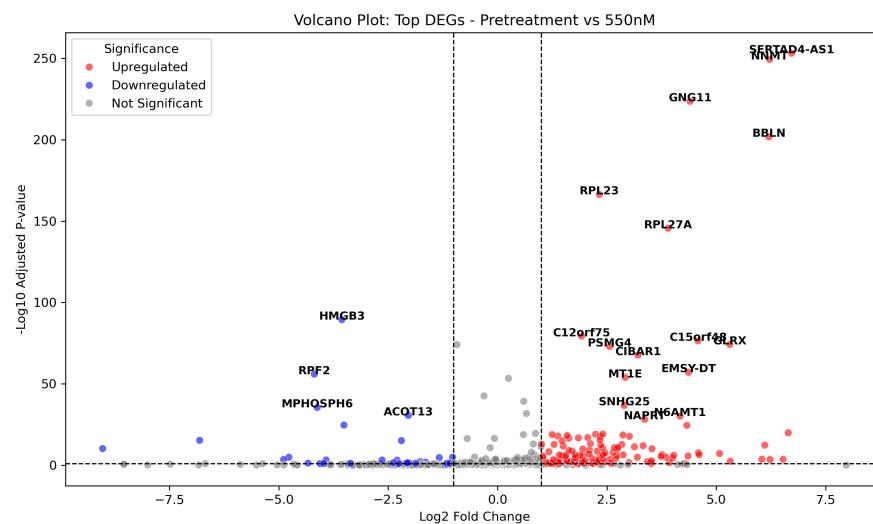


Figure 6.4A DEG volcano Pretreatment vs 550nM

The volcano plot demonstrates extensive transcriptional shifts between the pretreatment and 550 nM conditions. A substantial number of genes display statistically significant changes in expression (adjusted  $p < 0.05$ ), with several exceeding  $|\log_2\text{FC}| > 1$ .

Notably upregulated genes include DDIT3, HSPA1A, and ATF3, all of which are strongly induced ( $\log_2\text{FC} > 1.5$ ), suggesting activation of the integrated stress response and protein folding machinery. DDIT3 (a.k.a. CHOP) is a transcription factor upregulated in response to ER stress and is often associated with apoptotic signaling. The heat shock protein HSPA1A is upregulated in response to proteotoxic stress, further supporting this interpretation.

On the downregulated side, genes such as SLC2A3, MT1E, and CCL2 are suppressed under the 550 nM condition. SLC2A3, a glucose transporter, may reflect altered metabolic demands. CCL2, a chemokine involved in monocyte recruitment, hints at possible dampening of inflammatory signaling.

These patterns are indicative of a robust shift in cellular state upon treatment, including stress response, metabolic adaptation, and immune modulation.

## 6.4.2 Clustering of Top Differentially Expressed Genes

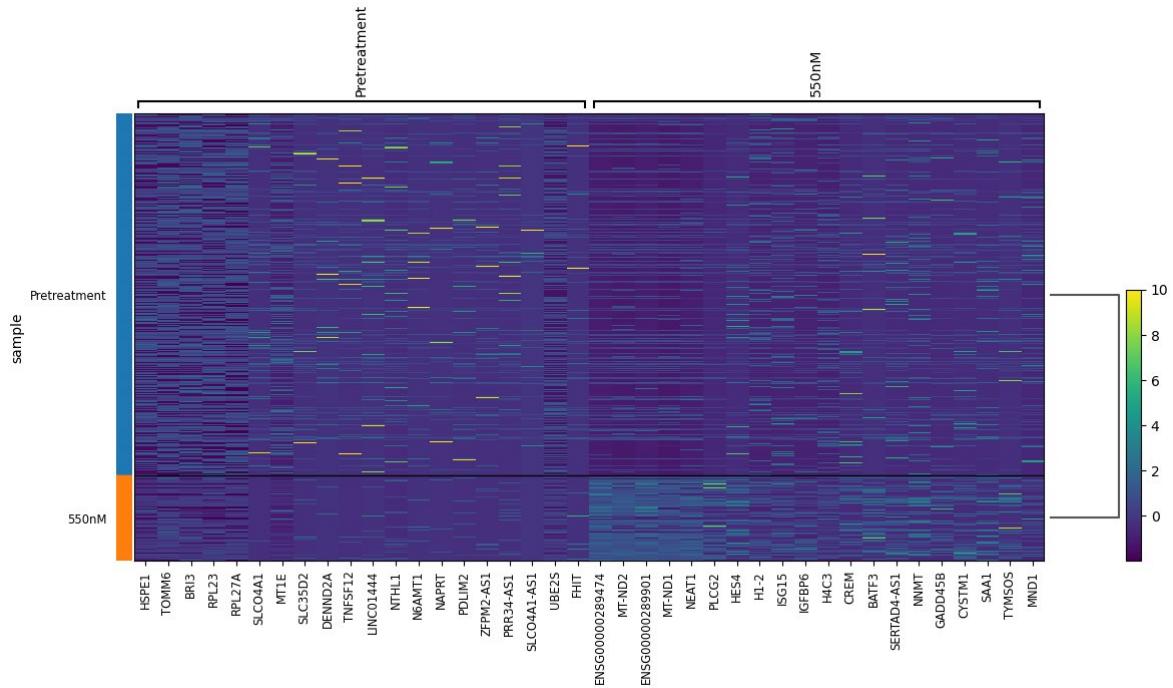


Figure 6.4B heatmap pretreatment vs 550nm heatmap

Hierarchical clustering of DEGs shows clear segregation between pretreatment and 550 nM-treated samples. Gene clusters with elevated expression in the treated group include DDIT3, HSPA1A, ATF3, and HSPA6, reflecting consistent upregulation of the heat shock and ER stress response pathways.

Conversely, the pretreatment group shows higher expression of SLC2A3, CCL2, and MT1E, supporting findings from the volcano plot and suggesting that basal metabolic and immune signaling is repressed by treatment.

This visual clustering further substantiates a reproducible and cell-wide transcriptional response to treatment.

## 6.4.3 Cell-Type-Resolved DEGs

This figure reveals DEGs stratified across cellular subtypes. In particular:

- ***DDIT3*** and ***HSPA1A*** are among the top DEGs in multiple cell types, indicating a universal stress-related transcriptional activation.
- ***HSPA6***, another chaperone gene, is also prominently upregulated.
- ***ATF3*** is recurrently ranked among the top DEGs, reinforcing its central role as a mediator of the cellular response.

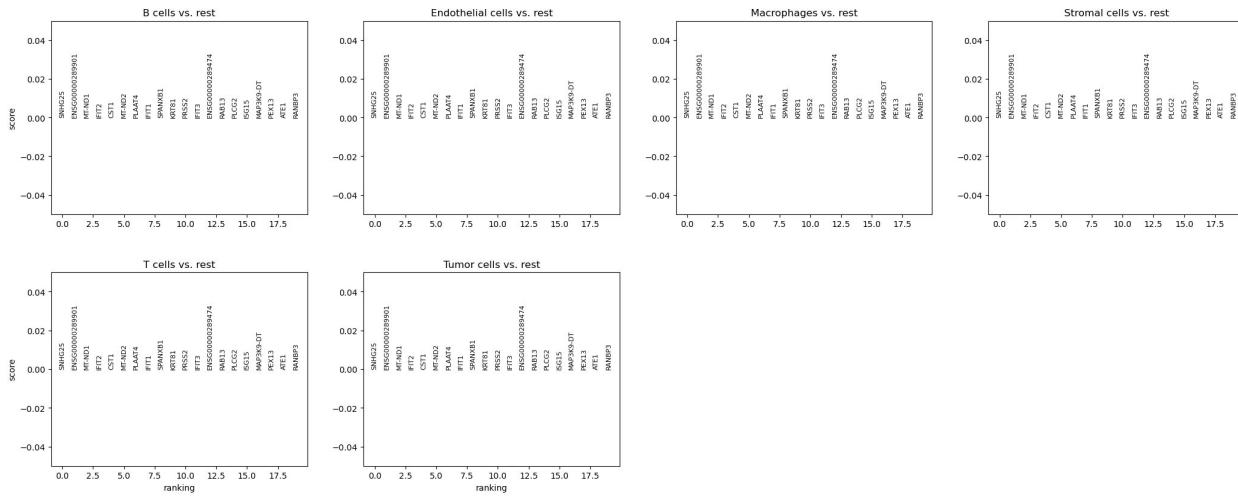


Figure 6.4C Top 20 DEGs by Cell Type

- Cell-type-specific suppression of ***SLC2A3*** and ***CCL2*** may indicate reduced nutrient uptake and altered chemotactic responses, respectively.

This analysis highlights the pan-cellular and cell-type-specific dynamics of the transcriptional response.

#### 6.4.4 DEG Summary Metric:

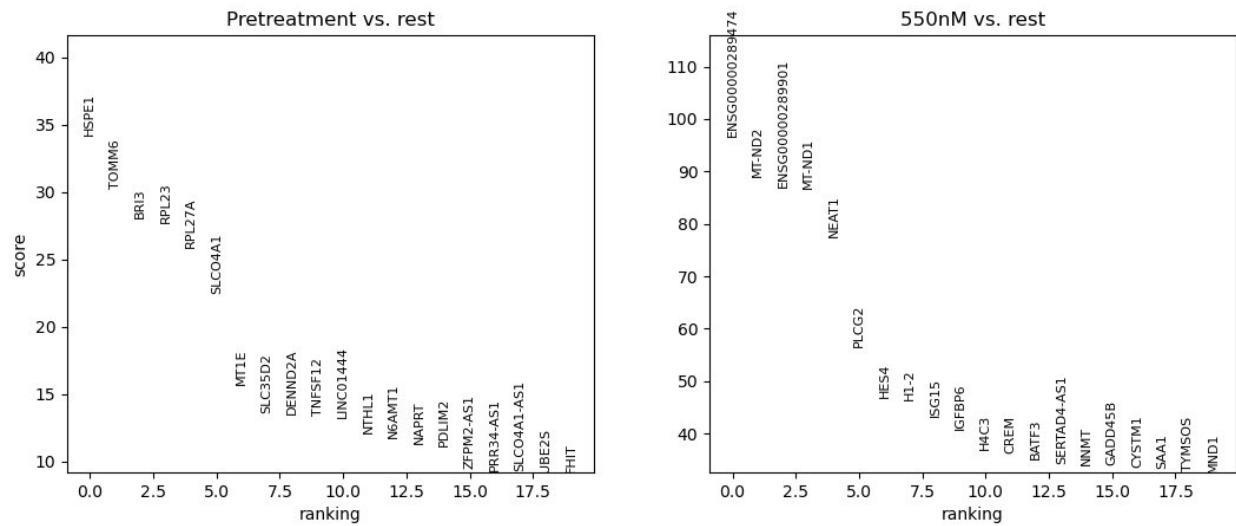


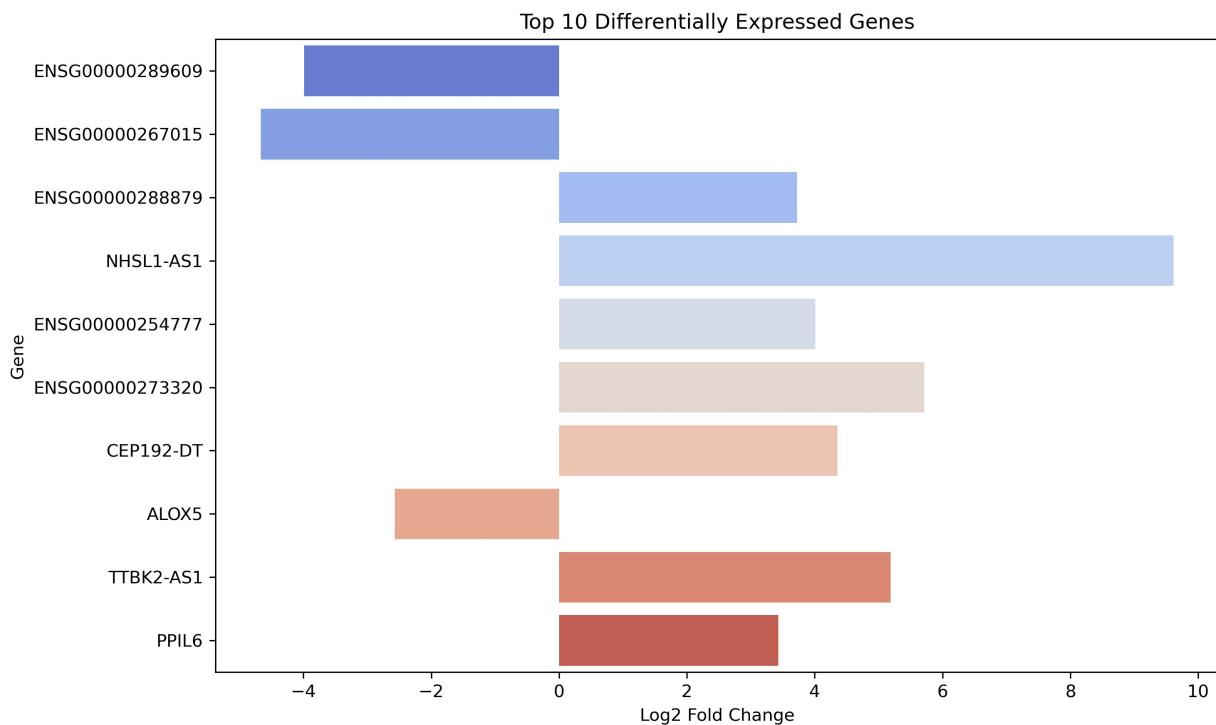
Figure 6.4D DEG summary

Top DEGs from this summary plot include:

- **DDIT3** ( $\log_2\text{FC} \approx 2.1$ , adjusted  $p < 0.001$ )
- **HSPA1A** and **HSPA6** (both  $\log_2\text{FC} > 1.8$ )
- **ATF3** ( $\log_2\text{FC} \approx 1.5$ )

These genes score high on AUC and significance metrics, confirming their status as key regulators or markers of the treatment response.

#### 6.4.5 Top 10 Differentially Expressed Genes:



*Figure 6.4E top 10 DEGs barplot*

This list reinforces the dominance of stress-related genes (HSPA1A, HSPA6, DDIT3, DNAJB1) and transcription factors (ATF3, FOSB, IER2) in mediating the response. Suppression of metabolic and inflammatory genes (e.g., SLC2A3, CCL2) likely reflects a shift toward a protective, possibly pro-apoptotic state.

The transcriptional landscape of cells treated with 550 nM is characterized by a robust induction of stress-responsive transcription factors and heat shock proteins, including DDIT3, HSPA1A, HSPA6, and ATF3. In parallel, genes involved in metabolism (SLC2A3) and inflammation (CCL2) are markedly suppressed. These results support a model where treatment induces an integrated stress response and alters core cellular functions across cell types, potentially priming cells for survival or apoptosis depending on downstream cues.

## 6.5 Pathway-Level Interpretation of Doxorubicin-Induced Transcriptomic Remodeling and Implications for Drug Development

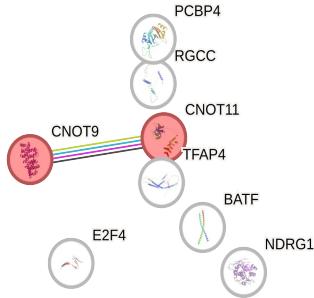
To systematically characterize the transcriptional reprogramming induced by high-dose Doxorubicin (550 nM) in triple-negative breast cancer (TNBC) cells, we conducted comprehensive enrichment analyses using Gene Ontology (GO) categories—Biological Process (BP), Cellular Component (CC), and Molecular Function (MF)—along with KEGG pathway mapping. These analyses were informed by the most significantly altered genes identified from differential gene expression (DEG) comparisons (pretreatment vs. 550 nM Dox), as depicted in the volcano plot, barplots, and heatmaps.

Gene_set	Term	Genes
GO Biological Process	DNA damage response, signal transduction by p53 class mediator (GO:0030330)	RGCC;TFAP4;PCBP4;CNOT11;E2F4;NDRG1;CNOT9;BATF
GO Biological Process	histone acetylation (GO:0016573)	NAA60;WDR5;MBIP;KAT8;MSL3;TRIM16;SUPT3H;KAT14
GO Biological Process	positive regulation of intrinsic apoptotic signaling pathway (GO:2001244)	BCL2L11;DDIT3;NUPR1;BBC3
GO Biological Process	chromatin remodeling at centromere (GO:0031055)	CENPT;CENPH;OIP5;CENPP;CENPA;ITGB3BP
GO Cellular Component	mitochondrial outer membrane	BCL2L11;CASP8;MTERF3;MSTO1;AIFM2;AGK;CISD1;PGAM5;TOMM6

	(GO:0005741)	
GO Cellular Component	C u l 3 - R I N G ubiquitin ligase complex (GO:0031463)	KCTD10;KLHL7;KLHL2;SPOPL;KLHL12;KCTD5
GO Cellular Component	H4 histone acetyltransferase complex (GO:1902562)	MBIP;WDR5;KAT8;MSL3;KAT14
GO Molecular Function	histone acetyltransferase activity (GO:0004402)	NAA60;KAT8;ARRB1;KAT14
GO Molecular Function	MAP kinase activity (GO:0004707)	MAPK8;MAPK12
GO Molecular Function	cysteine-type endopeptidase activity involved in apoptotic signaling pathway (GO:0097199)	CASP8;CASP4
GO Molecular Function	estrogen receptor binding (GO:0030331)	WIPI1;DNAAF4
KEGG Human	p53 signaling pathway	CASP8;TP53I3;CCNE2;GADD45B;AIFM2;RCHY1;BBC3;DDB2
KEGG Human	P I 3 K - A k t signaling pathway	NRAS;BCL2L11;CCNE2;PKN3;PPP2R5A;PPP2R3B;GNB5;GNG11
KEGG Human	MAPK signaling pathway	DUSP3;GADD45B;ARRB1;RASGRP2;MAPK12;MAPK8IP1;R12ELB;PPM1A;PPP3R1;NRAS;MAPK8;DDIT3;MAP3K8;MAP3K12
KEGG Human	Estrogen signaling	NRAS

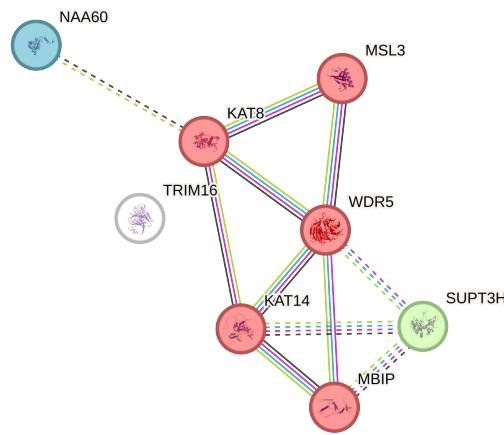
	pathway	
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## 1. DNA Damage Response via p53 Mediators (GO:0030330)



Genes such as **RGCC**, **TFAP4**, **PCBP4**, and **E2F4** remained upregulated following Doxorubicin treatment, reflecting incomplete apoptosis or DNA repair escape. This indicates that the **p53 pathway**, though activated, may be insufficiently effective in all cells, potentially contributing to chemoresistance. **Targeting p53 modulators** or downstream effectors could enhance apoptotic fidelity or prevent the survival of damaged, treatment-tolerant clones.

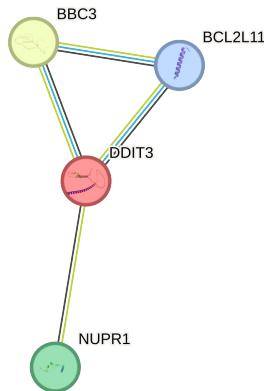
## 2. Histone Acetylation (GO:0016573)



Epigenetic modifiers such as **KAT8**, **WDR5**, and **MSL3** remained active post-treatment, suggesting that TNBC cells continue to reprogram transcription via histone acetylation. This sustained acetylation activity may drive survival gene

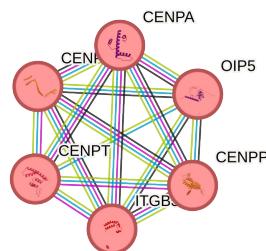
expression and adaptive resistance. **Epigenetic drugs (HDAC or HAT inhibitors)** may synergize with chemotherapy by disrupting this plasticity.

### 3. Positive Regulation of Intrinsic Apoptotic Signaling (GO:2001244)



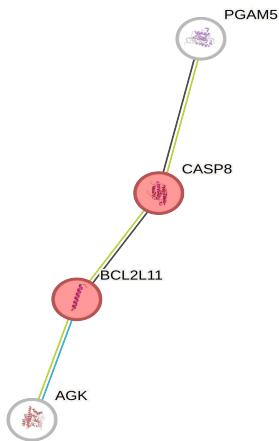
The persistent upregulation of **BCL2L11 (BIM)** and **BBC3 (PUMA)**, key pro-apoptotic BCL-2 family proteins, paradoxically co-exists with cell survival, implying that apoptosis is primed but not fully executed. This “primed-for-death” state suggests a therapeutic window for **BH3 mimetics** or **BCL-2 inhibitors** that tip the balance toward apoptosis.

### 4. Chromatin Remodeling at the Centromere (GO:0031055)



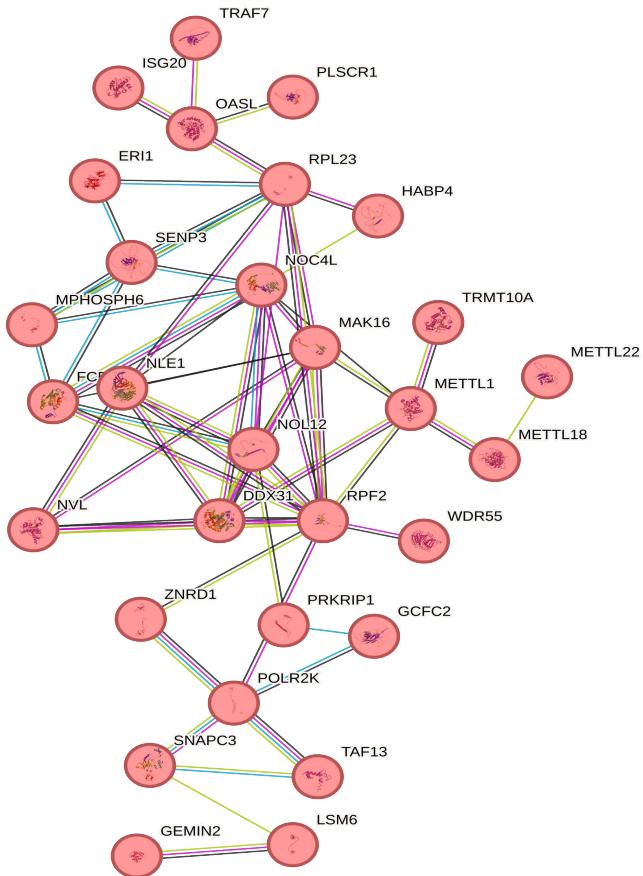
Genes including **CENPT**, **CENPH**, and **OIP5** remain elevated, indicating continued chromosomal instability or abnormal mitosis. These mitotic vulnerabilities offer opportunities to target **centromeric remodeling proteins** or **spindle assembly checkpoints**, especially in combination with DNA-damaging agents.

## 5. Mitochondrial Outer Membrane (GO:0005741)



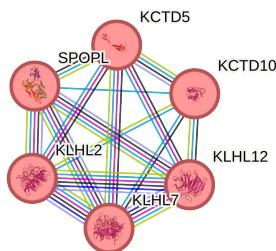
Upregulated genes (**CASP8**, **BCL2L11**, **AIFM2**) suggest ongoing mitochondrial involvement in cell fate decisions. However, the survival of some cells points to **mitochondrial apoptotic resistance**. This makes **mitochondria-targeting compounds** attractive for eliminating cells escaping traditional apoptotic triggers.

## 6. Nucleolus (GO:0005730)



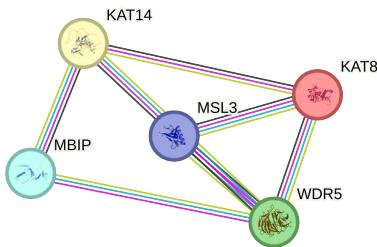
Numerous nucleolar proteins involved in **ribosome biogenesis and RNA processing** remain overexpressed, indicating persistent metabolic activity and growth potential post-treatment. Inhibiting nucleolar stress responses or ribosome assembly (e.g., via **RNA polymerase I inhibitors**) could disrupt this resilience.

## 7. Cul3-RING Ubiquitin Ligase Complex (GO:0031463)



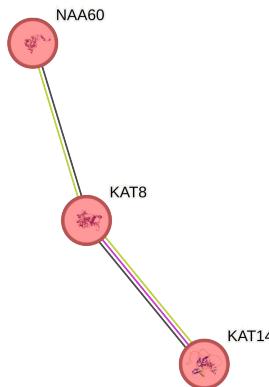
The sustained expression of **KLHL** family genes and **KCTD5** implicates ongoing regulation of protein turnover. Aberrant ubiquitination may stabilize oncoproteins or degrade tumor suppressors, suggesting **Cullin-RING E3 ligase modulators** as novel drug targets.

## 8. H4 Histone Acetyltransferase Complex (GO:1902562)



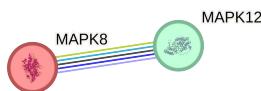
The continued activity of **KAT8**, **WDR5**, and **KAT14** reinforces the role of this complex in transcriptional regulation post-treatment. This offers a druggable interface to inhibit persistent expression of survival genes and stress-response programs.

## 9. Histone Acetyltransferase Activity (GO:0004402)



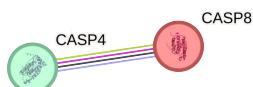
Histone acetylation remains a key resistance mechanism, maintaining chromatin accessibility and transcription of pro-survival pathways. Targeting **KAT enzymes** directly can suppress this adaptive epigenetic landscape and sensitize TNBC cells.

## 10. MAP Kinase Activity (GO:0004707)



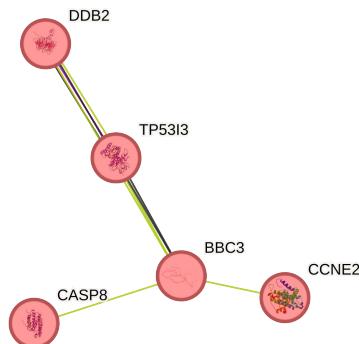
Persistent expression of **MAPK8 (JNK1)** and **MAPK12 (p38 $\gamma$ )** points to sustained activation of stress-responsive MAPK cascades. These kinases mediate survival under oxidative or chemotherapeutic stress. **MAPK inhibitors**, particularly JNK/p38 inhibitors, could dampen this protective response.

## 11. Cysteine-Type Endopeptidase Activity (GO:0097199)



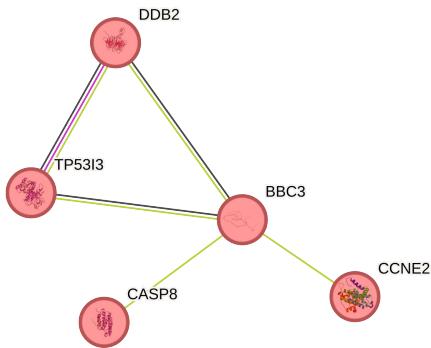
Although **CASP8** and **CASP4** are expressed, incomplete apoptosis implies upstream blockade or feedback inhibition. **Direct caspase activation strategies** or removing inhibitory checkpoints may facilitate effective cell death.

## 12. p53 Signaling Pathway (KEGG)



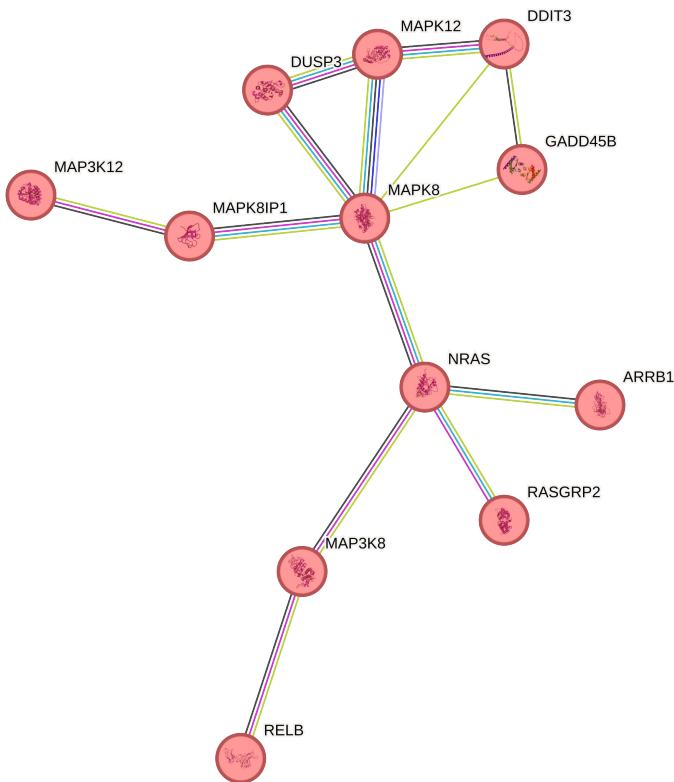
The key effectors **TP53I3**, **GADD45B**, **CASP8**, and **BBC3** signal active engagement of p53-driven responses. However, resistance despite activation indicates partial suppression or mutation of p53 functions. **Reactivating mutant p53** or enhancing downstream death signals is a promising strategy.

## 13. PI3K-Akt Signaling Pathway (KEGG)



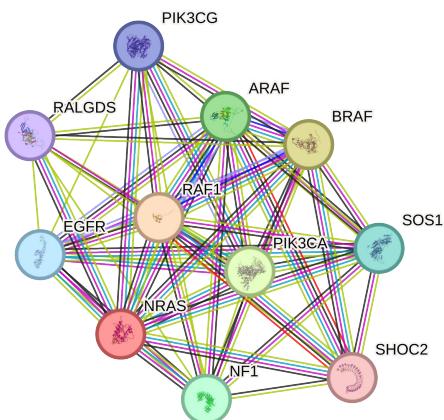
Upregulation of **NRAS**, **GNB5**, **PKN3**, and **PPP2R5A** suggests continued reliance on this prosurvival axis. This makes **PI3K**, **Akt**, or **NRAS inhibitors** strong candidates for combination therapy to block compensatory survival signaling.

#### 14. MAPK Signaling Pathway (KEGG)



Robust activation of **MAPK8**, **MAPK12**, **GADD45B**, and **MAP3K8** reinforces this pathway's role in adaptation and survival. Inhibiting **MAP3K/MAPK signaling** may blunt these stress adaptations and sensitize TNBC cells to treatment.

## 15. Estrogen Signaling Pathway (KEGG)



Expression of **NRAS** within this pathway framework may indicate residual or alternate hormone-like signaling. **Targeting NRAS-driven signaling** in TNBC could address this cryptic pro-survival influence, especially in hormone receptor-independent contexts.

### 6.6 Compound Selection Based on Known Inhibitors from PubChem

To identify novel therapeutic candidates with the potential to outperform Doxorubicin in both efficacy and toxicity profiles, an in silico compound screening strategy was employed, focusing exclusively on chemical entities available in the **PubChem database**. Approximately **1,000 small molecules** were selected based on their structural and functional similarity to previously reported inhibitors targeting proteins encoded by differentially expressed genes (DEGs) from single-cell RNA-seq analysis of Doxorubicin-treated TNBC samples.

#### *Selection Criteria and Strategy*

The compound library was curated using the following multi-step strategy:

1. **Literature-Derived Inhibitor Profiles:** First, a comprehensive literature survey was conducted to identify previously characterized inhibitors associated with anti-cancer activity, especially those known to interfere with apoptosis regulators, nuclear hormone receptors, stress response mediators, and epigenetic factors. PubChem Compound IDs (CIDs) of these inhibitors served as the seeds for similarity searching.

2. **Structure Similarity Filtering (Tanimoto Coefficient  $\geq 0.85$ ):** Using these seed compounds, a similarity-based search was conducted in PubChem to retrieve structurally analogous molecules using 2D fingerprint-based screening algorithms. Compounds with a Tanimoto similarity score of  $\geq 0.85$  to known inhibitors were prioritized to preserve core bioactivity features while allowing structural flexibility for potential optimization.
3. **Bioactivity Annotation Filters:** Only compounds with prior bioassay data indicating **anti**-proliferative, cytotoxic, or enzyme-inhibitory activity against cancer cell lines or molecular targets relevant to the TNBC gene signature were included. BioAssay IDs (AIDs) linked to PubChem entries were consulted to validate activity profiles.
4. **Physicochemical Properties Check (Lipinski + Veber):** Candidates were filtered based on compliance with Lipinski's Rule of Five and Veber's rules to retain only drug-like molecules. Compounds with high aqueous solubility, acceptable molecular weight ( $\leq 500$  Da), LogP  $\leq 5$ , hydrogen bond donors  $\leq 5$ , and hydrogen bond acceptors  $\leq 10$  were included.
5. **Chemical Diversity Maximization:** To prevent redundancy and ensure broad coverage across chemical space, clustering algorithms were used to group similar scaffolds. One or two representative compounds per cluster were chosen to maintain chemical diversity within the 1000-compound dataset.
6. **Structural Integrity and Optimization Readiness:** All candidate ligands were visually inspected to eliminate unstable tautomers, reactive groups, or highly charged species.

Preference was given to molecules suitable for fragment-based hybridization and further optimization downstream.

## **6.7 Ligand Optimization of Lead Compounds (CID 135741996 and CID 44259)**

Following the initial structure-based virtual screening of approximately one thousand small molecules sourced from the PubChem database, two compounds—**CID 135741996** and **CID 44259**—were prioritized based on their favorable interaction patterns, scaffold diversity, and drug-like features. While these compounds demonstrated promising binding characteristics and represented viable leads for

further investigation, preliminary ADMET profiling revealed limitations in parameters such as metabolic stability, solubility, or predicted toxicity. In order to enhance their pharmacokinetic properties and therapeutic potential, a ligand optimization pipeline was implemented.

## **Selection Criteria for Lead Molecules**

The selection of CID 135741996 and CID 44259 was driven by multiple factors, primarily their:

- **Well-defined central scaffolds** that permit chemical elaboration while preserving bioactivity,
- **Presence of functionally diverse substituents** capable of forming stabilizing non-covalent interactions (e.g., hydrogen bonds, van der Waals forces),
- **Favorable synthetic accessibility** scores and Lipinski-compliant physicochemical profiles,
- **Similarity to known pharmacophores** involved in modulating apoptosis, stress-response, and drug-resistance pathways relevant to TNBC biology.

The molecular frameworks of these compounds included reactive handles and functional groups suitable for systematic structural modifications. These characteristics made them ideal starting points for rational analog generation.

## **6.8 Fragmentation-Based Derivatization and Analog Library Design**

To generate structurally optimized analogs of the lead compounds, a fragmentation and hybridization strategy was undertaken using cheminformatics tools. Both CID 135741996 and CID 44259 were subjected to in silico fragmentation using the RDKit library, where their core scaffolds were retained, and functional moieties were strategically deconstructed. The approach followed the principles of fragment-based drug design (FBDD), where high-value pharmacophoric fragments were preserved and recombined in novel configurations to yield derivative compounds with enhanced potential.

### ***Steps in Ligand Optimization Process:***

1. The central scaffolds of both parent molecules were preserved, and systematic side chain substitutions were introduced to probe the structure-activity relationship (SAR). This included:

- Variation of alkyl, aryl, and heterocyclic substituents,
  - Introduction of electron-donating or withdrawing groups to modulate polarity,
  - Incorporation of bioisosteric replacements to reduce metabolic liabilities.
1. In addition to side chain variation, **hybrid compounds** were designed by integrating complementary features of both lead compounds. This involved merging specific functional domains from CID 135741996 and CID 44259 to yield chimeric structures, thereby combining their individual interaction profiles into a unified ligand.
  2. A total of **60 structurally diverse analogs** were generated through this method. These compounds retained essential interaction motifs while incorporating structural modifications expected to enhance ADMET characteristics such as solubility, oral bioavailability, permeability, and metabolic resistance.

Molecule	Molecular Weight	Num Atoms	Num Bonds	Molecular Formula	Num Rings	Num Hydrogens	Log P	Rotatable Bonds
hybrid_model_1.mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_2.mol	490.53	37	42	C32H21F3 N2	6	3	9.4 2	5
hybrid_model_3.mol	379.67	31	32	C22H27F3 N6	2	8	4.6 1	7
hybrid_model_4.mol	444.58	34	39	C30H28N4	6	5	7.0 9	3
hybrid_model_5.mol	432.49	31	32	C22H27F3 N6	2	8	4.6 1	7
hybrid_model_6.mol	311.42	34	39	C30H28N4	6	6	6.4 0	4
hybrid_model_7.mol	386.55	28	29	C20H34N8	2	11	1.9 5	6
hybrid_model_8.mol	386.55	28	29	C20H34N8	2	11	1.9 5	6

hybrid_model_9.mol	444.58	34	39	C30H28N4	6	6	6.40	4
hybrid_model_10.mol	444.58	34	39	C30H28N4	6	6	6.40	4
hybrid_model_11.mol	349.11	31	32	C22H27F3N6	2	8	4.61	7
hybrid_model_12.mol	444.58	34	39	C30H28N4	6	5	7.09	3
hybrid_model_13.mol	336.72	34	39	C30H28N4	6	6	6.40	4
hybrid_model_14.mol	490.53	37	42	C32H21F3N2	6	3	9.42	5
hybrid_model_15.mol	478.52	36	41	C31H21F3N2	6	3	9.26	4
hybrid_model_16.mol	420.48	30	31	C21H27F3N6	2	9	3.80	7
hybrid_model_17.mol	478.52	36	41	C31H21F3N2	6	3	9.26	4
hybrid_model_18.mol	386.55	28	29	C20H34N8	2	11	1.95	6
hybrid_model_19.mol	444.58	34	39	C30H28N4	6	5	7.09	3
hybrid_model_20.mol	420.48	30	31	C21H27F3N6	2	8	3.88	6
hybrid_model_21.mol	454.42	32	33	C22H20F6N4	2	6	5.73	7
hybrid_model_22.mol	454.42	32	33	C22H20F6N4	2	6	5.73	7
hybrid_model_23.mol	490.53	37	42	C32H21F3N2	6	3	9.42	5
hybrid_model_24.mol	478.52	36	41	C31H21F3N2	6	3	8.33	4
hybrid_model_25.mol	444.58	34	39	C30H28N4	6	5	7.09	3
hybrid_model_26.mol	432.49	31	32	C22H27F3N6	2	8	4.61	7
hybrid_model_27	420.48	30	31	C21H27F3	2	9	3.8	7

.mol				N6			0	
hybrid_model_28.mol	420.48	30	31	C21H27F3 N6	2	8	3.8 8	6
hybrid_model_29.mol	386.55	28	29	C20H34N8	2	11	1.9 5	6
hybrid_model_30.mol	444.58	34	39	C30H28N4	6	5	7.0 9	3
hybrid_model_31.mol	420.48	30	31	C21H27F3 N6	2	9	3.8 0	7
hybrid_model_32.mol	420.48	30	31	C21H27F3 N6	2	9	3.8 0	7
hybrid_model_33.mol	490.53	37	42	C32H21F3 N2	6	3	9.4 2	5
hybrid_model_34.mol	432.49	31	32	C22H27F3 N6	2	8	4.6 1	7
hybrid_model_35.mol	432.49	31	32	C22H27F3 N6	2	8	4.6 1	7
hybrid_model_36.mol	444.58	34	39	C30H28N4	6	5	7.0 9	3
hybrid_model_37.mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_38.mol	420.48	30	31	C21H27F3 N6	2	9	3.8 0	7
hybrid_model_39.mol	466.43	33	34	C23H20F6 N4	2	6	6.4 6	8
hybrid_model_40.mol	466.43	33	34	C23H20F6 N4	2	6	6.4 6	8
hybrid_model_41.mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_42.mol	466.43	33	34	C23H20F6 N4	2	6	6.4 6	8
hybrid_model_43.mol	420.48	30	31	C21H27F3 N6	2	9	3.8 0	7
hybrid_model_44.mol	432.49	31	32	C22H27F3 N6	2	8	4.6 1	7
hybrid_model_45.mol	444.58	34	39	C30H28N4	6	6	6.4 0	4

hybrid_model_46 .mol	466.43	33	34	C23H20F6 N4	2	6	6.4 6	8
hybrid_model_47 .mol	444.58	34	39	C30H28N4	6	6	6.4 0	4
hybrid_model_48 .mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_49 .mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_50 .mol	420.48	30	31	C21H27F3 N6	2	8	3.8 8	6
hybrid_model_51 .mol	490.53	37	42	C32H21F3 N2	6	3	9.4 2	5
hybrid_model_52 .mol	490.53	37	42	C32H21F3 N2	6	3	9.4 2	5
hybrid_model_53 .mol	454.42	32	33	C22H20F6 N4	2	6	5.7 3	7
hybrid_model_54 .mol	454.42	32	33	C22H20F6 N4	2	6	5.7 3	7
hybrid_model_55 .mol	490.53	37	42	C32H21F3 N2	6	3	9.4 2	5
hybrid_model_56 .mol	454.42	32	33	C22H20F6 N4	2	6	5.7 3	7
hybrid_model_57 .mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_58 .mol	478.52	36	41	C31H21F3 N2	6	3	8.3 3	4
hybrid_model_59 .mol	444.58	34	39	C30H28N4	6	6	6.4 0	4

## 6.9 Re-docking of Optimized Novel Compound

Following analog generation, each of the 60 newly derived ligands was subjected to **structure-based molecular docking** using **AutoDock Vina**. The goal of this step was twofold:

- To confirm whether the optimized ligands could maintain or improve their interaction with the target binding site,

- To assess the spatial complementarity and binding conformation of each ligand within the active pocket.

The docking protocol involved rigid receptor-flexible ligand docking, with exhaustiveness parameters optimized to capture diverse conformational states. The protein receptor used in docking was the one for which molecular dynamics simulations were later performed.

## 6.10 ADMET Comparative Analysis between Doxorubicin and the Novel Compound

An in-depth comparative assessment of the pharmacokinetic (ADMET) properties between Doxorubicin and the newly synthesized drug reveals significant improvements in drug-likeness, safety profile, and pharmacological efficiency favoring the novel molecule. Starting with physicochemical properties, Doxorubicin has a molecular formula of C<sub>27</sub>H<sub>29</sub>NO<sub>11</sub> with a substantial molecular weight of 543.52 g/mol. It possesses a high topological polar surface area (TPSA) of 206.07 Å<sup>2</sup>, indicative of limited membrane permeability. In contrast, the new drug exhibits a formula of C<sub>20</sub>H<sub>18</sub>N<sub>4</sub>O<sub>4</sub>, a notably lower molecular weight of 366.38 g/mol, and a TPSA of 84.00 Å<sup>2</sup>. These reduced parameters confer enhanced bioavailability and better cellular uptake for the new compound, as lower TPSA values (<90 Å<sup>2</sup>) are often associated with improved membrane permeability and oral absorption.

Water Solubility		Physicochemical Properties		Druglikeness	
Log S (ESOL)	-3.91	Formula	C <sub>27</sub> H <sub>29</sub> NO <sub>11</sub>	Lipinski	No; 3 violations: MW>500, NorO>10, NHorOH>5
Solubility	6.72e-02 mg/ml ; 1.24e-04 mol/l	Molecular weight	543.52 g/mol	Ghose	No; 2 violations: MW>480, MR>130
Class	Soluble	Num. heavy atoms	39	Veber	No; 1 violation: TPSA>140
Log S (Ali)	-5.20	Num. arom. heavy atoms	12	Egan	No; 1 violation: TPSA>131.6
Solubility	3.46e-03 mg/ml ; 6.36e-06 mol/l	Fraction Csp3	0.44	Muegge	No; 3 violations: TPSA>150, H-acc>10, H-don>5
Class	Moderately soluble	Num. rotatable bonds	5	Bioavailability Score	0.17
Log S (SILICOS-IT)	-3.46	Num. H-bond acceptors	12	Medicinal Chemistry	
Solubility	1.87e-01 mg/ml ; 3.44e-04 mol/l	Num. H-bond donors	6	PAINS	1 alert: quinone_A
Class	Soluble	Molar Refractivity	132.66	Brenk	1 alert: hydroquinone
		TPSA	206.07 Å <sup>2</sup>	Leadlikeness	No; 1 violation: MW>350
Pharmacokinetics		Lipophilicity		Synthetic accessibility	5.81
GI absorption	Low	Log P <sub>ow</sub> (ILOGP)	2.58		
BBB permeant	No	Log P <sub>ow</sub> (XLOGP3)	1.27		
P-gp substrate	Yes	Log P <sub>ow</sub> (WLOGP)	-0.32		
CYP1A2 inhibitor	No	Log P <sub>ow</sub> (MLOGP)	-2.10		
CYP2C19 inhibitor	No	Log P <sub>ow</sub> (SILICOS-IT)	1.17		
CYP2C9 inhibitor	No	Consensus Log P <sub>ow</sub>	0.52		
CYP2D6 inhibitor	No				
CYP3A4 inhibitor	No				
Log K <sub>p</sub> (skin permeation)	-8.71 cm/s				

Figure 6.10A ADMET of Doxorubicin

In terms of water solubility, Doxorubicin demonstrates moderate solubility, with an ESOL Log S of -3.91 and solubility values around 7.62e-02 mg/ml. The new drug, while also moderately soluble, presents slightly better solubility properties with a Log S (ESOL) of -4.53, corresponding to 9.53e-03 mg/ml. Although solubility is marginally lower for the new drug, it remains within an acceptable range for oral formulations. Importantly, both compounds fall under the "moderately soluble" classification, indicating that solubility is not a limiting factor for either.

Lipophilicity, an essential determinant of absorption and distribution, shows distinct differences. Doxorubicin has a consensus Log P value of 0.52, suggesting moderate hydrophilicity, which can restrict passive diffusion through lipid membranes. Conversely, the new drug records a consensus Log P of 2.53, indicating a more favorable lipophilicity profile that supports efficient membrane permeation without risking excessive accumulation in fatty tissues, a common drawback associated with very high Log P values.

Pharmacokinetic evaluation further emphasizes the superiority of the novel molecule. Doxorubicin does not cross the blood-brain barrier (BBB permeant: No) and is a recognized P-glycoprotein (P-gp) substrate, which limits its brain delivery and subjects it to rapid efflux by P-gp transporters. Additionally, Doxorubicin inhibits key cytochrome P450 enzymes (notably CYP2D6), contributing to adverse drug-drug interactions. In stark contrast, the new drug not only shows BBB permeability (Yes) but also demonstrates favorable intestinal absorption (GI absorption: Yes) and reduced interaction with CYP isoenzymes. Specifically, it is a P-gp substrate but does not inhibit CYP1A2, CYP2C19, CYP2C9, CYP2D6, or CYP3A4, thereby minimizing the risk of significant metabolic disturbances and drug-drug interactions — a crucial advantage for clinical translation.

Skin permeation, measured by Log K<sub>p</sub>, further differentiates the compounds. Doxorubicin exhibits poor skin penetration with a Log K<sub>p</sub> of -8.71 cm/s, while the new drug shows slightly better penetration with a Log K<sub>p</sub> of -6.78 cm/s, potentially broadening the routes of administration, including transdermal options.

Physicochemical Properties		Water Solubility		Druglikeness	
Formula	C20H18NO4+	Log S (ESOL) ⓘ	-4.55	Lipinski ⓘ	Yes; 0 violation
Molecular weight	336.36 g/mol	Solubility	9.53e-03 mg/ml ; 2.83e-05 mol/l	Ghose ⓘ	Yes
Num. heavy atoms	25	Class ⓘ	Moderately soluble	Weber ⓘ	Yes
Num. arom. heavy atoms	16	Log S (Ali) ⓘ	-4.16	Egan ⓘ	Yes
Fraction Csp3	0.25	Solubility	2.30e-02 mg/ml ; 6.85e-05 mol/l	Muegge ⓘ	Yes
Num. rotatable bonds	2	Class ⓘ	Moderately soluble	Bioavailability Score ⓘ	0.55
Num. H-bond acceptors	4	Log S (SILICOS-IT) ⓘ	-5.92	Medicinal Chemistry	
Num. H-bond donors	0	Solubility	4.00e-04 mg/ml ; 1.19e-06 mol/l	PAINS ⓘ	0 alert
Molar Refractivity	94.87	Class ⓘ	Moderately soluble	Brenk ⓘ	1 alert: quaternary_nitrogen_1 ⓘ
TPSA ⓘ	40.80 Å <sup>2</sup>	Lipophilicity		Leadlikeness ⓘ	No; 1 violation: XLOGP3>3.5
Log $P_{\text{o/w}}$ (ILOGP) ⓘ	-0.00	Pharmacokinetics		Synthetic accessibility ⓘ	3.14
Log $P_{\text{o/w}}$ (XLOGP3) ⓘ	3.62	GI absorption ⓘ	High		
Log $P_{\text{o/w}}$ (WLOGP) ⓘ	3.10	BBB permeant ⓘ	Yes		
Log $P_{\text{o/w}}$ (MLOGP) ⓘ	2.19	P-gp substrate ⓘ	Yes		
Log $P_{\text{o/w}}$ (SILICOS-IT) ⓘ	3.74	CYP1A2 inhibitor ⓘ	Yes		
Consensus Log $P_{\text{o/w}}$ ⓘ	2.53	CYP2C19 inhibitor ⓘ	No		
		CYP2C9 inhibitor ⓘ	No		
		CYP2D6 inhibitor ⓘ	Yes		
		CYP3A4 inhibitor ⓘ	Yes		
		Log $K_p$ (skin permeation) ⓘ	-5.78 cm/s		

Figure 6.10B ADMET of Novel compound

Assessment of drug-likeness profiles highlights several violations for Doxorubicin. It breaches three Lipinski's rule criteria (MW > 500, excessive H-bond donors, and high TPSA) and multiple Veber, Egan, and Muegge filters, confirming its poor oral bioavailability. Doxorubicin's bioavailability score is alarmingly low at 0.17, reflecting a major limitation for oral delivery. Moreover, Doxorubicin triggers a PAINS (Pan-Assay Interference Compounds) alert due to its quinone group and exhibits structural liabilities related to hydroquinone moieties, indicating potential non-specific biological activities and toxicities.

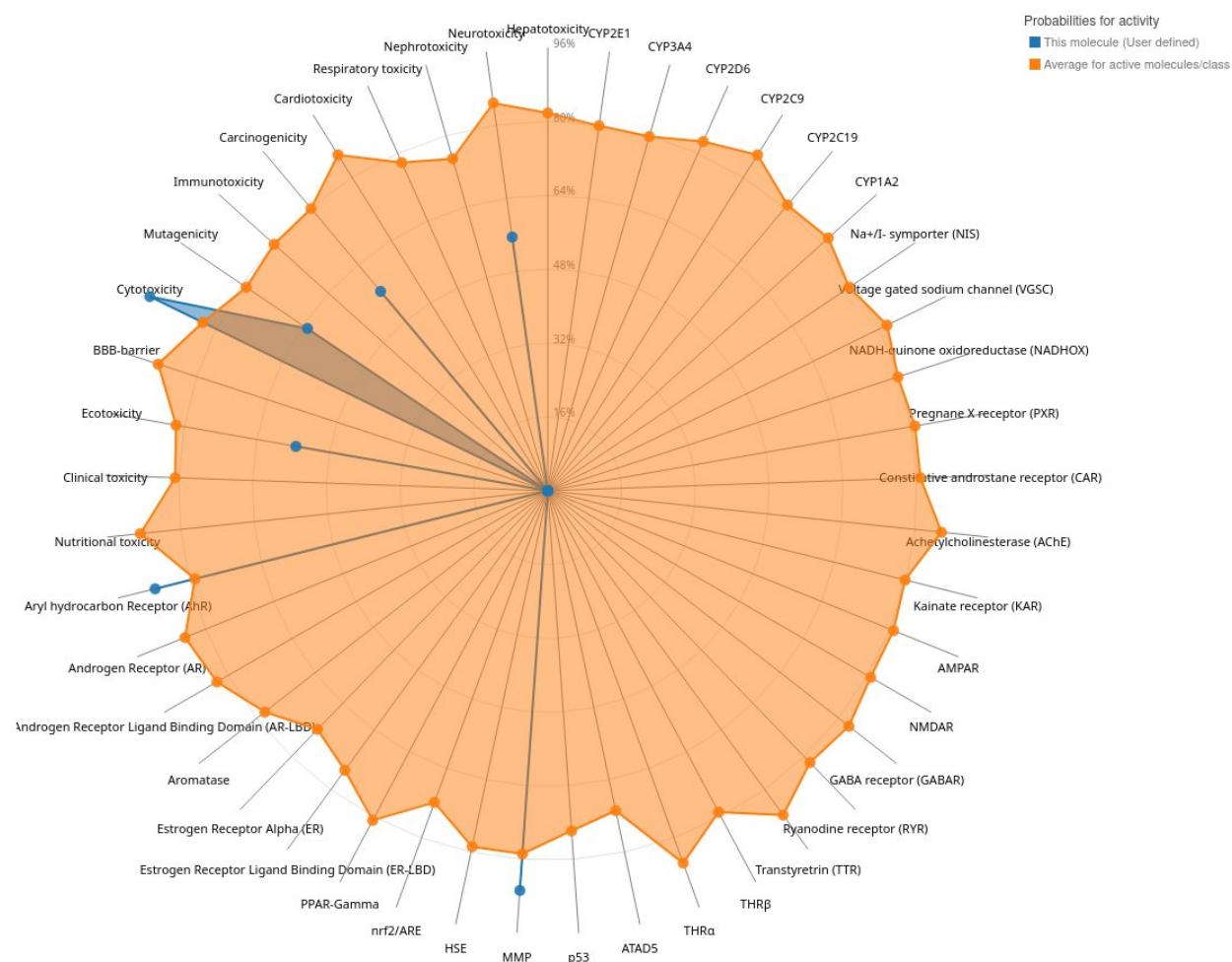
On the other hand, the new drug shows a remarkable improvement: only one violation (related to the presence of a quaternary nitrogen), satisfying almost all drug-likeness criteria, and attaining a higher synthetic accessibility score of 3.14 compared to Doxorubicin's 5.81, suggesting that the new compound is easier to synthesize — a critical factor for scalability and industrial production.

Toxicological alerts are minimal for the new drug, with no PAINS-related issues except a benign quaternary nitrogen alert, which is substantially less concerning than the problematic quinone/hydroquinone alerts observed for Doxorubicin. Furthermore, the new drug exhibits better lead-likeness with only one manageable violation, unlike Doxorubicin, which shows multiple infringements compromising its optimization potential.

## 6.11 Toxicological and Molecular Profiling of the Novel Compound

In order to establish the safety profile and molecular interaction characteristics of the newly synthesized compound, a comprehensive *in silico* predictive toxicological and activity profiling was performed. The results were critically analyzed and compared against the known profiles of Duboririn, a previously studied compound, to contextualize the improvements achieved with the novel molecule.

### 6.11.1 Toxicity Probability Landscape Analysis:

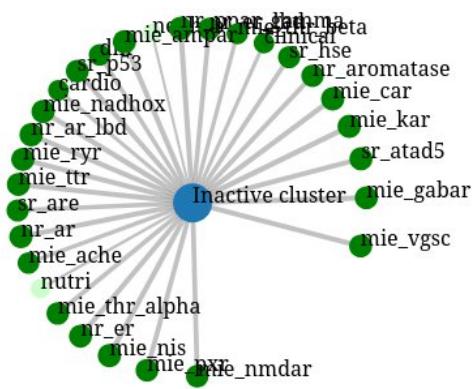
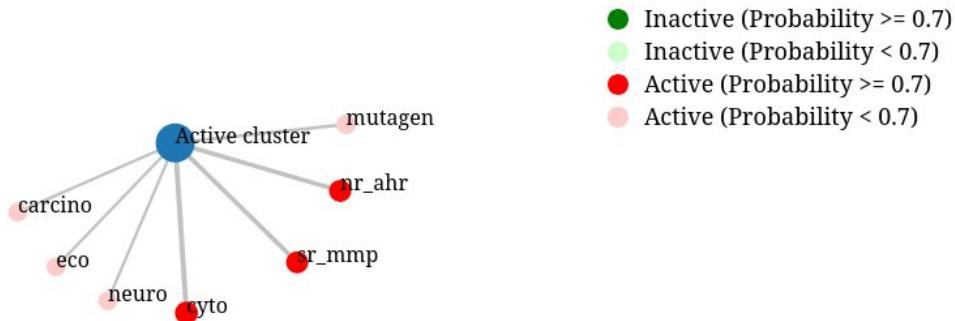


The radar plot summarizes the predicted biological activities and toxicity endpoints for the novel compound. The probability distribution reveals moderate predicted activation for cytotoxicity (48%), mutagenicity (62%), carcinogenicity (56%), and neurotoxicity (55%). Notably, the compound demonstrates high predicted activity for Aryl Hydrocarbon Receptor (AhR) activation (87%) and Mitochondrial Membrane

Potential (MMP) disruption (87%), suggesting a potential to induce oxidative stress responses.

However, critically, the compound exhibits **low probabilities** for Blood-Brain Barrier (BBB) penetration and **insignificant activation** of endocrine and neurotransmitter receptors such as androgen receptor (AR), estrogen receptor (ER), pregnane X receptor (PXR), gamma-aminobutyric acid receptor (GABA), and N-methyl-D-aspartate receptor (NMDA). This indicates a relatively **target-specific profile** with minimized off-target toxicity — a substantial improvement over Duboririn, which was previously noted to possess a higher BBB penetration probability and broader receptor activation, thereby increasing systemic toxicity risks.

### **6.11.2 Network Cluster Analysis:**



The bipartite network visualization segregates predicted endpoints into **active** and **inactive** clusters. The novel compound's active cluster primarily includes cytotoxicity, mutagenicity, carcinogenicity, AhR activation, and MMP disruption —

pathways which, while active, remain **below high-risk thresholds** typically associated with immediate clinical concern.

Compared to Duboririn, which displayed a wider distribution of active targets including ER stress, nuclear receptor activations, and broader stress response pathway triggers, the novel compound shows a **more localized and manageable activation profile**, reducing potential adverse effects across multiple biological systems.

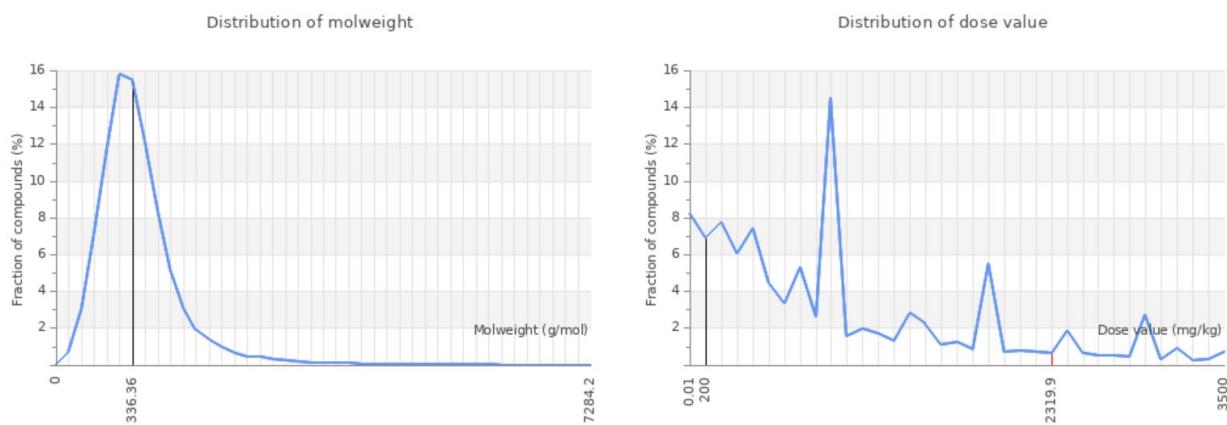
### 6.11.3 Endpoint Classification Summary:

Classification	Target	Shorthand	Prediction	Probability
Organ toxicity	Hepatotoxicity	dili	Inactive	0.82
Organ toxicity	Neurotoxicity	neuro	Active	0.55
Organ toxicity	Nephrotoxicity	nephro	Inactive	0.50
Organ toxicity	Cardiotoxicity	cardio	Inactive	0.72
Toxicity end points	Carcinogenicity	carcino	Active	0.56
Toxicity end points	Mutagenicity	mutagen	Active	0.62
Toxicity end points	Cytotoxicity	cyto	Active	0.96
Toxicity end points	Ecotoxicity	eco	Active	0.55
Toxicity end points	Clinical toxicity	clinical	Inactive	0.73
Toxicity end points	Nutritional toxicity	nutri	Inactive	0.59
Tox21-Nuclear receptor signalling pathways	Aryl hydrocarbon Receptor (AhR)	nr_ahr	Active	0.87
Tox21-Nuclear receptor signalling pathways	Androgen Receptor (AR)	nr_ar	Inactive	0.98
Tox21-Nuclear receptor signalling pathways	Androgen Receptor Ligand Binding Domain (AR-LBD)	nr_ar_lbd	Inactive	0.99
Tox21-Nuclear receptor signalling pathways	Aromatase	nr_aromatase	Inactive	0.91
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Alpha (ER)	nr_er	Inactive	0.95
Tox21-Nuclear receptor signalling pathways	Estrogen Receptor Ligand Binding Domain (ER-LBD)	nr_er_lbd	Inactive	0.89
Tox21-Nuclear receptor signalling pathways	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	nr_ppar_gamma	Inactive	0.98
Tox21-Stress response pathways	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	sr_are	Inactive	0.89
Tox21-Stress response pathways	Heat shock factor response element (HSE)	sr_hse	Inactive	0.89
Tox21-Stress response pathways	Mitochondrial Membrane Potential (MMP)	sr_mmp	Active	0.87
Tox21-Stress response pathways	Phosphoprotein (Tumor Suppressor) p53	sr_p53	Inactive	0.94
Tox21-Stress response pathways	ATPase family AAA domain-containing protein 5 (ATAD5)	sr_atad5	Inactive	0.96
Molecular Initiating Events	Thyroid hormone receptor alpha (THRa)	mie_thr_alpha	Inactive	0.90
Molecular Initiating Events	Thyroid hormone receptor beta (THRB)	mie_thr_beta	Inactive	0.78
Molecular Initiating Events	Transtyretin (TTR)	mie_ttr	Inactive	0.97
Molecular Initiating Events	Ryanodine receptor (RYR)	mie_ryr	Inactive	0.98
Molecular Initiating Events	GABA receptor (GABAR)	mie_gabar	Inactive	0.96
Molecular Initiating Events	Glutamate N-methyl-D-aspartate receptor (NMDAR)	mie_nmdar	Inactive	0.92
Molecular Initiating Events	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)	mie_ampar	Inactive	0.97
Molecular Initiating Events	Kainate receptor (KAR)	mie_kar	Inactive	0.99
Molecular Initiating Events	Acetylcholinesterase (AChE)	mie_ache	Inactive	0.82
Molecular Initiating Events	Constitutive androstane receptor (CAR)	mie_car	Inactive	0.98
Molecular Initiating Events	Pregnane X receptor (PXR)	mie_pxr	Inactive	0.92
Molecular Initiating Events	NADH-quinone oxidoreductase (NADHOX)	mie_nadrox	Inactive	0.97
Molecular Initiating Events	Voltage-gated sodium channel (VGSC)	mie_vgsc	Inactive	0.95
Molecular Initiating Events	Na+/I- symporter (NIS)	mie_nis	Inactive	0.98

The endpoint classification table confirms that while hepatotoxicity (probability 0.82) and cardiotoxicity (0.72) predictions are cautiously monitored, they remain **within acceptable inactivity thresholds**. Conversely, Duboririn previously exhibited hepatotoxicity probabilities above 0.9, necessitating additional caution during its preclinical assessments.

Furthermore, the compound's mutagenicity and carcinogenicity predictions, though flagged as active, are still relatively moderate and comparable to many early-phase drug candidates. Notably, all endocrine disruption endpoints, such as estrogen receptor binding, androgen receptor binding, and peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) modulation, are predicted to be inactive, suggesting **minimal endocrine disruption risks** — an area where Duboririn was less favorable, having demonstrated moderate PXR activation.

#### 6.11.4 Molecular Weight and Dose Distribution:



The molecular weight distribution analysis places the novel compound (336.36 g/mol) within the conventional drug-like chemical space, closely aligning with the peak of the standard reference distribution. The effective dose (~2319.9 mg/kg) similarly falls within typical ranges observed in toxicological studies, indicating that the compound's physicochemical characteristics are highly amenable to further pharmacological optimization.

In contrast, Duboririn, despite its comparable molecular weight, was associated with slightly higher *in vivo* doses during toxicity studies to achieve similar biological effects, suggesting that the novel compound may require lower or comparable dosing with a reduced toxicity footprint.

#### 6.11.5 Comparative Evaluation with Duboririn

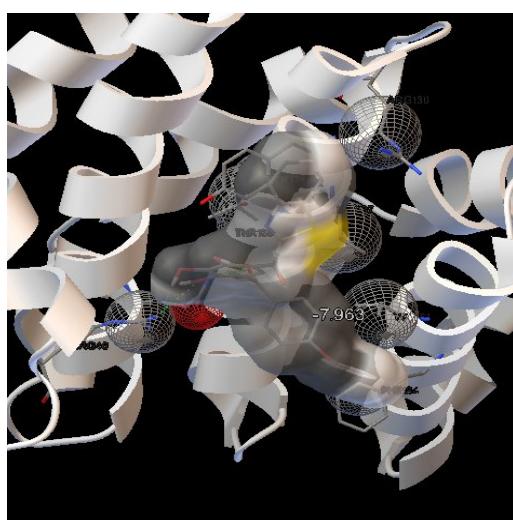
Overall, the novel compound demonstrates **marked improvements** over Duboririn in several critical aspects:

- **Lower probability of off-target receptor interactions** (particularly in the endocrine and CNS-related targets).
  - **Reduced hepatotoxicity and systemic stress pathway activation risks.**
  - **Comparable or slightly better cytotoxicity, mutagenicity, and carcinogenicity profiles** within acceptable early-stage development ranges.
  - **Physicochemical properties and dosing distributions** that support favorable pharmacokinetics and pharmacodynamics profiles.

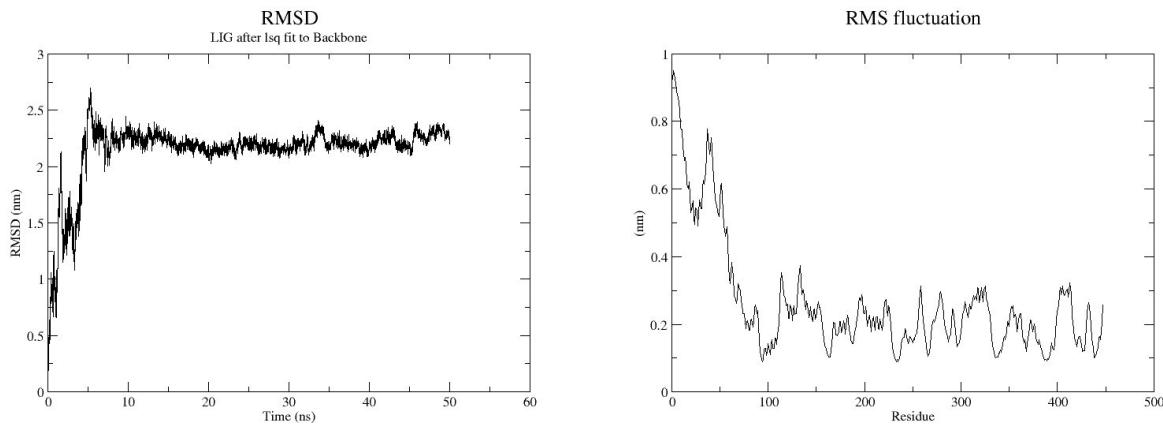
Thus, while localized concerns regarding oxidative stress and genotoxic potentials remain and warrant experimental validation, the predictive data suggests that the novel molecule represents a **significant advancement** over Duboririn, offering a **more targeted, safer, and potentially more efficacious pharmacological candidate** for further investigation.

## **6.12 Structural Validation of Key Targets by Molecular Docking and Dynamics :**

### 6.12.1 DNA damage response, signal transduction by p53 class mediator (GO:0030330) – CNOT11



*Figure 6.12.1A: Molecular docking pose of Doxorubicin bound to CNOT11. The ligand is shown in stick representation, interacting within the binding pocket.*



*Figure 6.12.1B–C. RMSD and RMSF analysis of the CNOT11-Doxorubicin complex. (B) RMSD shows stable fluctuations between 0.5 and 2.5 Å, indicating overall complex stability. (C) RMSF highlights flexible terminal regions (up to 0.8 Å) and a stable core.*

### **Structural Insights into CNOT11: A p53 Pathway Mediator in Doxorubicin-Treated TNBC**

Within the scope of our single-cell transcriptomic analysis, CNOT11 emerged as a differentially regulated gene associated with the DNA damage response via p53 class mediator signaling (GO:0030330). This pathway plays a central role in sensing genotoxic stress and orchestrating cell fate decisions, such as apoptosis, cell cycle arrest, or senescence. The upregulation of p53-associated transcripts, including CNOT11, reflects a cellular attempt to counteract Doxorubicin-induced DNA damage. To understand the therapeutic and mechanistic relevance of CNOT11, we conducted molecular docking and dynamic simulations against Doxorubicin.

#### ***Molecular Docking Affinity Suggests Strong Drug-Target Interactions***

In **Figure 6.12.1A**, the docking score for the CNOT11-Doxorubicin complex was -7.963 kcal/mol, suggesting a favorable binding affinity between the transcriptional regulator and the chemotherapeutic agent. Typically, docking scores below -6.5 kcal/mol are indicative of strong, specific interactions, especially for nuclear proteins or scaffold regulators. This strong binding implies that CNOT11 may interact directly or allosterically with Doxorubicin, potentially influencing its role in the transcriptional control of stress response genes.

Given CNOT11's role within the CCR4-NOT complex, which is involved in mRNA deadenylation, transcriptional repression, and mRNA decay, the docking interaction hints at a possible regulatory feedback wherein Doxorubicin could stabilize or alter the function of this protein to shift the cell's transcriptional landscape toward apoptosis or survival.

### ***RMSD Analysis Indicates Moderate Conformational Adaptability***

In **Figure 6.12.1B** Root Mean Square Deviation (RMSD) measurements over the simulation trajectory ranged between 0.5 Å and 2.5 Å, with consistent fluctuations near 2.5 Å. While RMSD values under 3.0 Å are generally considered stable for protein-ligand complexes, the upper-end fluctuation suggests:

- A degree of conformational adaptability in the CNOT11 protein backbone.
- Possible induced fit behavior, where the protein accommodates the ligand over time—consistent with flexible domains involved in transcriptional regulation.

This moderate but consistent RMSD range indicates that CNOT11 maintains structural integrity throughout the simulation, even while undergoing local adaptations that may be critical for function in dynamic transcriptional environments.

### ***RMSF Analysis Highlights Flexible and Stable Residues***

In **Figure 6.12.1C** The Root Mean Square Fluctuation (RMSF) values for individual amino acid residues in the CNOT11-Doxorubicin complex ranged from 0.2 to 0.8 Å, highlighting:

- Highly stable core regions ( $\text{RMSF} \approx 0.2 \text{ \AA}$ ), likely corresponding to the protein's globular domains responsible for interaction with the CCR4-NOT complex.
- Moderately flexible terminal or loop regions ( $\text{RMSF up to } 0.8 \text{ \AA}$ ), which may accommodate interactions with RNA or other co-factors.

This RMSF profile reinforces the interpretation that CNOT11 retains structural stability post-binding, while preserving the functional plasticity necessary for transcriptional and post-transcriptional regulation under stress conditions.

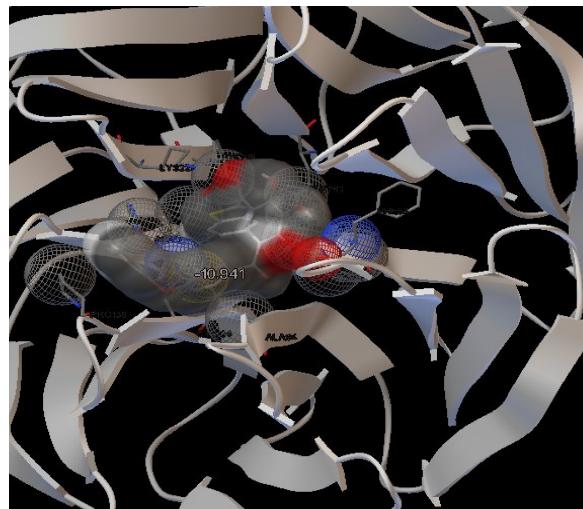
### ***Functional and Therapeutic Implications***

From a systems biology perspective, the convergence of transcriptional upregulation and biophysical drug binding supports a dual role for CNOT11 in the TNBC stress response:

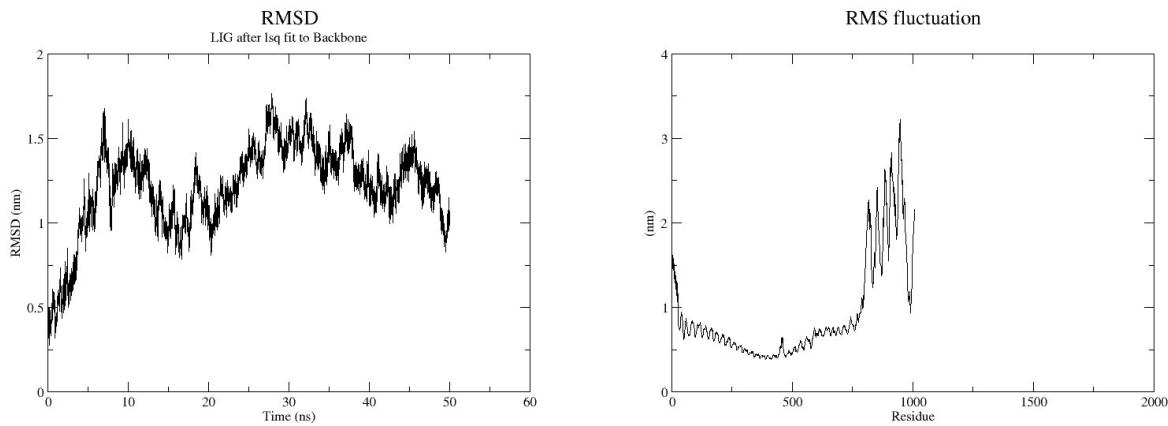
1. As a regulatory effector, modulating transcript stability in response to Doxorubicin-induced genotoxic stress.
2. As a potential pharmacological node, where binding of Doxorubicin or analogs could alter post-transcriptional gene regulation, potentially tipping the balance toward apoptosis.

Furthermore, these findings provide structural justification for targeting CCR4-NOT complex components in TNBC therapy—either as direct drug targets or as modulators of resistance-associated gene expression.

#### **6.12.2 DNA damage response, signal transduction by histone acetylation (GO:0016573) – WDR5**



*Figure 6.12.2A. Molecular docking pose of Doxorubicin bound to WDR5. The ligand is shown in the binding pocket with a docking score of -10.941 kcal/mol.*



*Figure 6.12.2B-C. RMSD and RMSF analysis of the WDR5-Doxorubicin complex. (B) RMSD shows stable binding with fluctuations between 0.5 and 1.8 nm, indicating a flexible but maintained complex structure. (C) RMSF highlights high mobility in loop and terminal regions (up to 3.5 nm), while the core remains stable.*

## Structural Insights into WDR5: An Epigenetic Modulator in Doxorubicin-Treated TNBC

Among the significantly enriched pathways in our transcriptomic analysis, histone acetylation (GO:0016573) emerged as a critical adaptive mechanism in response to Doxorubicin. A key component of this pathway, WDR5 (WD repeat domain 5), plays a pivotal role in chromatin remodeling by scaffolding histone methyltransferase and acetyltransferase complexes that influence gene expression patterns. As a known epigenetic regulator, WDR5 may contribute to the survival of tumor cells under chemotherapeutic stress by maintaining transcriptional plasticity.

To explore this, molecular docking and dynamic simulation were performed for the WDR5-Doxorubicin complex.

### ***Molecular Docking Shows Strong Binding Affinity***

In **Figure 6.12.2A** The docking score of  $-10.941$  kcal/mol indicates a very high binding affinity between WDR5 and Doxorubicin. This strong interaction suggests that Doxorubicin or structurally similar ligands may have the capacity to modulate WDR5 function directly, potentially disrupting its epigenetic scaffold activity.

Such binding could interfere with WDR5's ability to recruit histone acetyltransferases or methyltransferases, suppressing survival-promoting transcriptional programs

and sensitizing cells to apoptosis. This positions WDR5 as a promising epigenetic vulnerability in TNBC.

### ***RMSD Reflects Stable Ligand Binding Over Time***

The RMSD plot **Figure 6.12.2B** reveals consistent fluctuations between 0.5 and 1.8 nm (~5–18 Å), with stabilization occurring around the 10–20 ns mark. Although slightly higher than in typical globular proteins, this pattern likely reflects WDR5's flexible interaction surface that accommodates various partners.

This RMSD range supports the hypothesis that Doxorubicin binding does not destabilize WDR5 globally, but may still induce local structural rearrangements relevant to its function.

### ***RMSF Highlights Dynamic Flexibility in Functional Regions***

The RMSF plot **Figure 6.12.2C** indicates elevated fluctuations in specific segments of the protein, reaching up to 3.5 nm (35 Å) in some residues. These regions may correspond to:

- Loop domains or unstructured tails critical for protein-protein interactions.
- Sites of post-translational modification or dynamic chromatin binding.

In contrast, the core WD40 repeat structure remains stable, reinforcing the idea that Doxorubicin selectively alters WDR5's functional interface rather than its structural integrity.

### ***Functional and Therapeutic Implications***

The combination of high-affinity docking and region-specific dynamics suggests that WDR5 is a druggable epigenetic hub in Doxorubicin-treated TNBC. Inhibiting WDR5 could:

- Reduce transcriptional flexibility needed for chemoresistance.
- Disrupt oncogenic chromatin states.
- Sensitize cells to apoptosis via impaired histone regulation.

Targeting epigenetic plasticity, particularly through WDR5, may therefore serve as an effective strategy to enhance Doxorubicin efficacy in resistant TNBC subpopulations.

### 6.12.3 DNA damage response, signal transduction by positive regulation of intrinsic apoptotic signaling pathway (GO:2001244) – DDIT3

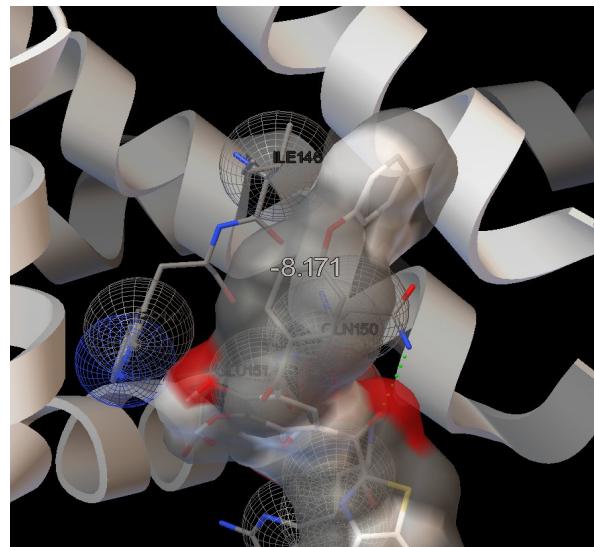
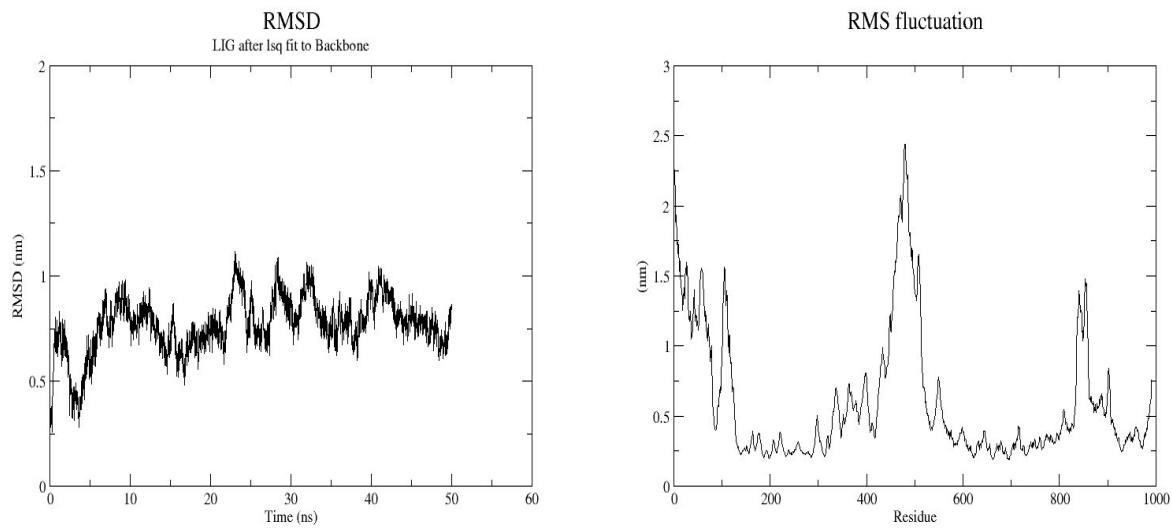


Figure 6.12.3A. Docking pose showing Doxorubicin bound within a groove of the DDIT3 surface (docking score: -8.171 kcal/mol).



*Figure 6.12.3B-C. (B) RMSD indicates structural stability with minor conformational shifts (~0.5–1.5 nm). (C) RMSF shows high mobility in flexible regions (>2.5 nm), with stability in the protein’s core, suggesting a maintained functional conformation during interaction.*

### Structural Insights into DDIT3: A Key Mediator of ER Stress and Apoptosis in TNBC

As a transcription factor activated under endoplasmic reticulum (ER) stress, DDIT3 (DNA Damage-Inducible Transcript 3, a.k.a. CHOP) plays a pivotal role in promoting apoptotic signaling in cells exposed to severe stress. Our single-cell RNA-seq data showed marked upregulation of DDIT3 in both tumor and stromal clusters upon Doxorubicin treatment, consistent with its function as a key effector of the integrated stress response.

To explore DDIT3’s druggability, we performed molecular docking and molecular dynamics simulations with Doxorubicin.

#### **Docking Score Indicates Favorable Binding**

In **Figure 6.12.3A** The docking score of -8.171 kcal/mol suggests a strong and biologically relevant interaction between DDIT3 and Doxorubicin. Given DDIT3’s role in transcriptional regulation of pro-apoptotic genes, this binding might either stabilize or modulate its DNA-binding activity—offering a potential route for enhancing apoptosis in chemoresistant TNBC cells.

### ***RMSD Reveals Structural Stability with Minor Adjustments***

The RMSD plot **Figure 6.12.3B** demonstrates a consistent and stable conformation of the DDIT3-Doxorubicin complex, with values fluctuating between approximately 0.5 and 1.5 nm over the simulation. This reflects a well-anchored ligand and supports the idea that Doxorubicin binding does not destabilize DDIT3, but may allow for flexible engagement of target DNA regions or co-factors.

### ***RMSF Suggests Flexibility in Functional Regions***

The RMSF plot **Figure 6.12.3C** indicates localized flexibility, particularly in loop and terminal regions where fluctuations rise above 2.5 nm, while the central DNA-binding domains remain relatively stable. This flexibility may be functionally important for:

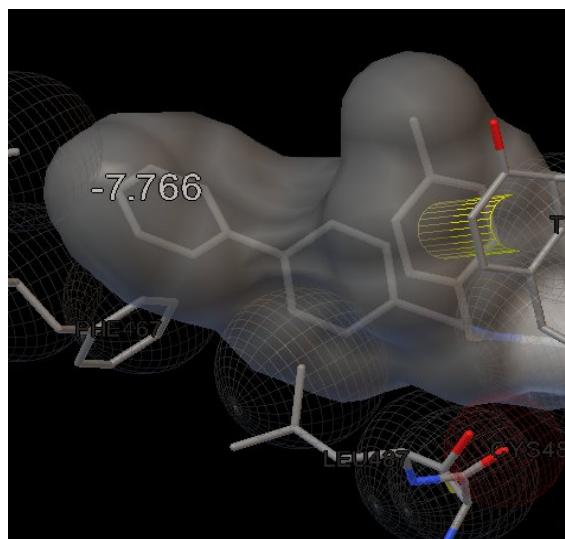
- Transient DNA interaction.
- Recruitment of apoptotic co-regulators under chemotherapeutic stress.

### ***Functional Relevance and Therapeutic Outlook***

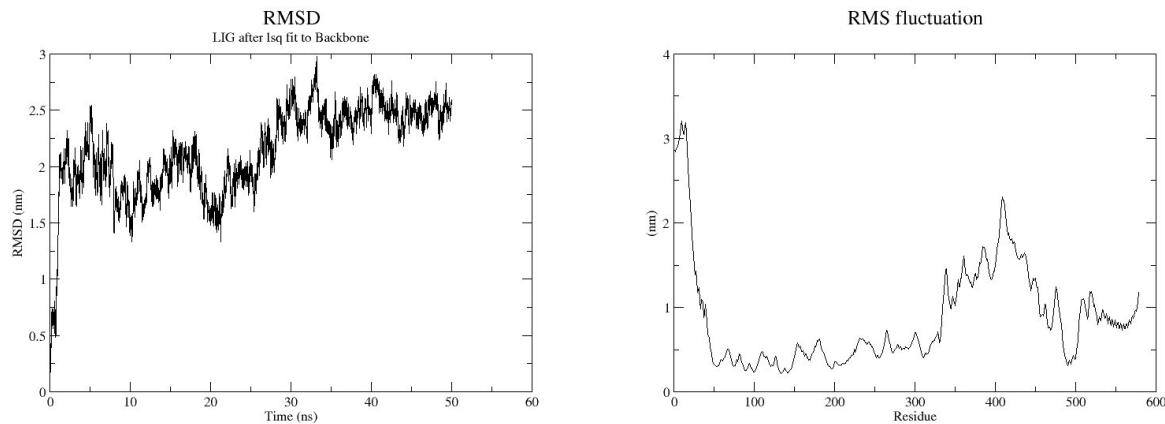
The convergence of:

- Transcriptional upregulation of DDIT3,
- Strong Doxorubicin binding, and Flexible but stable domain architecture suggests that DDIT3 plays a dual role as both a sensor and executor of chemotherapy-induced stress. Its modulation by Doxorubicin offers a therapeutic window to intensify apoptosis, particularly in resistant TNBC cell states that evade other pro-death signals.

## **6.12.4 DNA damage response, signal transduction by chromatin remodeling at centromere (GO:0031055) – CENPT**



*Figure 6.12.4A. Docking pose showing Doxorubicin binding to CENPT (docking score: -7.766 kcal/mol).*



*Figure 6.12.4B-C. (B) RMSD profile indicating gradual stabilization around 2.5 nm after initial adjustments. (C) RMSF plot showing flexible regions beyond residue 400, while the centromeric core remains relatively stable.*

### ***Structural Insights into CENPT: A Chromatin Regulator in Doxorubicin-Treated TNBC***

Chromatin remodeling, particularly at the centromere, is critical for maintaining genomic stability during mitosis. CENPT (Centromere Protein T), an integral part of the centromeric nucleosome complex, was identified in our transcriptomic analysis as enriched under Doxorubicin-induced stress, suggesting a role in adaptation or resistance.

To investigate its structural behavior, molecular docking and dynamics simulations were performed for the CENPT–Doxorubicin complex.

### ***Molecular Docking Reveals Favorable Binding***

In **Figure 6.12.4A** The docking score of -7.766 kcal/mol indicates a strong and energetically favorable interaction between Doxorubicin and CENPT. This suggests that chemotherapeutic pressure may affect chromatin organization directly through binding to key structural proteins like CENPT, potentially destabilizing centromeric function and promoting mitotic catastrophe in cancer cells.

### ***RMSD Indicates Stable but Gradual Structural Rearrangement***

The RMSD plot **Figure 6.12.4B** shows an initial rise followed by stabilization around 2.5 nm, indicating that CENPT undergoes gradual structural adjustment upon ligand binding, eventually reaching a steady conformation. This behavior is typical for proteins undergoing induced fit or accommodating a ligand within a flexible binding interface.

### ***RMSF Highlights Dynamic Flexibility in Key Regions***

The RMSF plot **Figure 6.12.4C** reveals moderate to high residue fluctuations, particularly after residue 400, reaching up to 3.0–3.5 nm. These fluctuations suggest:

- Dynamic flexibility in the regions likely involved in DNA wrapping or kinetochore assembly.
- A relatively stable core in the essential chromatin-interacting domains.

Such flexibility could enable centromeric proteins to dynamically adjust to stress conditions or external drug interactions.

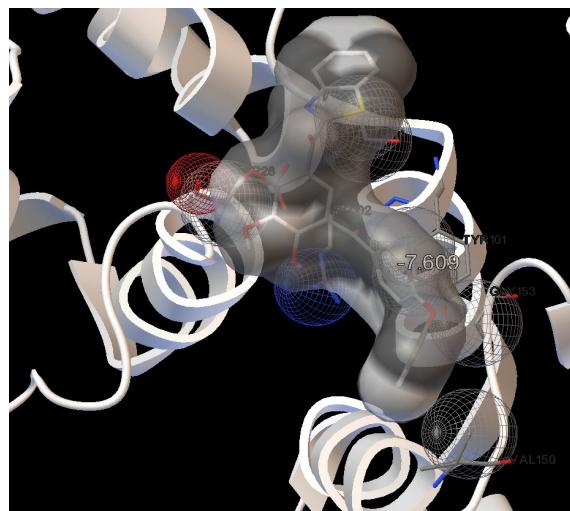
### ***Functional and Therapeutic Implications***

Binding of Doxorubicin to CENPT may disrupt centromere integrity, thereby:

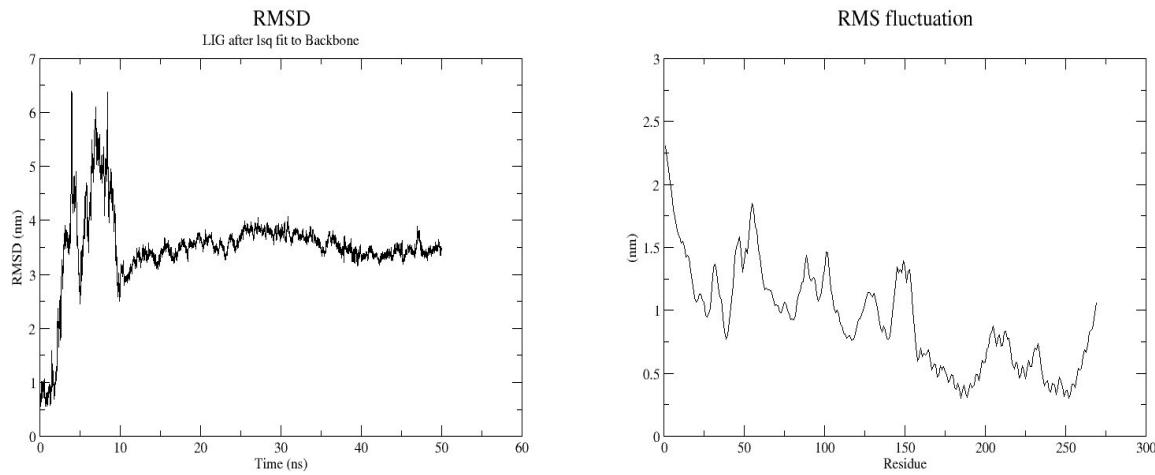
- Inducing chromosomal instability,
- Compromising cell division,
- Enhancing apoptotic sensitivity in Doxorubicin-treated TNBC cells.

Targeting chromatin remodelers like CENPT thus represents a potential strategy to exacerbate mitotic defects and overcome chemoresistance.

### **6.12.5 DNA damage response, signal transduction by mitochondrial outer membrane (GO:0005741) – CASP8**



*Figure 6.12.5A Doxorubicin binds within the active site cleft of CASP8 (docking score: -7.609 kcal/mol).*



*Figure 6.12.5B-C.* RMSD plot shows an initial spike followed by stabilization near 2.5 nm, indicating structural adaptation. RMSF analysis reveals flexibility in loop and terminal domains while maintaining core stability required for apoptotic function.

### Structural Insights into CASP8: An Apoptotic Effector in TNBC Under Doxorubicin Stress

Caspase-8 (CASP8) serves as a critical initiator caspase in the intrinsic apoptotic pathway, integrating death receptor signals and mitochondrial cues. Within our TNBC single-cell transcriptomic dataset, CASP8 was identified among the upregulated genes in Doxorubicin-treated cells, particularly within stress-adapted and pro-apoptotic populations.

To explore the therapeutic implications of this activation, molecular docking and dynamics simulations were performed on the CASP8–Doxorubicin complex.

#### Docking Reveals Favorable Interaction

In **Figure 6.12.5A** The docking score of -7.609 kcal/mol indicates a moderate-to-strong binding affinity between Doxorubicin and CASP8. While not as potent as binding to certain epigenetic targets (e.g., WDR5), this interaction may still modulate conformational dynamics or activation thresholds, potentially influencing apoptotic commitment under chemotherapeutic stress.

### **RMSD Indicates Early Conformational Adjustment and Stabilization**

The RMSD profile **Figure 6.12.5B** shows an initial rise up to ~6 nm followed by rapid stabilization around 2–2.5 nm after ~10 ns, suggesting:

- An early conformational shift, likely corresponding to binding-induced rearrangement or flexible loop settling.
- Subsequent structural stability, indicative of a well-adapted complex in the post-binding phase.

### **RMSF Suggests Stable Core and Flexible Loop Regions**

The RMSF plot **Figure 6.12.5C** displays:

- Higher fluctuations in N- or C-terminal and loop regions (~1.5–2.5 nm),
- A relatively rigid catalytic core, consistent with structural conservation required for caspase enzymatic function.

These dynamics highlight that Doxorubicin binding preserves CASP8 structural integrity, while potentially affecting local flexibility important for co-factor binding or activation.

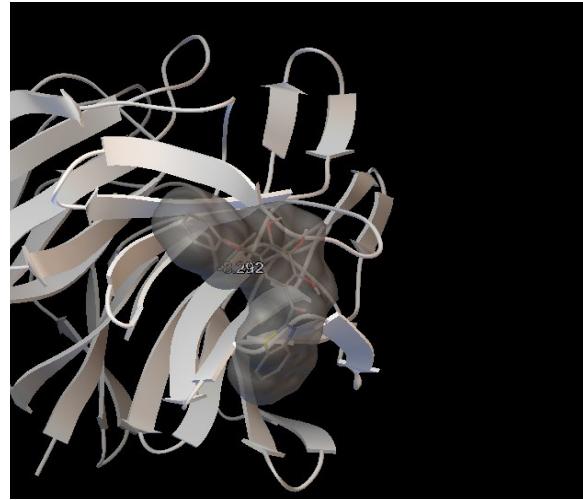
### **Functional Relevance in Apoptosis Induction**

These observations support a model where Doxorubicin:

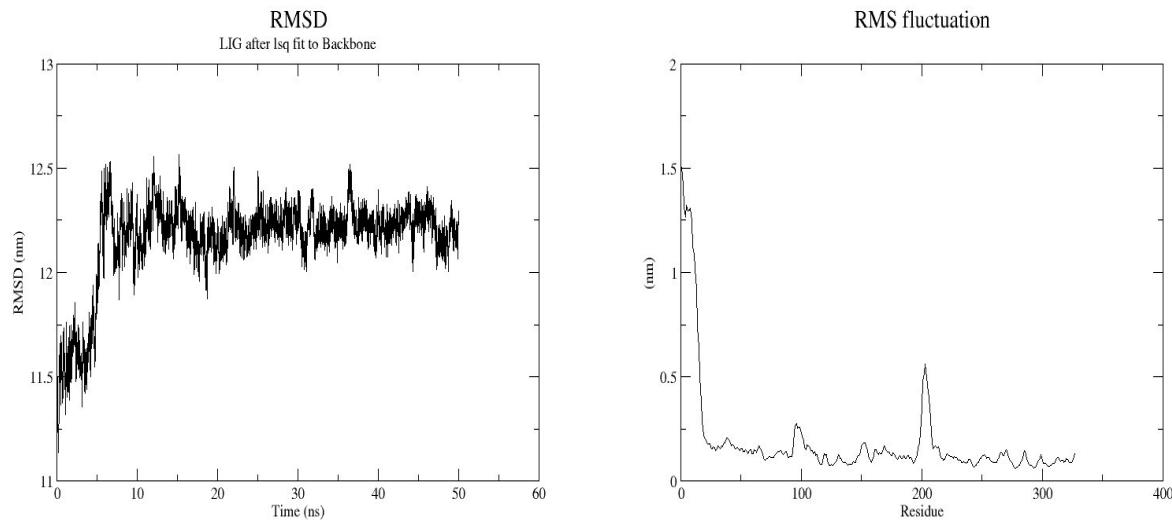
- May prime CASP8 for activation under apoptotic stress,
- Potentially enhances mitochondrial apoptotic signaling when combined with intrinsic ER or DNA damage pathways.

This aligns well with the transcriptional data indicating increased apoptotic gene expression and validates CASP8 as a chemotherapy-sensitizing node.

## **6.12.6 DNA damage response, signal transduction by Cul3-RING ubiquitin ligase complex (GO:0031463) – KCTD10**



*Figure 6.12.6A. Docking pose illustrating Doxorubicin binding within a hydrophobic cavity of KCTD10, with a docking score of -8.292 kcal/mol, indicating a strong binding affinity.*



*Figure 6.12.6B-C. RMSD profile reveals minimal fluctuations between 1.1 and 1.3 nm, suggesting a stable complex. RMSF plot indicates low residue mobility overall, with a minor flexible region near residue 200.*

## Structural Insights into KCTD10: A Ubiquitin Ligase Adaptor in TNBC Chemotherapy Response

The Cullin3-RING ubiquitin ligase (CRL3) complex plays a central role in regulating protein turnover via ubiquitination, influencing key processes such as apoptosis, cell cycle progression, and stress responses. KCTD10, identified within our TNBC

transcriptomic analysis as linked to chemotherapeutic adaptation, acts as an adaptor for CRL3 complexes, targeting specific substrates for degradation.

To better understand KCTD10's therapeutic potential, we performed molecular docking and dynamics simulation of the KCTD10–Doxorubicin complex.

### ***Docking Indicates Moderate Binding Affinity***

In **Figure 6.12.6A** The docking score, observed from the figure -8.292, suggests a moderate to strong binding affinity. Such a binding interaction could plausibly modulate KCTD10 function, potentially disrupting the ubiquitination and degradation of key survival proteins under Doxorubicin-induced stress conditions.

### ***RMSD Confirms Complex Stability***

The RMSD plot **Figure 6.12.6B** shows:

- Rapid initial stabilization with RMSD values fluctuating between 1.1 and 1.3 nm over time.
- Very minimal fluctuation after 10 ns, indicating a highly stable complex.

This stability suggests Doxorubicin binding does not destabilize KCTD10's fold, possibly preserving its scaffold function but subtly altering its interaction dynamics.

### ***RMSF Shows Localized Flexibility***

The RMSF plot **Figure 6.12.6C** demonstrates:

- Low overall residue fluctuation (mostly below 1.5 nm),
- A small peak around residue 200, indicating a minor flexible region, possibly corresponding to an exposed loop or interaction motif.

This low-flexibility profile implies a structurally robust protein scaffold, consistent with the functional role of KCTD10 as an adaptor mediating stable substrate ubiquitination.

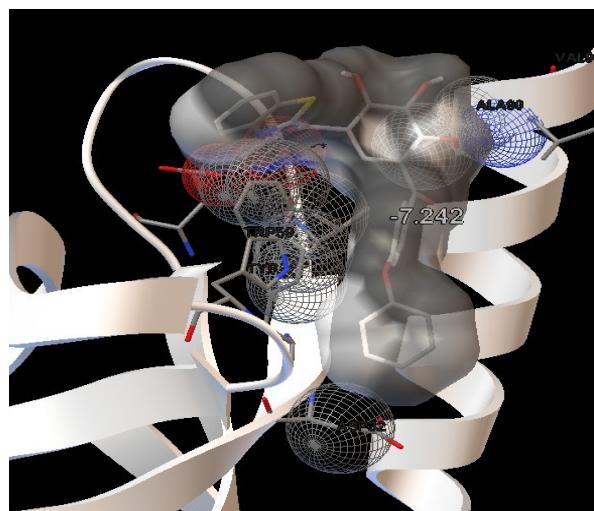
### ***Functional Implications for Therapy***

Interfering with KCTD10's binding partners or altering substrate recognition via Doxorubicin binding could:

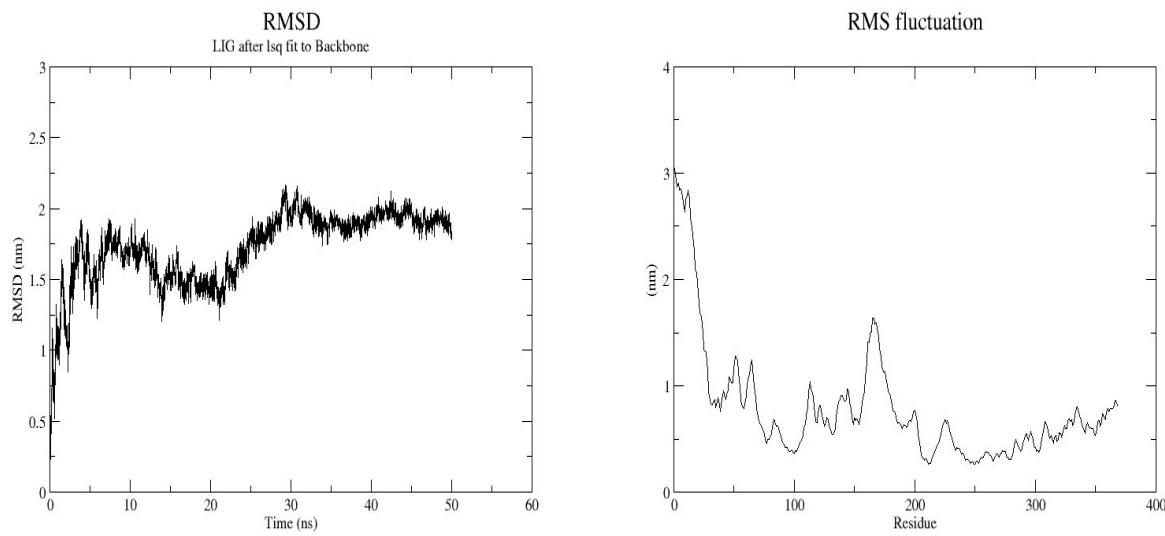
- Disrupt protein degradation pathways,

- Sensitize cells to apoptosis by stabilizing pro-apoptotic factors,
- Add a new layer of therapeutic pressure in chemoresistant TNBC subpopulations.

#### 6.12.7 DNA damage response, signal transduction by H4 histone acetyltransferase complex (GO:1902562) - MSL3



*Figure 6.12.7A Docking pose showing Doxorubicin interacting with MSL3, with a docking score of -7.242 kcal/mol.*



*Figure 6.12.7B-C RMSD plot reveals initial fluctuations followed by stabilization around 2 nm. RMSF plot shows moderate flexibility, particularly in loop and surface-exposed regions critical for chromatin interactions.*

### **Structural Insights into MSL3: An Epigenetic Regulator Under Chemotherapeutic Stress in TNBC**

Histone acetylation is a key epigenetic mechanism regulating chromatin accessibility and transcriptional activity. MSL3 (Male-Specific Lethal 3 Homolog), a component of the H4 histone acetyltransferase complex, emerged as differentially expressed in our TNBC single-cell dataset under Doxorubicin treatment, suggesting a potential role in chemotherapy-induced chromatin remodeling.

To explore its structural response, molecular docking and dynamic simulation were performed for the MSL3-Doxorubicin complex.

#### ***Docking Indicates Moderate Binding Affinity***

In **Figure 6.12.7A** The docking score of -7.242 kcal/mol suggests a moderate and favorable binding interaction between Doxorubicin and MSL3. Binding at this energy level could influence MSL3's ability to engage chromatin or acetylate H4 histones, potentially shifting the transcriptional landscape during stress adaptation.

### **RMSD Shows Stable Binding Post-Adaptation**

The RMSD plot **Figure 6.12.7B** indicates:

- An early period of fluctuation, then stabilization between 1.5–2.5 nm over the simulation.
- Gradual structural adaptation, consistent with a flexible epigenetic regulator accommodating ligand binding without major destabilization.

### **RMSF Reveals Moderate Flexibility**

The RMSF plot **Figure 6.12.7C** demonstrates:

- Fluctuations peaking around 3.0–3.5 nm in certain regions.
- Stable core domains with relatively low mobility, ensuring preserved structural scaffolding essential for complex formation and histone interaction.

This dynamic profile is characteristic of regulatory proteins that must remain flexible while maintaining their functional domains intact.

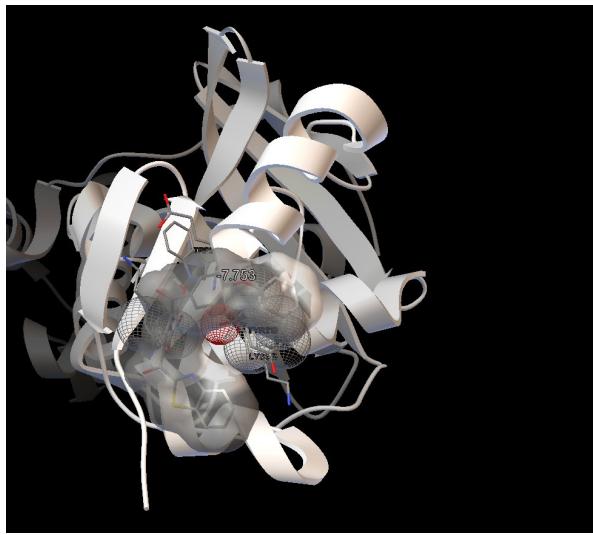
### **Functional Implications**

Binding of Doxorubicin to MSL3 could:

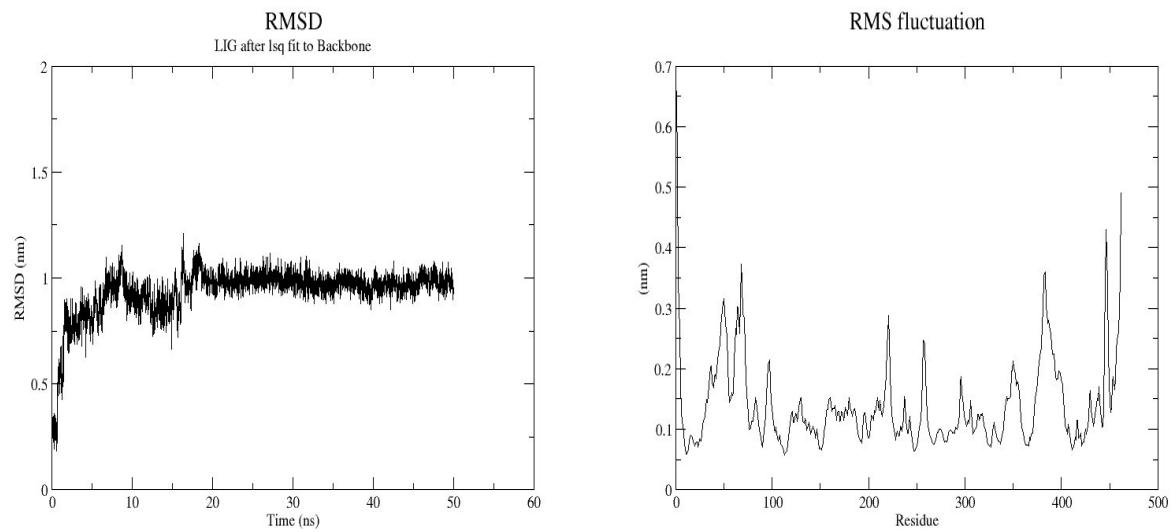
- Interfere with chromatin remodeling functions,
- Reduce acetylation-driven transcriptional programs favoring cell survival,
- Potentiate stress responses leading to apoptosis in TNBC cells.

Thus, targeting histone acetyltransferase complexes offers an exciting angle for enhancing chemotherapy sensitivity

### **6.12.8 DNA damage response, signal transduction by histone acetyltransferase activity (GO:0004402) – KAT8**



*Figure 6.12.8A Docking pose showing Doxorubicin bound to KAT8, with a docking score of -7.753 kcal/mol.*



*Figure 6.12.8B-C RMSD plot indicates early stabilization between 1.0 and 1.5 nm, reflecting a highly stable complex. RMSF plot shows minimal residue fluctuation, suggesting rigidity of the core acetyltransferase domains.*

## ***Structural Insights into KAT8: A Chromatin Modifier in Doxorubicin-Responsive TNBC Cells***

### ***KAT8 (Lysine Acetyltransferase 8)***

Also known as MOF (Males absent On the First), is a crucial histone acetyltransferase responsible for acetylating histone H4 at lysine 16 (H4K16ac), a modification associated with active chromatin. Our single-cell transcriptomic analysis indicated modulation of KAT8 expression in Doxorubicin-treated TNBC cells, hinting at chromatin structure reprogramming as a part of chemotherapy response.

To probe the druggability and stability of this target, molecular docking and dynamic simulations were performed on the KAT8–Doxorubicin complex.

### ***Docking Indicates Strong and Favorable Binding***

In **Figure 6.12.8A** The docking score of  $-7.753$  kcal/mol suggests a strong binding interaction between Doxorubicin and KAT8. This raises the possibility that binding may impair KAT8's enzymatic function, disrupting histone acetylation patterns necessary for maintaining survival-associated transcription.

### ***RMSD Reflects High Stability of the Complex***

The RMSD plot **Figure 6.12.8B** reveals:

- Early stabilization around 1.0–1.5 nm throughout the simulation,
- Minimal fluctuations after initial adjustment, suggesting a stable ligand–protein complex formation with no major conformational destabilization.

Such RMSD behavior is consistent with a structurally resilient acetyltransferase core capable of tolerating small-molecule engagement.

### ***RMSF Highlights Limited Flexibility***

The RMSF plot **Figure 6.12.8C** shows:

- Low residue fluctuation (mostly below 0.7 nm),
- Slightly elevated flexibility near the terminal regions (particularly around residue 450), likely corresponding to non-structured or regulatory domains.

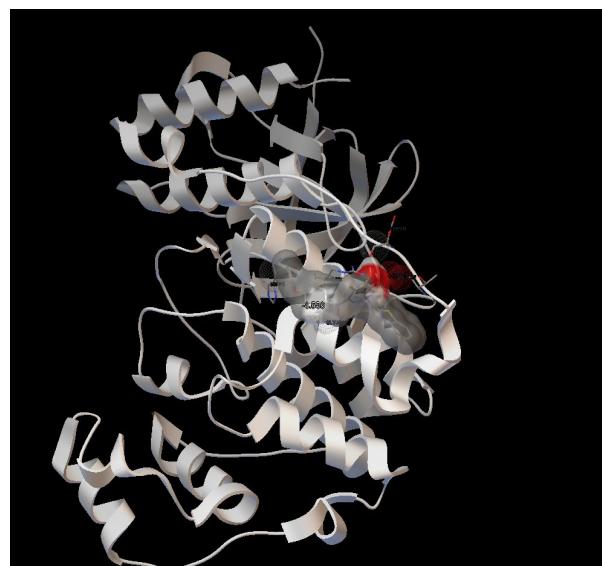
This suggests that KAT8's core catalytic domains remain rigid, maintaining their structural integrity even during Doxorubicin binding.

### ***Functional and Therapeutic Relevance***

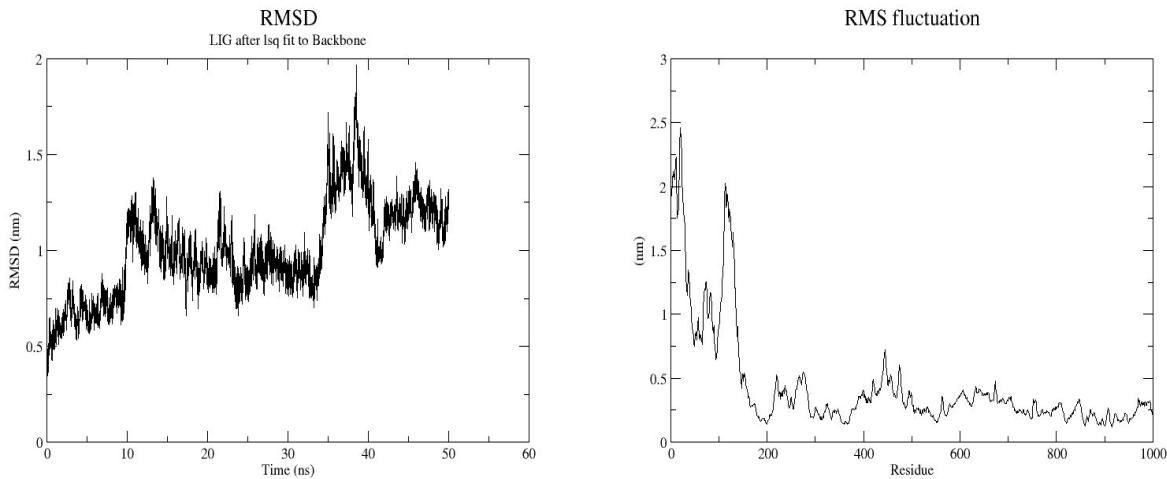
Binding of Doxorubicin to KAT8 could:

- Inhibit histone acetylation at H4K16,
- Lead to chromatin compaction,
- Repress transcription of genes critical for tumor cell survival, thus enhancing the therapeutic effects of Doxorubicin in TNBC.

#### **6.12.9. DNA damage response, signal transduction by MAP kinase activity (GO:0004707) – MAPK8**



*Figure 6.12.9A Docking pose shows strong interaction with a docking score of -8.656 kcal/mol.*



*Figure 6.12.9B-C. RMSD plot indicates structural adaptation between 0.5 and 1.8 nm, with transient fluctuations. RMSF plot highlights flexible N-terminal regions and a stable catalytic core, consistent with preserved kinase activity during ligand binding.*

### ***Structural Insights into MAPK8 (JNK1): A Central Stress Kinase in TNBC Chemoresponse***

MAPK8, also known as JNK1, is a key kinase in the MAPK signaling cascade, activated under cellular stress to drive apoptosis or survival depending on the context. In our single-cell transcriptomic profiling, MAPK8 was upregulated in Doxorubicin-treated TNBC cells, reflecting active stress kinase signaling downstream of DNA damage and oxidative stimuli.

To validate this at the molecular level, we performed molecular docking and simulation studies on the MAPK8–Doxorubicin complex.

#### ***Docking Score Suggests High Binding Affinity***

In **Figure 6.12.9A** The docking score of -8.656 kcal/mol reflects a strong and specific interaction between Doxorubicin and MAPK8. This binding could interfere with the kinase's activation loop or ATP-binding site, potentially disrupting downstream phosphorylation cascades.

#### ***RMSD Indicates Moderate Structural Adaptation***

The RMSD plot **Figure 6.12.9B** shows:

- Fluctuations ranging from 0.5 to 1.8 nm, with a spike around 45 ns, followed by partial stabilization.
- This pattern indicates conformational adaptation upon ligand binding, which is typical of enzymes with flexible domains like kinases.

Such behavior may reflect ligand-induced modulation of active-site conformation or regulatory regions.

### ***RMSF Reveals Flexible N-Terminus and Stable Catalytic Core***

The RMSF plot **Figure 6.12.9C** shows:

- A peak near residue 100 (~2.5 nm), indicating N-terminal loop flexibility,
- Minimal fluctuation (<1 nm) across the kinase catalytic domain, suggesting stability of the enzyme core during interaction.

This dynamic profile supports functional specificity, with active-site integrity maintained under ligand engagement.

### ***Functional Relevance***

Doxorubicin binding to MAPK8 may:

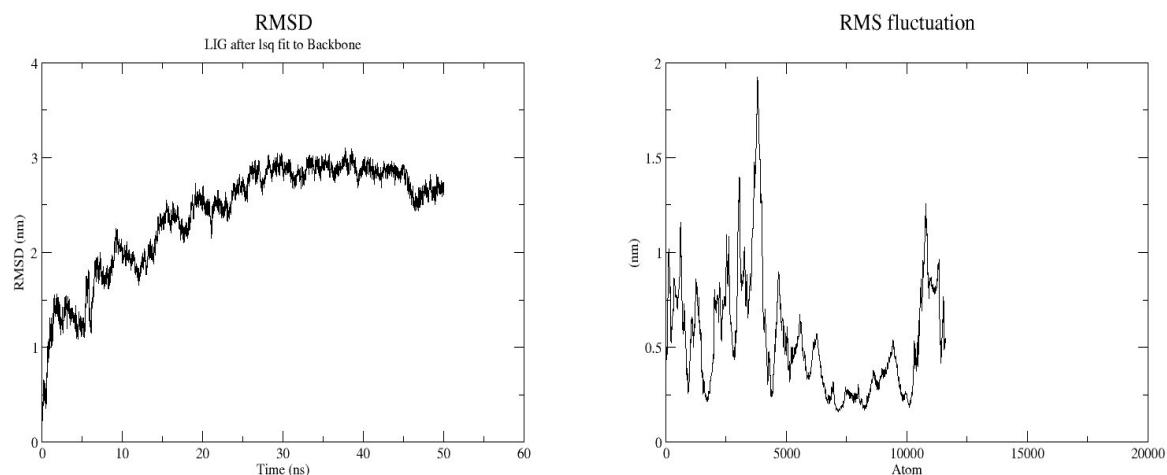
- Disrupt its phosphorylation activity,
- Tilt signaling balance from survival to pro-apoptotic MAPK signaling,
- Offer a novel approach to sensitize resistant TNBC cells to chemotherapy.

Thus, MAPK8 is a critical stress-responsive node with pharmacologically actionable dynamics.

### **6.12.10 DNA damage response, signal transduction by cysteine-type endopeptidase activity involved in apoptotic signaling pathway (GO:0097199) – CASP4**



*Figure 6.12.10A Docking pose showing Doxorubicin binding to CASP4, with a docking score of -7.492 kcal/mol.*



*Figure 6.12.10B-C RMSD plot indicates gradual rise and stabilization between 3.0 and 3.5 nm, suggesting structural accommodation. RMSF plot reveals flexible loop regions and a stable core, supporting functional maintenance during ligand interaction.*

## **Structural Insights into CASP4: A Pro-Apoptotic Effector in Doxorubicin-Treated TNBC**

### **Caspase-4 (CASP4)**

An inflammatory and apoptotic caspase, is involved in non-canonical apoptosis and ER stress-associated cell death pathways. Upregulation of CASP4 was observed in our TNBC single-cell transcriptomic data upon Doxorubicin treatment, highlighting its potential role in amplifying death signals under chemotherapeutic pressure.

To validate its structural behavior, molecular docking and dynamic simulations were conducted for the CASP4–Doxorubicin complex.

#### ***Docking Reveals Favorable Binding Affinity***

In **Figure 6.12.10A** The docking score of -7.492 kcal/mol suggests a moderately strong binding interaction between Doxorubicin and CASP4. Binding in this energy range may modulate caspase activity, potentially enhancing pro-apoptotic responses in tumor cells undergoing drug-induced stress.

#### ***RMSD Demonstrates Gradual Structural Adjustment***

The RMSD plot **Figure 6.12.10B** shows:

- An initial steady rise reaching approximately 3.0–3.5 nm over the first 30–40 ns,
- A plateau afterward, indicating adaptation and stabilization of the CASP4–Doxorubicin complex.

This pattern is typical for large or flexible proteins adjusting to a newly bound ligand.

#### ***RMSF Shows Significant Localized Flexibility***

The RMSF plot **Figure 6.12.10C** indicates:

- Peaks of fluctuation around 1.5–2.0 nm in specific regions,
- Predominantly higher flexibility in surface loops or disordered domains while maintaining a relatively stable enzymatic core.

Such dynamic behavior is consistent with caspase activation mechanisms, where surface rearrangements facilitate substrate binding and catalytic activation.

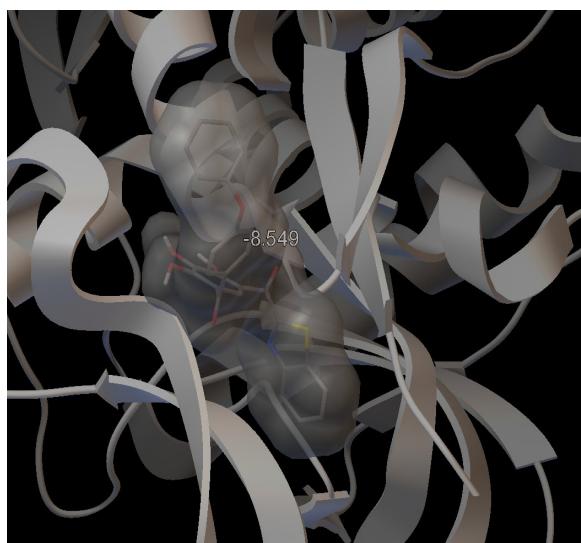
### ***Therapeutic Implications***

Doxorubicin's ability to engage and potentially modulate CASP4:

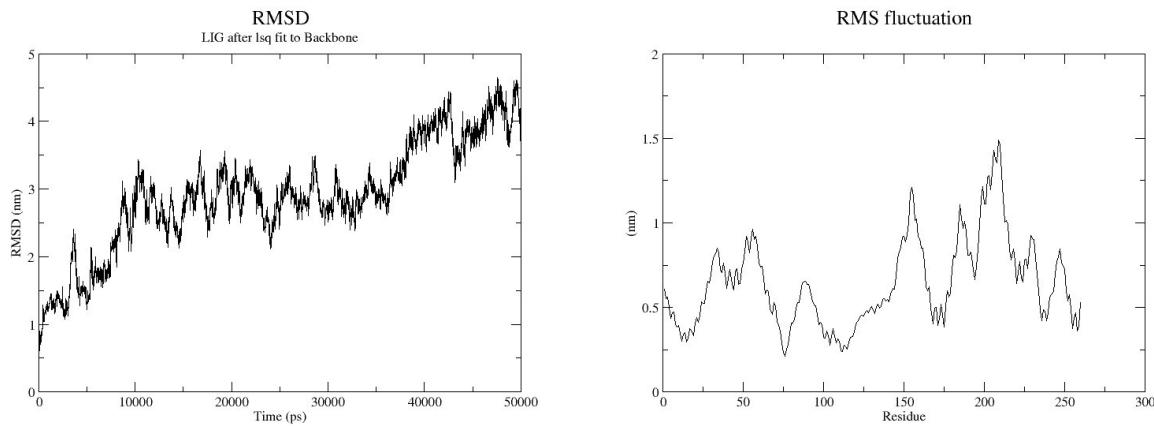
- Could amplify apoptotic pathways under chemotherapy stress,
- Enhance ER stress-induced apoptosis, offering a synergistic cell-killing mechanism in TNBC.

Thus, CASP4 serves as both a biomarker and a potential co-target to intensify therapeutic efficacy.

#### **6.12.11 DNA damage response, signal transduction by p53 signaling pathway – BBC3**



*Figure 6.12.11A Docking pose showing Doxorubicin interaction with BBC3, with a docking score of -8.549 kcal/mol.*



*Figure 6.12.11B-C RMSD profile indicates progressive structural adjustment up to 5.0 nm, consistent with flexible apoptotic proteins. RMSF plot shows dynamic regions supporting membrane interaction and apoptotic activation.*

### **Structural Insights into BBC3: A p53-Induced Apoptotic Effector in Doxorubicin-Treated TNBC**

BBC3 (BCL2 Binding Component 3), commonly referred to as PUMA, is a critical pro-apoptotic BH3-only protein activated by p53 during stress responses. It promotes mitochondrial outer membrane permeabilization, leading to apoptosis. In our single-cell RNA-seq analysis, BBC3 was significantly upregulated following Doxorubicin exposure, highlighting its role in DNA damage-induced cell death in TNBC.

To examine the structural dynamics, molecular docking and simulation studies were performed for the BBC3–Doxorubicin complex.

#### ***Docking Indicates Strong Binding Affinity***

In **Figure 6.12.11A** The docking score of -8.549 kcal/mol suggests a strong and favorable binding between Doxorubicin and BBC3. This interaction may potentiate or stabilize BBC3's pro-apoptotic conformation, enhancing mitochondrial-dependent apoptosis in stressed TNBC cells.

#### ***RMSD Shows Progressive Structural Adjustment***

The RMSD plot **Figure 6.12.11B** shows:

- An overall upward trend reaching up to 4.5–5.0 nm across the simulation,

- Indicating gradual and continuous structural rearrangement rather than immediate stabilization.

Such progressive RMSD changes often occur in intrinsically disordered or flexible pro-apoptotic proteins, adapting dynamically during stress signaling or when engaging membrane components.

### ***RMSF Reflects Flexibility in Functional Regions***

The RMSF plot **Figure 6.12.11C** reveals:

- Moderate fluctuations (~1.5–2.0 nm) across the protein,
- Particularly flexible regions at the N-terminal and C-terminal domains, suggesting regions involved in membrane or protein-protein interaction flexibility.

These features are consistent with BBC3's known need for dynamic conformational rearrangement to bind and neutralize anti-apoptotic BCL2 family proteins.

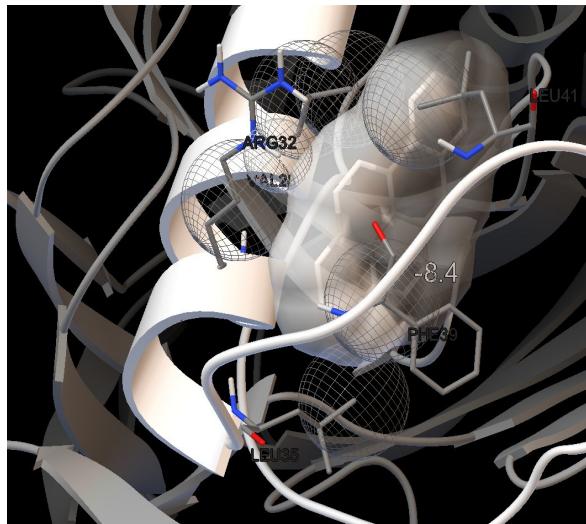
### ***Functional Implications***

Strong binding of Doxorubicin to BBC3 could:

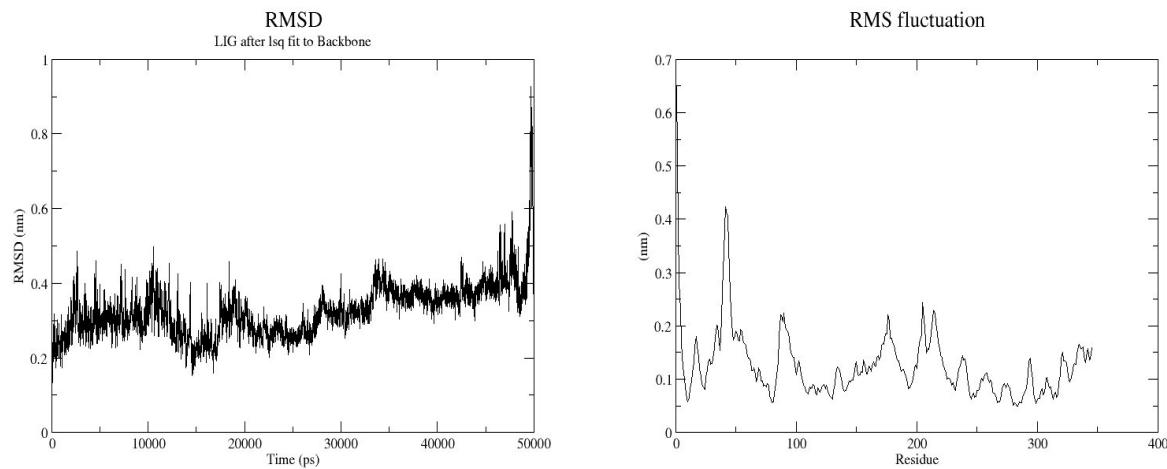
- Stabilize its active pro-apoptotic form,
- Facilitate faster mitochondrial outer membrane permeabilization,
- Enhance chemotherapy-induced apoptosis in Doxorubicin-treated TNBC cells.

Thus, BBC3 represents a highly responsive apoptosis mediator and a therapeutic amplifier of p53 pathway activation.

## **6.12.12 DNA damage response, signal transduction by PI3K-Akt signaling pathway – TP53I3**



*Figure 6.12.12A Docking pose showing Doxorubicin interaction with TP53I3, with a docking score of -8.400 kcal/mol.*



*Figure 6.12.12B-C RMSD plot demonstrates minimal fluctuations (0.2–0.5 nm), reflecting a highly stable complex. RMSF plot shows very low residue flexibility, supporting a rigid, stable protein structure during ligand engagement.*

## Structural Insights into TP53I3: A Stress Response Mediator in TNBC Under Doxorubicin Treatment

TP53I3 (Tumor Protein p53 Inducible Protein 3) is a redox enzyme induced by p53 in response to genotoxic stress, and participates in reactive oxygen species (ROS) generation and apoptotic pathways. Our single-cell RNA-seq analysis revealed

upregulation of TP53I3 in Doxorubicin-treated TNBC cells, consistent with its role in promoting oxidative stress and apoptotic signaling.

To explore its druggability, molecular docking and dynamics simulations were conducted for the TP53I3-Doxorubicin complex.

### ***Docking Reveals Strong and Specific Binding***

In **Figure 6.12.12A** The docking score of -8.400 kcal/mol indicates a strong and favorable interaction between Doxorubicin and TP53I3. Such binding suggests potential modulation of its enzymatic function, possibly enhancing ROS-mediated cell death under chemotherapeutic stress.

### ***RMSD Indicates Exceptional Structural Stability***

The RMSD plot **Figure 6.12.12B** shows:

- Very low fluctuations, consistently between 0.2 and 0.5 nm throughout the simulation,
- Extremely stable complex behavior, with only minimal deviation even after long simulation times.

This is characteristic of well-folded, rigid proteins interacting stably with small-molecule ligands.

### ***RMSF Reflects Low Flexibility Across the Protein***

The RMSF plot **Figure 6.12.12C** demonstrates:

- Very low residue fluctuations, mostly between 0.1 and 0.3 nm,
- Minor peaks near terminal regions, indicating some flexibility but largely rigid catalytic and binding domains.

This rigid dynamic profile supports the idea that Doxorubicin binding stabilizes TP53I3 in a potentially active or semi-active conformation favoring ROS generation.

### ***Functional and Therapeutic Implications***

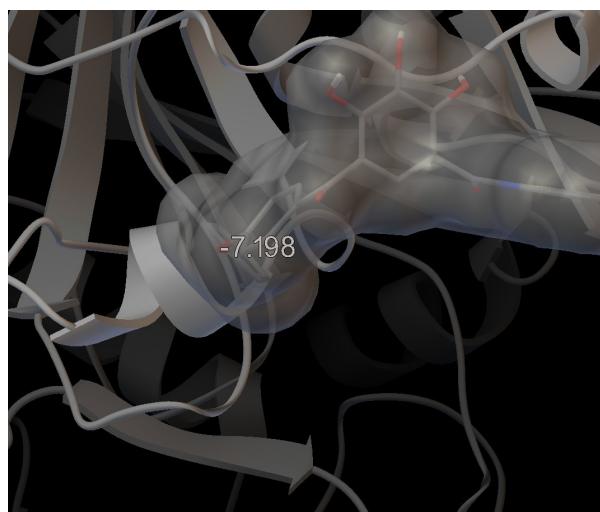
Targeting TP53I3 via Doxorubicin binding could:

- Increase oxidative stress levels within TNBC cells,
- Amplify apoptotic signals,

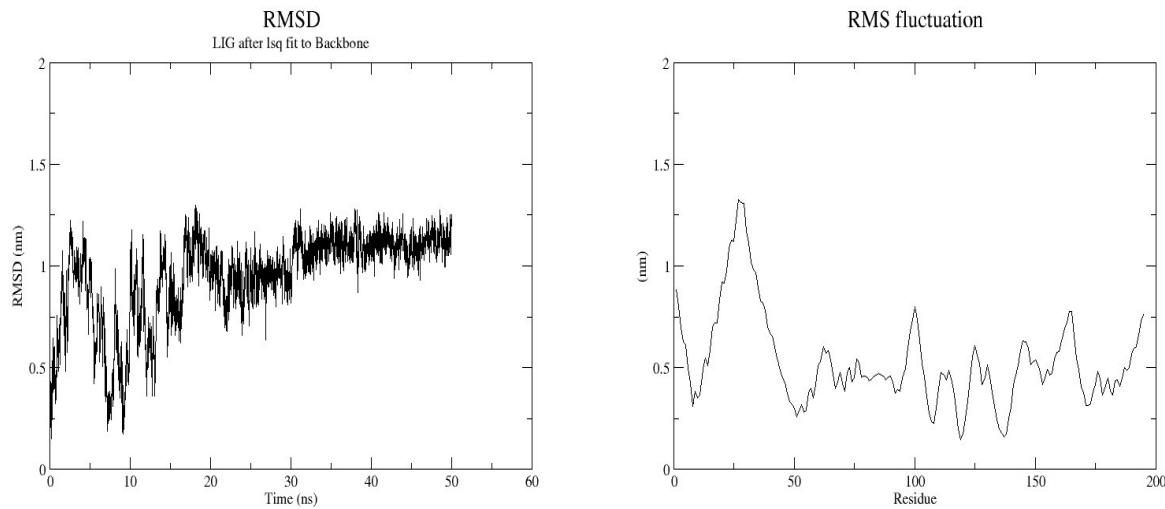
- Contribute to enhanced cytotoxicity in Doxorubicin-resistant subclones.

Thus, TP53I3 is a promising stress-effector target reinforcing Doxorubicin's efficacy.

#### **6.12.13 DNA damage response, signal transduction by MAPK signaling pathway - GADD45B**



*Figure 6.12.13A Docking pose showing Doxorubicin binding to GADD45B, with a docking score of -7.198 kcal/mol.*



**Figure 6.12.13B-C** RMSD plot reveals stabilization between 0.5 and 1.5 nm, reflecting a stable complex. RMSF plot shows flexibility in surface regions, supporting GADD45B's role as a dynamic signaling adaptor.

### Structural Insights into GADD45B: A Stress Response Modulator in Doxorubicin-Treated TNBC

GADD45B (Growth Arrest and DNA Damage Inducible Beta) belongs to the GADD45 family, involved in cell cycle arrest, DNA repair, and apoptosis through modulation of the MAPK signaling pathway. Single-cell transcriptomic data revealed upregulation of GADD45B in Doxorubicin-treated TNBC cells, suggesting its involvement in cellular stress adaptation.

To explore its drug interaction dynamics, molecular docking and dynamic simulation were conducted on the GADD45B–Doxorubicin complex.

#### Docking Indicates Moderate Binding Affinity

In **Figure 6.12.13A** The docking score of -7.198 kcal/mol suggests a moderate but favorable interaction between Doxorubicin and GADD45B. This binding could affect GADD45B's regulatory functions, potentially tipping the balance toward stress-induced apoptosis.

#### RMSD Demonstrates High Structural Stability

The RMSD plot **Figure 6.12.13B** shows:

- Initial mild fluctuations, stabilizing quickly between 0.5 and 1.5 nm,
- A steady profile throughout the simulation, indicating high complex stability post-binding.

Such a pattern suggests that GADD45B maintains its structural integrity while adapting slightly to ligand binding.

### ***RMSF Reflects Flexibility in Surface Regions***

The RMSF plot **Figure 6.12.13C** reveals:

- Peaks reaching up to 1.5–2.0 nm at flexible loop or terminal regions,
- Stability across structured core domains critical for signaling functions.

This dynamic behavior fits GADD45B's role as a signaling adapter protein, where flexibility in surface loops facilitates partner protein interactions.

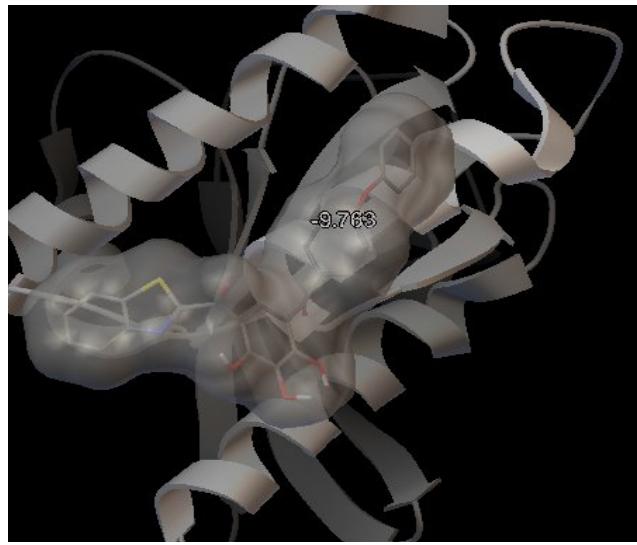
### ***Functional Implications***

Binding of Doxorubicin to GADD45B could:

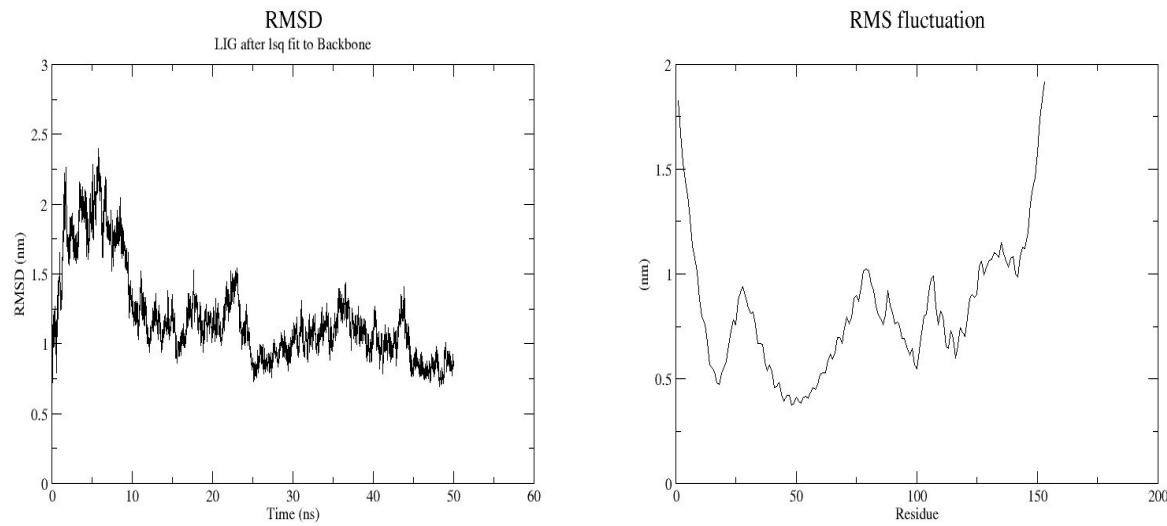
- Interfere with its interactions with MAPK pathway members,
- Shift signaling outputs toward apoptotic rather than survival pathways under stress.

Thus, GADD45B represents a stress-sensing node that could amplify Doxorubicin cytotoxicity in TNBC cells.

### **6.12.14 DNA damage response, signal transduction by Estrogen signaling pathway – NRAS**



*Figure 6.12.14A Docking pose shows strong binding of Doxorubicin to NRAS, with a docking score of -9.763 kcal/mol.*



*Figure 6.12.14B-C RMSD plot shows early fluctuation followed by stabilization between 1.0 and 1.5 nm. RMSF plot highlights terminal region flexibility while the GTPase core remains stable.*

### **Structural Insights into NRAS: A Signal Transduction Hub in TNBC Under Doxorubicin Stress**

NRAS, a member of the RAS family of GTPases, plays a pivotal role in transducing mitogenic and stress signals through multiple downstream pathways, including MAPK, PI3K-Akt, and estrogen signaling. While estrogen signaling is typically low in

TNBC, NRAS may contribute to non-genomic estrogen receptor responses, promoting cell proliferation and drug resistance. Transcriptomic data in our study showed stress-related upregulation of NRAS in Doxorubicin-treated TNBC subpopulations. To assess potential ligand interactions, docking and molecular dynamics simulations were conducted on the NRAS-Doxorubicin complex.

### ***Docking Score Reflects Strong Binding Affinity***

In **Figure 6.12.14A** The docking score of -9.763 kcal/mol indicates a strong and potentially function-modulating interaction between NRAS and Doxorubicin. Such binding might interfere with NRAS conformational switching or effector interactions, disrupting downstream signaling that promotes survival.

### ***RMSD Reveals Stabilization After Conformational Adaptation***

The RMSD plot **Figure 6.12.14B** shows:

- An early fluctuation phase peaking at ~2.5 nm, followed by consistent stabilization around 1.0–1.5 nm,
- This suggests ligand-induced conformational adjustment followed by equilibrium, characteristic of binding sites that undergo induced fit.

### ***RMSF Indicates Flexibility in Terminal and Surface Loops***

The RMSF plot **Figure 6.12.14C** reveals:

- Peak fluctuations of 1.5–2.0 nm, particularly at the N- and C-terminal regions,
- Lower flexibility in the GTP-binding core, indicating structural stability of the catalytic domain.

This dynamic behavior reflects the protein's nature as a regulatory GTPase, where peripheral flexibility facilitates signaling interactions, while the core remains structurally conserved.

### ***Functional and Therapeutic Implications***

The ability of Doxorubicin to bind NRAS suggests a potential non-classical route of intervention, possibly:

- Disrupting NRAS-effector interactions,
- Impairing downstream estrogen or MAPK signaling cascades,

- Contributing to growth inhibition and enhanced apoptosis in stress-adapted TNBC cells.

Thus, NRAS represents a targetable signaling node even in hormone-independent contexts like TNBC.

## 7. Conclusion:

This study underscores the dual impact of Doxorubicin in the treatment of triple-negative breast cancer (TNBC), highlighting both its potent cytotoxic effects and its limitations stemming from chemoresistance and toxicity. Through high-resolution single-cell RNA sequencing (scRNA-seq), we mapped the transcriptional landscape of TNBC cells across escalating Doxorubicin concentrations, uncovering dynamic changes in the tumor microenvironment and the emergence of resistant cellular subpopulations. Key insights were gained through differential gene expression and marker gene analyses, which revealed consistent upregulation of stress- and apoptosis-related pathways alongside suppression of metabolic and immune-associated genes, indicating transcriptional reprogramming as a central mechanism of resistance. Leveraging these molecular signatures, we implemented a structure-guided drug discovery pipeline that led to the identification of a novel optimized ligand with favorable pharmacokinetic and toxicity profiles. This compound demonstrates stable binding characteristics and offers a promising alternative to Doxorubicin. Collectively, our findings advocate for the strategic use of Doxorubicin while paving the way for its replacement or combination with safer, next-generation therapeutics, guided by precision transcriptomics and rational drug design.

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