Identification of GITR-targeted Small Molecules via Virtual Screening for Cancer Immunotherapy and Autoimmune Disease Treatment

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Abstract

Both cancer and autoimmune disease are closely linked to immune system dysregulation: cancer is characterized by the uncontrolled proliferation of tumor cells due to a defective immune response, while autoimmune disease involves an overzealous immune system that attacks the patient's own cells. Small-molecule immunotherapy is a promising treatment for both diseases that warrants additional research. This study identified potential small-molecule binding candidates to the glucocortocoid-induced tumor necrosis factor receptor-related (GITR) protein, which mediates important T-cell costimulatory pathways. Modulation of GITR may either upregulate antitumor responses or temper an overactive immune system by altering T-cell activity. To discover small molecule candidates, multistep virtual screening was performed on the GITR-GITRL complex: first, binding sites on the GITR protein were identified using geometric (DoGSiteScorer), energy-based (FTSite), and machine-learning (Prankweb) methods to ensure that GITR is a feasible target for small-molecule immunotherapy. Pharmacophore-based virtual screening was then used to identify potential small-molecule modulators for the GITR protein using Pocketquery and ZINCPharmer. Lastly, the druggability of these compounds was validated using molecular docking (Swissdock), Lipinski's rule (SwissADME), and toxicity screening (Protox-3.0). After screening a small-molecule library containing over 18 million compounds, six promising small molecule candidates and their properties were identified. This study is valuable because it explores the potential applications of small-molecule immunotherapy on costimulatory T-cell receptors such as GITR and sets the framework for the development of new treatment options for cancer and autoimmune disease patients.

Keywords: Cancer immunotherapy, virtual screening, small molecules, immune checkpoint, costimulatory pathway, autoimmunity, TNFR superfamily, GITR

Acknowledgements

I would like to thank my mentor, Dr. Moustafa Gabr from Cornell University, for introducing me to these virtual screening technologies and guiding me throughout the research process as I collected my research data and prepared this manuscript.

Commitments on Academic Honesty and Integrity

We hereby declare that we

1. are fully committed to the principle of honesty, integrity and fair play throughout the competition.
2. actually perform the research work ourselves and thus truly understand the content of the work.
3. observe the common standard of academic integrity adopted by most journals and degree theses.
4. have declared all the assistance and contribution we have received from any personnel, agency, institution, etc. for the research work.
5. undertake to avoid getting in touch with assessment panel members in a way that may lead to direct or indirect conflict of interest.
6. undertake to avoid any interaction with assessment panel members that would undermine the neutrality of the panel member and fairness of the assessment process.
7. observe the safety regulations of the laboratory(ies) where we conduct the experiment(s), if applicable.
8. observe all rules and regulations of the competition.
9. agree that the decision of YHSA is final in all matters related to the competition.

We understand and agree that failure to honour the above commitments may lead to disqualification from the competition and/or removal of reward, if applicable; that any unethical deeds, if found, will be disclosed to the school principal of team member(s) and relevant parties if deemed necessary; and that the decision of YHSA is final and no appeal will be accepted.

(Signatures of full team below)

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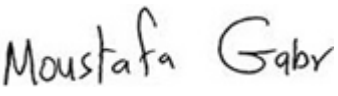
Name of team member: Henry Pei

X 

Name of supervising teacher: Moustafa Gabr

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1. Introduction

Cancer is a deadly disease characterized by a failure of the body's regulatory mechanisms to manage cell growth, which results in uncontrolled cancer cell proliferation (Hanahan & Weinberg, 2000). Cancer cells are capable of replicating infinitely, stimulating cell growth independently, evading growth suppressors and apoptosis, manipulating blood vessels to provide them with sustenance, and avoiding eradication by the body's immune system (Hanahan & Weinberg, 2011). The relentless growth of these cells significantly impairs bodily function through competition with healthy cells for resources and space (What Is Cancer, 2025). Moreover, cancer cells in malignant tumors invade different regions of the body through a process known as metastasis, which further exacerbates serious health risks posed to patients (What Is Cancer, 2021).

The prevention and treatment of cancer presents one of the most difficult healthcare obstacles to overcome during the 21st century, with nearly 20,000,000 cancer cases globally in 2022. In the same year, there were nearly 10,000,000 deaths caused by cancer, making it the 2nd most prevalent cause of death in the world (Bray et al., 2024). An estimated 2,041,910 new incidences of cancer and 618,120 cancer deaths will occur in the United States by the end of 2025 (Siegel et al., 2025).

Conventional cancer treatments include surgery, radiotherapy, and chemotherapy (Arruebo et al., 2011). However, these treatments often have harmful side effects and often cannot completely eradicate cancer tumors (Zafar et al., 2025). Treatments can be classified in two categories: neoadjuvant (administered before main treatment) and adjuvant (administered after main treatment) (Bilusic, 2022). In addition to these conventional cancer treatments, novel approaches like targeted therapy and immunotherapy are gaining traction. Targeted therapy inhibits essential proteins and pathways that facilitate tumor survival, while immunotherapy reinvigorates the body's immune system and allows it to recognize and kill cancer cells (Vanneman & Dranoff, 2012). Clinical immunotherapy treatment modalities include the use of monoclonal antibodies (mAbs), small molecules, cell therapy, oncolytic viruses, and the use of cancer vaccines (Liu et al., 2022).

Of these modalities, the use of mAbs is one of the most commonly used treatments. These antibodies are manufactured outside the body and are administered to eliminate cancer cells, both directly and indirectly (Hamdan & Cerullo, 2023). However, the use of small molecule drugs to reactivate immune cells to fight cancer cells is a promising field for ongoing research (Kerr & Chisholm, 2019). Cell therapy is another viable approach that entails the extraction, replication, and reinjection of patient T cells (Baruch et al., 2017). The use of oncolytic viruses and cancer vaccines as treatments are also under

investigation. Genetically modified oncolytic viruses are utilized to selectively lyse tumor cells (Santos Apolonio et al., 2021), while cancer vaccines facilitate immune system recognition of unique tumor cell identifiers (Lin et al., 2022). Since cancer is a complex disease with a multitude of hallmarks and factors that require consideration (Fouad & Aanei, 2017), many of these treatments are currently being administered in combination with each other to more effectively treat cancer (Singh et al., 2023).

One widely successful method of revitalizing the immune system to fight cancer cells is the administration of immune checkpoint inhibitors (Jenkins et al., 2018). Immune checkpoint proteins are found on T cell surfaces and work to inhibit T cell activation, which prevents an uncontrolled immune response that could potentially damage the host's self cells (Liu et al., 2022). However, some cancerous tumors have the capability to manipulate these immune checkpoints and deactivate the immune system, which facilitates tumor growth (Pardoll, 2012). Consequently, immune checkpoint inhibitor drugs have been developed to disrupt attempts to manipulate inhibitory checkpoints (Darvin et al., 2018). The most extensively researched immune checkpoint targets include PD-1 and CTLA-4 (He & Xu, 2020). Several existing mAb drugs such as Pembrolizumab and Nivolumab block the inhibitory function of the PD-1 protein, which reinvigorates T cell function and fosters a stronger antitumor immune response (Ohaegbulam et al., 2015).

In the same vein, costimulatory molecule pathways (such as OX40 and GITR) play an essential role in the tumor fighting process by facilitating the activation of T cells (Fu et al., 2020). The clinical development of T-cell agonists that activate costimulatory pathways and induce a stronger antitumor response is a highly researched topic (Choi et al., 2020). These costimulatory pathways are not only found in cancer research, however. They can also work in the opposite direction by playing a critical role in the treatment of autoimmune diseases (Sakowska et al., 2022). Autoimmune diseases are serious health conditions characterized by an overzealous immune system that mistakenly attacks the host's own cells (Autoimmune Diseases, 2025). Since these costimulatory pathways increase T cell activation, their inhibition can reduce T cell activity and hinder an autoimmune response. Thus, the development of therapeutic drugs that inhibit costimulatory pathways is an essential part of autoimmune disease research (Jung & Kim, 2022). This study is clinically relevant because it aims to facilitate the discovery of small molecule candidates that can either activate or inhibit costimulatory pathways.

The GITR/GITRL complex is one example of a costimulatory pathway that can modulate the immune system's response strength: the binding of the ligand GITRL to its cognate receptor GITR results in a strengthened antitumor response by contributing to the activation and proliferation of CD8+ and CD4+ effector T-cells (T-effs) and inhibiting regulatory T-cell (T-reg) activity. Thus, GITR agonists that

activate this pathway may have potential anticancer applications (Buzzatti et al., 2019). On the other hand, the inhibition of this pathway sustains T-reg cell activity while hindering T-eff cell activation, which can downregulate overactive immune responses caused by autoimmune disease. Therefore, GITR antagonists that block pathway activation may have potential autoimmune disease treatment applications (Tian et al., 2020).

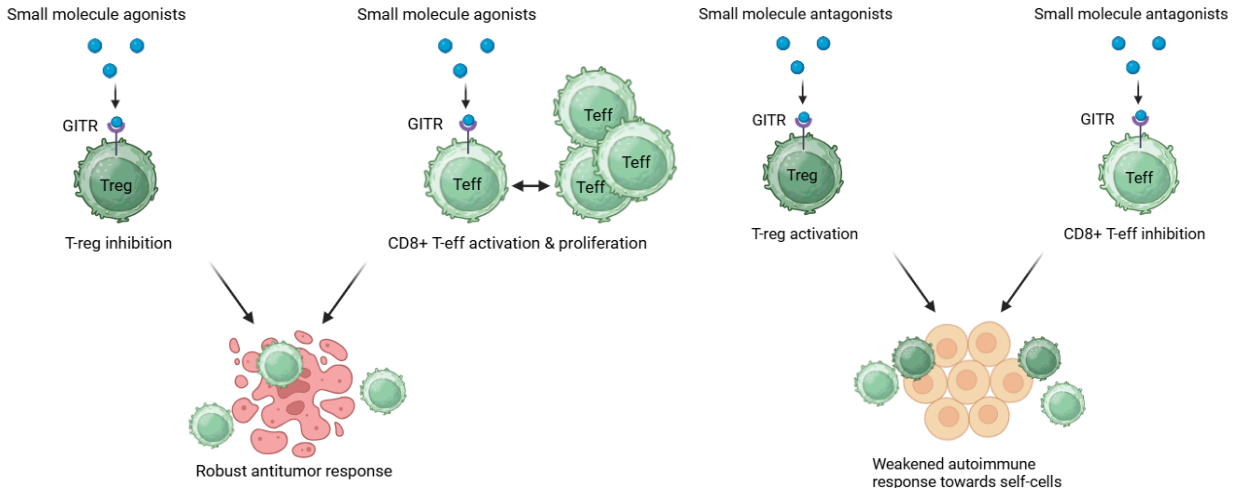

Figure 1. The effects of small molecule agonists on the GITR protein (left) and small molecule antagonists on the GITR protein (right) are shown.

However, ongoing research on the modulation of the GITR/GITRL costimulatory pathway primarily focuses on the applications of mAbs as opposed to small molecules. Multiple research studies investigating the role of DTA-1, an agonistic mAb targeting the GITR protein, discovered that the GITR agonist led to the downregulation of T-reg activity and the upregulation of T-eff activation and proliferation, which can contribute to a stronger antitumor response (Coe et al., 2010; Narumi et al., 2019). However, there are no reported attempts, to our knowledge, in the literature to explore the applications of GITR-targeted small molecules for either cancer immunotherapy or autoimmune disease treatment. Small molecule immunotherapy treatments confer several distinct advantages over mAb treatments, since they are administered orally, better penetrate the tumor microenvironment, circulate more quickly, decrease immunogenicity, and are more easily accessible (Adams et al., 2015; Cheng et al., 2025).

Previous research on small molecule cancer immunotherapy mainly targets immune checkpoint inhibitors instead of costimulatory pathways. For instance, small-molecule inhibitor drugs for the PD-1 immune checkpoint are currently being tested and developed, such as PDI-1, which has been shown to induce antitumor effects through the inhibition of PD-1 immune checkpoints, with effects comparable to

mAb-based immune checkpoint inhibitors (Wang et al., 2021). Moreover, some studies also discuss the applications of small molecule immunotherapy on costimulatory pathways. A research study on small molecule agonists of the OX40-OX40L complex (another costimulatory molecule with mechanisms similar to the GITR-GITRL complex) discovered that small molecules are capable of stimulating this costimulatory pathway and contributing to increased T-eff cell activity while suppressing T-reg cells (Song et al., 2014). This demonstrates that small-molecule cancer immunotherapy treatments targeting costimulatory pathways like GITR-GITRL can be viable for potential anticancer and autoimmune treatment applications.

With these factors in mind, this paper aims to cover this crucial research gap by utilizing comprehensive multi-step virtual screening to identify potential small molecule binding candidates to the GITR protein for the dual purpose of discovering lifesaving cancer immunotherapy treatments and autoimmune disease treatments. Computer-based screening was selected because it is a powerful technique that has the potential to save a significant amount of time and money in the initial steps of drug discovery. This is because the alternative, physical screening in the laboratory, is both time-consuming and costly (Giordano et al., 2022). We hypothesize that if multi-step virtual screening is performed on the GITR protein, then we will be able to efficiently identify small molecule candidates for potential cancer immunotherapy and autoimmune disease treatments.

2. Methods

2.1 Target protein structure:

In this experiment, we utilized the GITR-GITRL complex structure with the PDB ID 7KHD to complete all subsequent virtual screening steps. We chose to screen the activated complex instead of the individual GITR protein and GITRL ligand to more accurately determine the chemical characteristics that small molecule binders should emulate, since ligand-protein conformations can shift slightly when binding to each other. In order to conform to virtual screening requirements, a fixed protein crystal structure was utilized.

2.2 Analysis of binding sites in GITR:

Firstly, potential binding sites were located on the GITR protein to verify its nature as a promising protein target for small molecule immunotherapy. In this study, binding sites for the GITR protein were identified using three methods: geometric analysis with the help of DoGSiteScorer,

energetic analysis with the help of FTSite, and machine learning analysis with the help of Prankweb. Multiple computational techniques were utilized during binding site identification to ensure that there was strong evidence of GITR's potential to bind to small molecules before proceeding with further screening.

2.2a Geometric method (DoGSiteScorer):

DoGSiteScorer is a computational tool that identifies potential binding sites on protein targets by analyzing the protein's geometric and physical structure (Volkamer et al., 2012). To utilize this tool, we navigated to the Proteins Plus website, and entered the PDB code 7KHD, which corresponds to the GITR-GITRL complex. Binding site analysis with DoGSiteScorer then proceeded with the C and D chain of the complex, which corresponds to the GITR protein.

2.2b Energetic-based method (FTSite):

FTSite is an online tool that discovers potential binding sites on protein targets via energy calculations and the theory that small organic molecules can also interact with ligand binding sites (Ngan et al., 2012). To use this tool, we navigated to FTSite and entered the PDB code of 7KHD. The application was then run with chains C and D of the GITR complex.

2.2c Machine learning method (Prankweb):

Prankweb is a virtual technique that identifies potential binding sites on protein targets via P2Rank, a machine learning algorithm (Jendele et al., 2019). To utilize this method, we navigated to the Prankweb website and entered the PDB code of 7KHD. The application was then run with chains C and D of the GITR complex selected.

2.3 Pharmacophore-based virtual screening:

A pharmacophore is a 3-D map that depicts different chemical features vital to ligand-receptor interactions, the frequency of each feature, and the distance between each feature through the use of colored spheres (Seidel et al., 2020). These pharmacophores then undergo virtual screening to identify small molecule binding candidates that match the pharmacophore map (Voet et al., 2014). Virtual screening with pharmacophore maps is a very powerful tool that can be utilized as an alternative to or in conjunction with in vitro screening. In this study, the computational tools PocketQuery and ZINCPharmer were employed to generate two pharmacophore maps for the GITRL portion of the GITR-GITRL complex. 18 million small molecules compounds were then

imposed on these maps to assess the compatibility of potential small molecule binders to the GITR protein. PocketQuery and ZINCPharmer were chosen for their accessibility, ease of use, and online availability.

2.3a Target cluster identification (PocketQuery)

PocketQuery is an online tool that examines ligand-protein interactions and identifies groups of amino acid residues that show promise as potential models for small molecule therapy (Koes & Camacho, 2012a). In this experiment, key amino acids on the GITRL ligand that play a crucial role in binding interactions with the GITR protein were utilized for pharmacophore map generation. To utilize this tool, we navigated to the PocketQuery website and uploaded the protein structure for the GITR-GITRL complex. In this case, the PDB ID 7KHD was not used because it had not been analyzed yet by PocketQuery. Instead, the protein structure was manually uploaded and assigned the PocketQuery ID 5SKH9. After initial screening, we selected an amino acid cluster with a high druggability score from both chain A and chain B of the GITR-GITRL complex, which corresponds to two different regions of GITRL. Subsequently, we exported the two clusters identified to ZINCPharmer for further analysis.

2.3b Virtual screening (ZINCPharmer)

ZINCPharmer is an online tool that matches 18 million small molecules from the ZINC virtual library to a given pharmacophore to identify the most promising potential binders that best emulate the chemical properties outlined in the map. (Koes & Camacho, 2012b). Starting from a pharmacophore map exported to ZINCPharmer, we hid the ligand and receptor residues present in the viewing window. We then submitted the query to view potential small molecule candidates and sorted them according to their Root Mean Square Deviation (RMSD) values. Promising small molecule candidates that deviate minimally from the pharmacophore are assigned lower RMSD scores, while less promising candidates are assigned higher RMSD scores. The top ten small molecule compounds from each of the two clusters screened were selected for further testing.

2.4 Molecular docking

Molecular docking is a technique that simulates binding interactions between small molecules and target proteins. These simulations then display top clusters that highlight optimal binding alignments and evaluate the binding affinity of these conformations (Meng et al., 2011). In this experiment, we used Swissdock to simulate potential docking site interactions between 20 small

molecules and the GITR protein target. Swissdock primarily employs the Attracting Cavities method for small molecule docking simulations. This technique converts the rough protein target into a smooth landscape and employs geometric transformations and energy calculations to identify and score docking sites (Röhrig et al., 2023). These geometric and energetic calculations provide valuable insight when identifying the most promising small molecule binding candidates for the target protein, and are displayed as SwissParam scores.

2.4a Docking with attracting cavities (Swissdock)

Swissdock is an online tool that investigates interactions between small molecules and protein targets and sorts the viability of these interactions using their Gibbs free energy values (Grosdidier et al., 2011). Gibbs free energy values dictate the spontaneity of chemical interactions, with negative values corresponding to more favorable binding interactions between the selected small molecule compound and the GITR protein. To utilize Swissdock, we navigated to their website and pasted the Simplified Molecular Input Line Entry System (SMILES) code for each small molecule being tested, which can be found through ZINC15 database searches. Then, we prepared the ligand for screening and entered the PDB ID 7KHD. We chose to keep Chains C and D of the complex, which correspond to the GITR protein. Next, we prepared the protein target without keeping any heteroatoms. After that, we entered the numbers (-60, 2, 48) to set the search box center and entered the numbers (51, 51, 51) to set the search box size. We set the number of Random Initial Conditions to 1, checked the parameters, and began docking simulations. The top six promising small molecule compounds with the most negative SwissParam scores were then selected for further screening to verify druggability.

2.5 ADME and Lipinski's rule

Small molecule drugs must effectively demonstrate four crucial pharmacokinetic properties to be safe for oral administration: Absorption, Distribution, Metabolism, and Excretion (ADME). Due to the importance of ADME to drug discovery, a multitude of computational models predicting small molecules' ADME properties with well-established metrics have emerged (M. Honorio et al., 2013). Lipinski's Rule of Five is one such example of a metric that evaluates small molecules' ADME properties to determine its druggability. Lipinski's rule requires a given compound to have no more than five hydrogen bond donors, a calculated LogP value less than or equal to five, a molecular mass no more than 500 daltons (500 g/mol), and less than or equal to ten hydrogen bond acceptors (Lipinski et al., 2012). In this study, Lipinski's Rule of Five was used to evaluate

the druglikeness of the top six small molecule candidates with the help of the computational tool SwissADME.

2.5a Druglikeness evaluation (SwissADME)

SwissADME is an online tool that predicts the ADME properties of small molecules and evaluates their druggability potential with a variety of benchmarks, including Lipinski's Rule of Five (Daina et al., 2017). To use this tool, we navigated to the SwissADME website and pasted the SMILES code for each small molecule being tested, which can be found through ZINC15 database searches, and ran the simulation.

2.6 Toxicity screening

Screening for potential toxicities in small molecule compounds is essential to ensure patient safety, but experimental testing in the laboratory is both costly and time-consuming. Consequently, several computational approaches have been developed to predict the toxicity of these compounds in an efficient manner (Banerjee et al., 2018). These tools predict small molecule toxicities using a variety of evaluation metrics, including median lethal dose (LD50) values. Moreover, computational models screen for potential organ toxicity, toxicological endpoints, and toxicological pathways (Banerjee et al., 2024). In this study, Protox-3.0 was used to predict the toxicity values of the most promising small molecule compound to gauge its druggability potential.

2.6a Oral toxicity prediction (Protox-3.0)

Protox 3.0 is an online tool that predicts the potential toxicity of a given small molecule compound with several benchmarks, including the LD50 test (Banerjee et al., 2024). To utilize this technique, we navigated to the toxicity prediction section of the Protox 3.0 website. Then, we pasted the SMILES code for the small molecule being tested, which can be found through ZINC15 database searches. Finally, we selected all the toxicity prediction models and proceeded with examination.

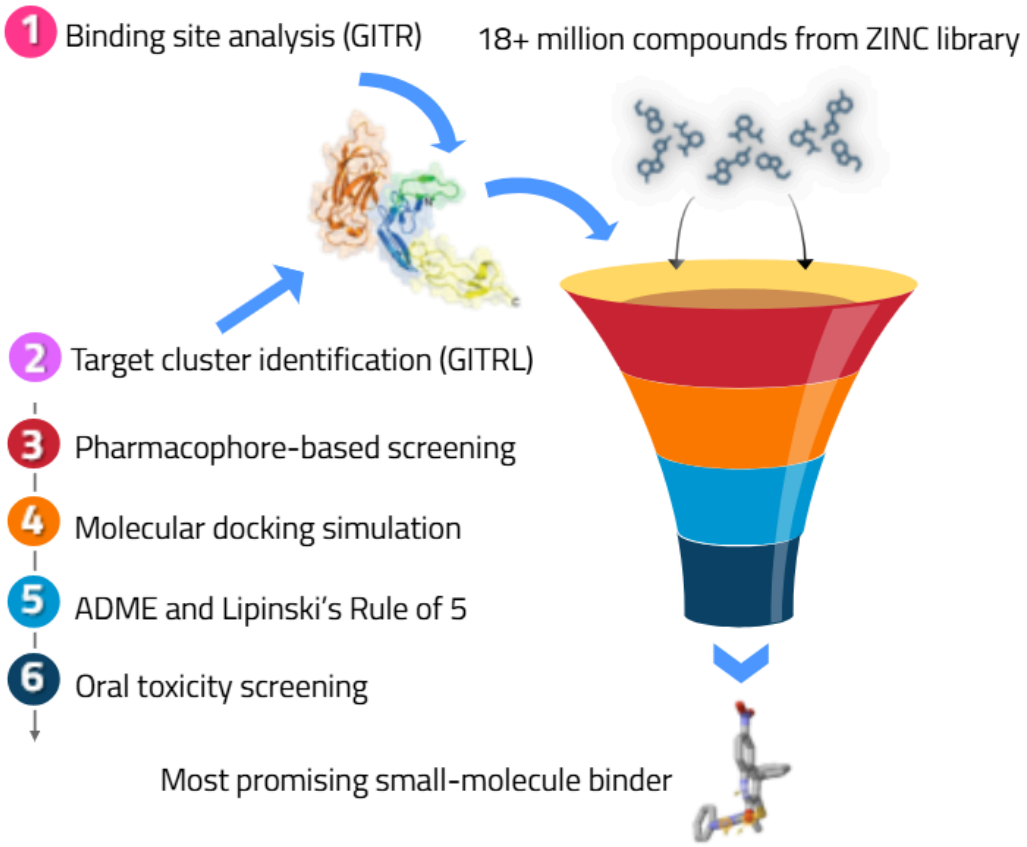

Figure 2. A visual depiction of the virtual screening workflow for finding small-molecule binding candidates to the GITR protein.

3. Results:

3.1 Analysis of binding sites in GITR:

Small molecule binding sites on GITR were analyzed using a collection of computational tools, including investigations of the geometric and physical properties of binding sites to assess their potential, the identification of areas of high binding affinity based on energetic calculations, and the use of machine learning algorithms such as P2Rank. This screening was conducted on the activated GITR-GITRL protein complex represented by PDB ID 7KHD, which contains 4 chains, labeled A, B, C, and D. Chains A and B correspond to GITRL, while Chains C and D correspond to GITR. Since the purpose of this study is to identify potential small molecule binders to the GITR protein, we performed binding site identification on Chains C and D only. All three

methods identified multiple potential binding sites on the GITR protein for small molecule modulators, demonstrating that the GITR protein is a promising target for small molecule immunotherapy. This step allows further screening for potential small molecule binding candidates to occur. The detailed results of binding site identification can be found below.

3.1a Geometric method (DoGSiteScorer):

DoGSiteScorer identified 5 potential binding sites for the GITR protein using the geometric method: two are located on Chain C, and three are located on Chain D. The binding site with the largest volume, surface area, and drug score is P_0 (located on Chain C), with a volume of 580.74 $Å^3$, a surface area of 960.32 $Å^2$, and a drug score of 0.78. The binding site with the second largest volume, surface area, and drug score is P_0 (located on Chain D), with a volume of 494.02 $Å^3$, a surface area of 650.41 $Å^2$, and a drug score of 0.74 (Table 1). From these results, it appears that higher binding site volumes and surface areas correspond to higher druggability potential. Diagrams of binding sites identified on the GITR protein by DoGSiteScorer can be found in Fig. 1.

Table 1. Binding sites in GITR identified using DoGSiteScorer (Geometric method):

Name	Volume ($Å^3$)	Surface Area ($Å^2$)	Drug Score
P_0 (Chain C)	580.74	960.32	0.78
P_1 (Chain C)	128.58	331.07	0.29
P_0 (Chain D)	494.02	650.41	0.74
P_1 (Chain D)	193.79	349.29	0.43
P_2 (Chain D)	103.94	263.07	0.21

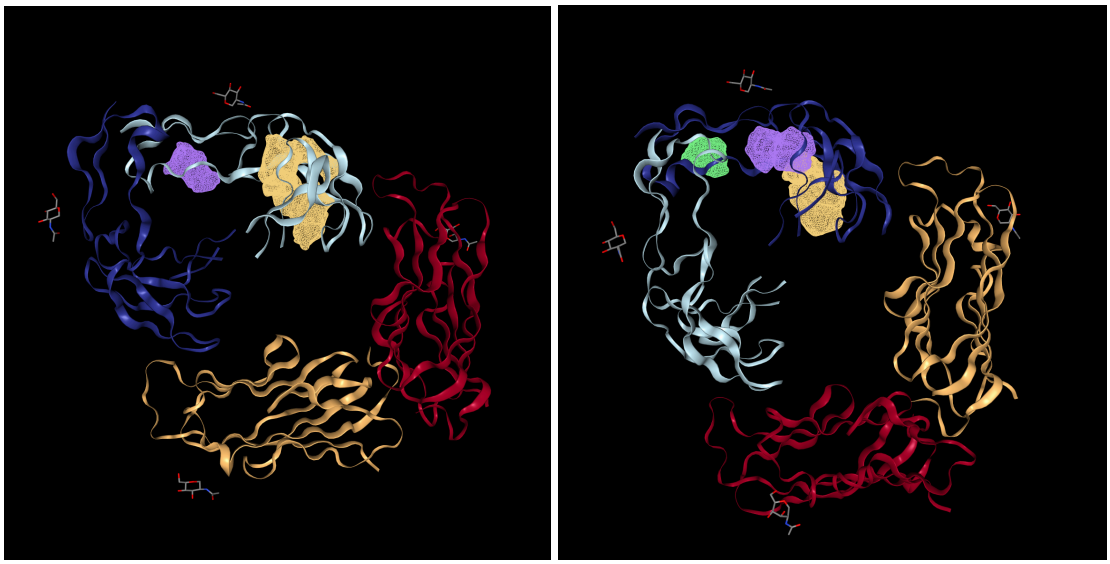

Figure 3. Binding sites in GITR are shown as green, purple, and yellow shaded regions as identified by DoGSiteScorer in Chain C (left) and Chain D (right).

3.1b Energetic-based method (FTSite):

FT site identified 3 potential binding sites for the GITR protein using the energetic-based method. A diagram of binding sites identified on the GITR protein by FTSite are shown (Fig. 2).

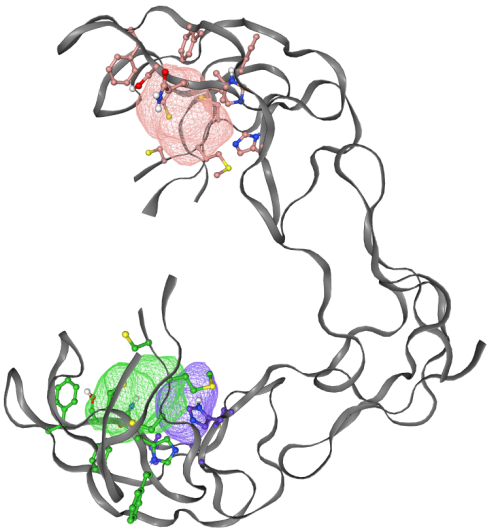

Figure 4. Binding sites for GITR in Chain C and Chain D are represented as red, green, and purple colored spheres as identified by FTSite.

3.1c Machine learning method (Prankweb):

Prankweb identified 9 potential binding sites for the GITR protein using the machine learning method. The binding site on GITR that shows the most promise has a Prankweb score of 4.82, a probability of 0.225, and contains 12 amino acid residues. The second most promising binding site has a score of 3.99, a probability of 0.168, and contains 13 amino acid residues (Table 2). Diagrams of binding sites identified on the GITR protein by Prankweb are shown in Fig. 3 and Fig. 4.

Table 2. Binding sites in GITR identified using Prankweb (Machine learning method):

Pocket Rank	Score	Probability	Amino Acid Residues
1	4.82	0.225	12
2	3.99	0.168	13
3	3.64	0.142	7
4	3.26	0.117	9
5	2.81	0.088	9
6	2.12	0.048	12
7	1.47	0.020	6
8	1.11	0.009	8
9	0.89	0.004	7

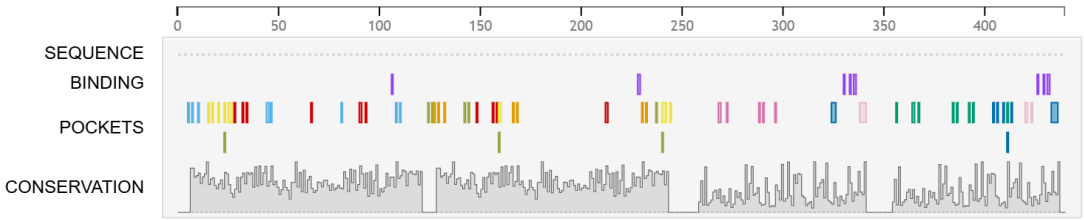

Figure 5. Amino acid residues that are part of potential binding sites in GITR are represented as colored dashes (dashes of the same color belong to the same binding site) based on the GITR protein's amino acid sequence in Prankweb.

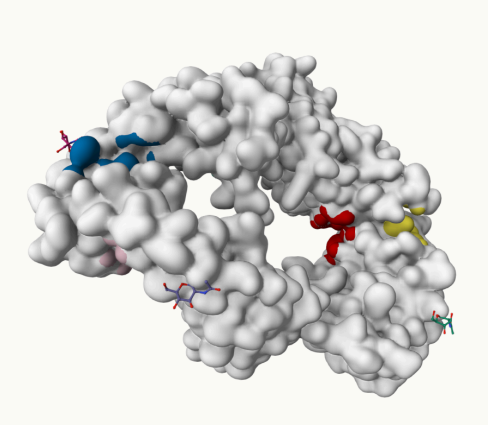

Figure 6. Binding sites in GITR in Chain C and Chain D are represented as colored regions as identified by Prankweb.

3.2 Pharmacophore-based virtual screening:

After we conducted binding site analysis on the GITR protein, we screened the GITRL protein portion of the combined GITR-GITRL complex using PocketQuery. Results yielded two pharmacophore maps highlighting portions of the ligand's chemical structure that are essential to binding interactions with the GITR protein. Then, millions of small molecule compounds were overlaid on these pharmacophore maps with the help of ZINCPharmer to identify promising candidates. We selected the top twenty promising small molecule compounds (ten from each cluster) to move on to the next stage of screening. Virtual screening was conducted on key amino acid clusters on the GITRL ligand instead of GITR because potential small molecule candidates should emulate the chemical properties of the GITRL's binding regions to effectively target the GITR protein receptor.

3.2a Target cluster identification (PocketQuery)

The first pharmacophore map identified using PocketQuery is located on Chain A, contains two amino acid residues, has a maximum distance of 9.5558 Å between each residue, and was assigned a druggability score of 0.730097. The second pharmacophore map identified using PocketQuery is located on Chain B, contains two amino acid residues, has a maximum distance of 9.4921 Å between each residue, and a druggability score of 0.6838 (Table 3). A higher druggability score indicates that the pharmacophore map is more promising than a pharmacophore map with a lower druggability score, since the chemical features outlined by the map more closely align with the ligand binding pocket. These scores range from 0 to 1. However,

it is important to note that values generated by computational methods are not perfectly precise and provide a general estimate of each pharmacophore map's druggability score. Diagrams of each amino acid cluster and their respective pharmacophore maps generated can be found in Fig. 5.

Table 3. Potential model clusters for small molecules in GITRL identified using PocketQuery:

Cluster	Chain	Amino Acid Residues	Distance	Score
1	A	2	9.5558	0.730097
2	B	2	9.4921	0.6838

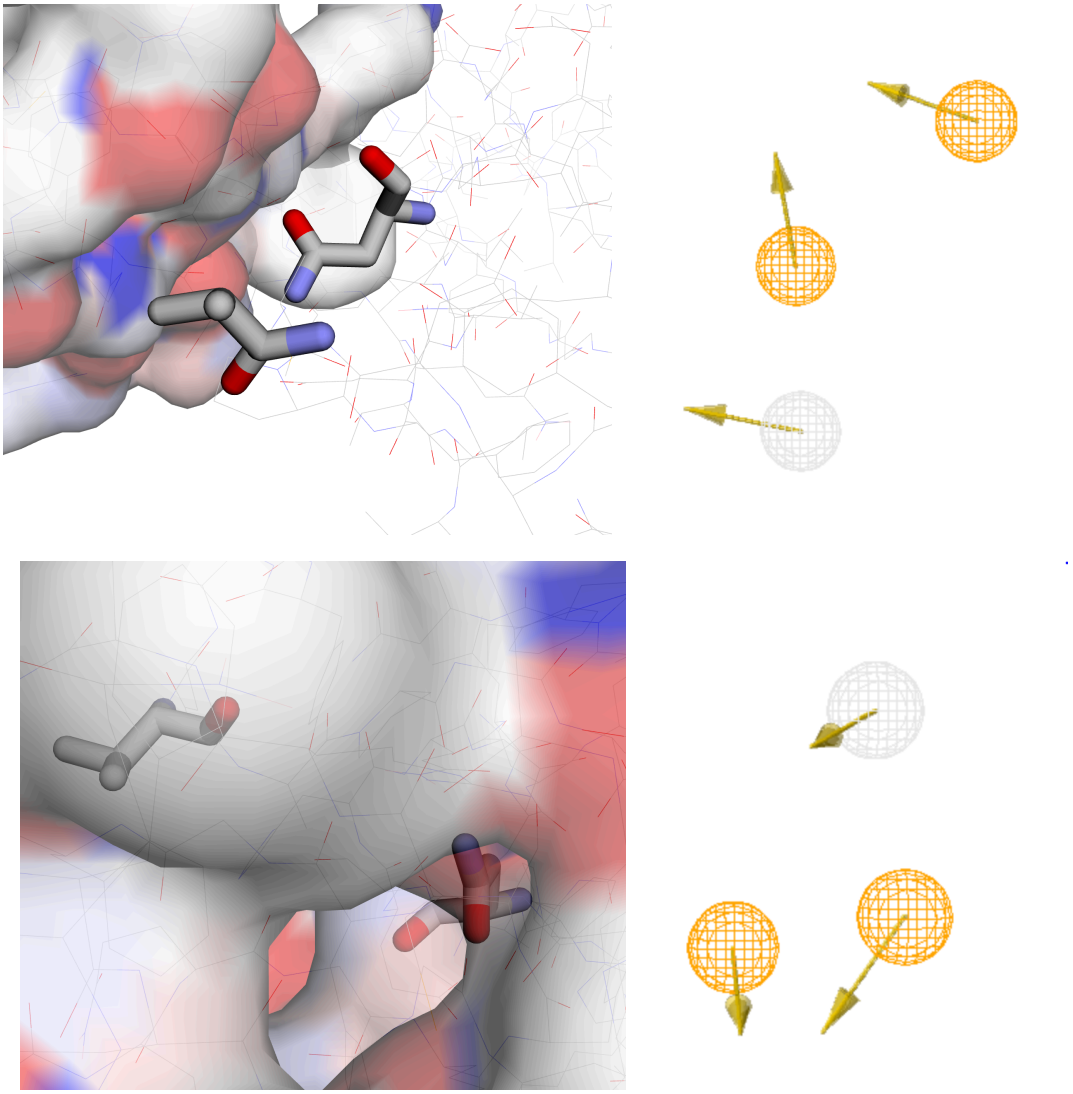

Figure 7. The first model cluster in GITRL is displayed as a 3-D structure by PocketQuery (top left) and as a pharmacophore diagram by ZINCPharmer (top right), and the second modle cluster in GITRL is displayed as a 3-D structure by PocketQuery (bottom left) and as a pharmacophore diagram by ZINCPharmer (bottom right). The gray circle corresponds to a hydrogen acceptor region, while the yellow circles correspond to hydrogen donor regions.

3.2b Virtual screening (ZINCPharmer)

The most promising small molecule binder for the first model cluster in GITRL is ZINC14199378, which has a RMSD score of 0.013 and a molar mass of 418 g/mol. The second most favorable small molecule binder for the first model cluster in GITRL is ZINC09633628, which has a RMSD score of 0.013 and a mass of 442 g/mol. The most promising small molecule binder for the second model cluster in GITRL is ZINC78563990, which has a RMSD score of 0.008 and a mass of 332 g/mol. The second most favorable small molecule binder for the second model cluster in GITRL is ZINC09366331, which has a RMSD score of 0.013 and a molar mass of 446 g/mol. A small molecule with a low RMSD value is more promising than a small molecule with a high RMSD value. These error scores range from 0 to 1. These computationally generated RMSD values provide a useful estimate of each molecule's fit to the pharmacophore maps, but results should nonetheless be verified using laboratory validation. More information on each small molecule compound for each cluster can be found below (Table 4 and Table 5). In addition, diagrams of each small molecule compound overlaid on each cluster's pharmacophore map can be found in Fig. 6 and Fig. 7.

Table 4. Small molecule candidates for the first model cluster in GITRL as identified by ZINCPharmer:

Name	RMSD (Error)	Mass (g/mol)
ZINC14199378	0.013	418
ZINC09633628	0.013	442
ZINC12921150	0.013	429
ZINC09548422	0.014	403
ZINC03357714	0.014	460
ZINC18136748	0.015	431
ZINC08882059	0.015	327

ZINC08882818	0.015	438
ZINC09366332	0.015	446
ZINC09482035	0.016	472

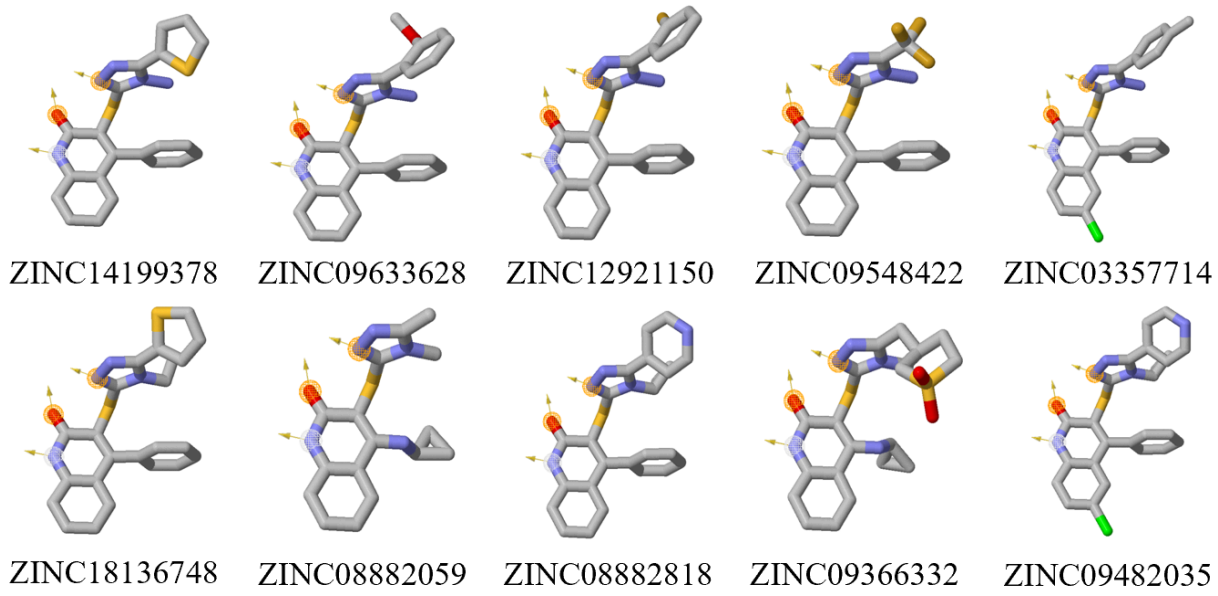

Figure 8. Small molecule candidates for the first model cluster in GITRL are overlaid on the first pharmacophore map as shown by ZINCPharmer.

Table 5. Small molecule candidates for the second model cluster in GITRL as identified by ZINCPharmer:

Name	RMSD (Error)	Mass (g/mol)
ZINC78563990	0.008	332
ZINC09366331	0.013	446
ZINC02405966	0.014	471
ZINC09041947	0.014	470
ZINC13775229	0.015	493
ZINC09310573	0.015	459
ZINC08825363	0.015	425
ZINC02782844	0.015	459

ZINC02782687	0.015	493
ZINC20808334	0.016	432

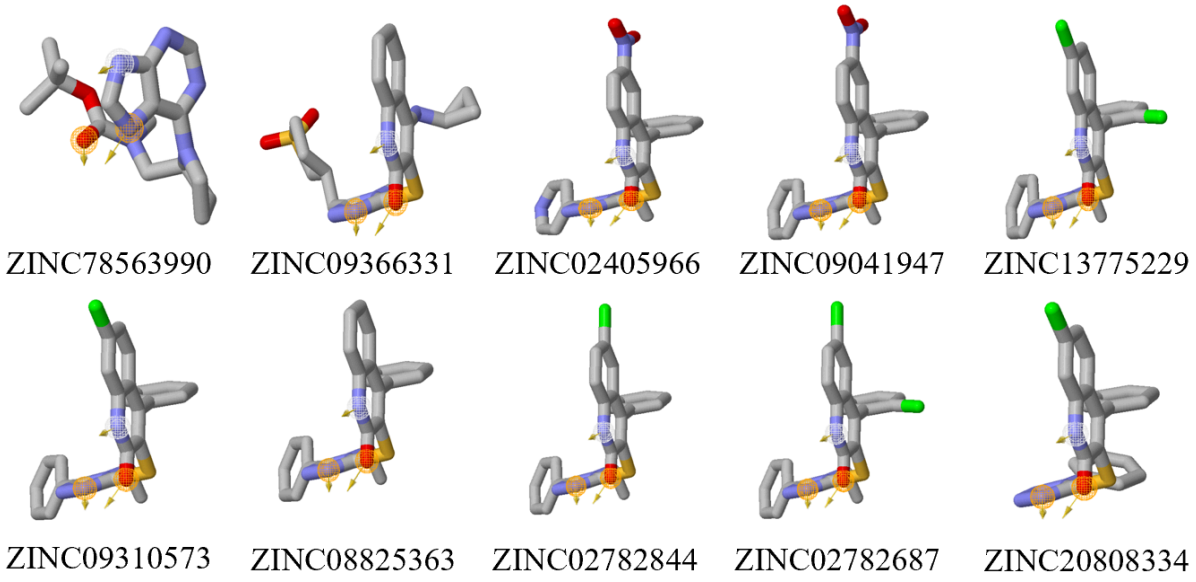

Figure 9. Small molecule candidates for the second model cluster in GITRL are overlaid on the second pharmacophore map as shown by ZINCPharmer.

3.3 Molecular docking

After pharmacophore map generation with PocketQuery and virtual screening with ZINCPharmer, we performed small molecule docking simulations between the GITR protein and each of the top 20 small molecule compounds virtually via Swissdock. The top six small molecule candidates with the most favorable interactions with the GITR protein were selected to move on to the next stage of virtual screening. Swissdock simulates molecular interactions between the small molecule and the GITR protein and determines if each conformation is geometrically and energetically favorable. These simulations are conducted within a search box specified by the user; in this case, we highlighted the entire GITR protein as the search region for docking simulations (Fig. 8). This method yields valuable predictions of each small molecule binder's efficacy while forgoing the need for laboratory testing.

3.3a Docking with attracting cavities (Swissdock)

The six most promising compounds identified were ZINC09041947, ZINC12921150, ZINC09366332, ZINC09482035, ZINC20808334, and ZINC02405966. These molecules were ranked using the SwissParam score provided by Swissdock. The more negative the SwissParam score, the more stable and efficient the binding interaction between the small molecule compound and the GITR protein. ZINC09041947's most promising cluster number was one, with one member and a SwissParam score of -7.9363. ZINC12921150's most promising cluster was one, with one member and a SwissParam score of -7.9264. ZINC09366332's most promising cluster was zero, with one member and a SwissParam score of -7.8711. All three of these small molecules show very strong potential to bind with the GITR protein efficiently. In addition, ZINC09482035, ZINC20808334, and ZINC02405966 also show promise, with SwissParam scores more negative than -7.84 each. Additional information on each small molecule compound (Table 6) and diagrams of each molecule overlaid on the GITR protein can be found in Fig. 9.

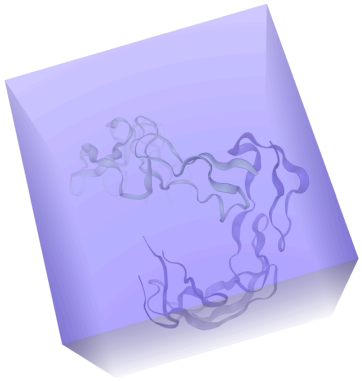

Figure 10. The search box for molecular docking on the GITR protein in Swissdock is displayed along with Chains C and D of the GITR-GITRL complex.

Table 6. The most promising clusters for docking interactions between small molecules and the GITR protein as identified by Swissdock:

Molecule ID	Cluster Number	Cluster Member	SwissParam Score
ZINC14199378	1	1	-7.8123
ZINC09633628	3	1	-7.8348
ZINC12921150	1	1	-7.9264
ZINC09548422	0	1	-7.5029

ZINC03357714	0	1	-7.7748
ZINC18136748	2	1	-7.8330
ZINC08882059	2	1	-7.1899
ZINC08882818	7	1	-7.2719
ZINC09366332	0	1	-7.8711
ZINC09482035	0	1	-7.8669
ZINC78563990	2	1	-7.1608
ZINC09366331	2	1	-7.7804
ZINC02405966	0	1	-7.8469
ZINC09041947	1	1	-7.9363
ZINC13775229	2	1	-7.6457
ZINC09310573	0	1	-7.7246
ZINC02782844	1	1	-7.5973
ZINC02782687	0	1	-7.7870
ZINC08825363	1	1	-7.7859
ZINC20808334	1	1	-7.8599

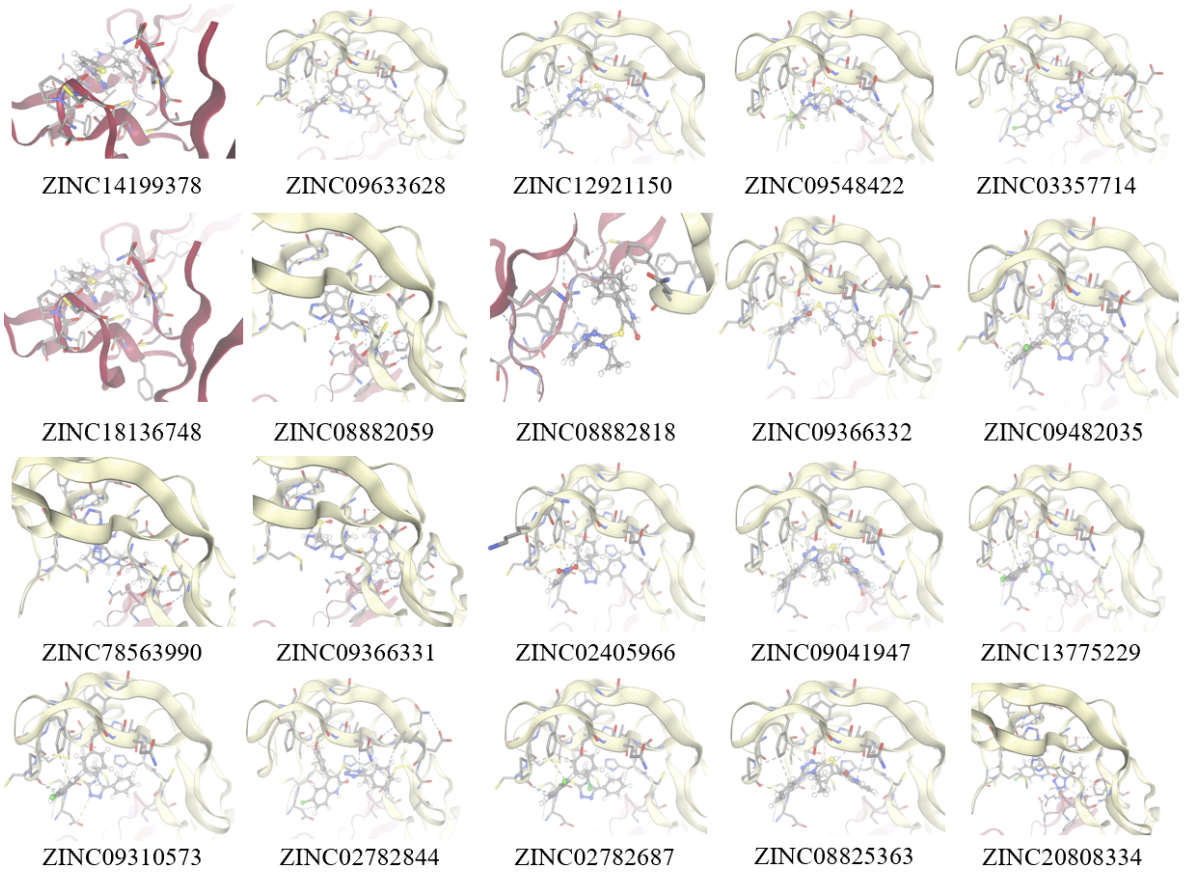

Figure 11. The most promising clusters for docking interactions between 20 small molecule candidates (gray structures) and the GITR protein (red and yellow strands) are displayed by Swissdock.

3.4 ADME and Lipinski's Rule

We screened the top six small molecule binding candidates to the GITR protein for druglikeness using Lipinski's rule. Lipinski's rule tests each small molecule for essential properties, including its capabilities for absorption, distribution, metabolism, and excretion. This step works to ensure that the small molecule has strong druggability potential and can efficiently permeate the tumor environment when administered to patients while also being metabolized and excreted relatively quickly to ensure patient safety. We then selected the most promising small molecule that passed Lipinski's rule as a final candidate for toxicity screening.

3.4a Druglikeness evaluation (SwissADME)

All six of the compounds screened followed Lipinski's Rule, meaning that they all show strong drug potential (Table 7). The most promising compound identified using Swissdock, ZINC09041947, has 1 hydrogen bond donor, a calculated LogP of 2.76, a molecular mass of 469.52g/mol, and 5 hydrogen bond acceptors. The second most promising compound identified using Swissdock, ZINC12921150, has 2 hydrogen bond donors, a calculated LogP of 2.67, a molecular mass of 429.47g/mol, and 4 hydrogen bond acceptors. The third most promising compound identified using Swissdock, ZINC09366332, has 2 hydrogen bond donors, a calculated LogP of 1.84, a molecular mass of 445.56g/mol, and 5 hydrogen bond acceptors.

Table 7. The most promising binding candidates to GITR and their druglikeness evaluations using Lipinski's rule as identified by SwissADME:

Compound	Hydrogen Bond Donors	Calculated LogP	Molecular Mass (g/mol)	Hydrogen Bond Acceptors	Druglikeness (Lipinski's Rule)
ZINC09041947	1	2.76	469.52	5	Yes
ZINC12921150	2	2.67	429.47	4	Yes
ZINC09366332	2	1.84	445.56	5	Yes
ZINC09482035	1	2.85	471.96	4	Yes
ZINC20808334	1	3.63	431.90	4	Yes
ZINC02405966	1	2.47	470.50	6	Yes

3.5 Toxicity Screening

Lastly, we checked the most promising small molecule binder to the GITR protein for potential toxicity using the online tool Protox-3.0. The purpose of this software is to assess the potential organ toxicities, toxicological endpoints, and toxicological pathways associated with each small molecule. This step confirms each binder's druggability potential by ensuring that small molecule candidates identified using virtual screening are safe for oral administration and produce minimal toxic side effects in patients.

3.5a Oral toxicity prediction (Protox-3.0)

Protox 3.0 predicted a LD50 score of 1000mg/kg and a toxicity class of four for ZINC09041947, the most promising small molecule binder to GITR (Table 8). The toxicity class scores range from one to six, with one being the most toxic and six being the least toxic, demonstrating that ZINC09041947 is a relatively safe compound. In addition, the inactive types of toxicity for the compound far outnumbered the active types of toxicity; the darker colored circles represent predictions with confidence scores over 70%, while the lighter colored circles represent predictions with confidence scores under 70% (Fig. 10). Furthermore, the toxicity radar chart for ZINC09041947 demonstrates that the small molecule binder has lower toxicity values for nearly all toxicity types when compared to the average toxicity levels of similar active molecules, showing that it has a high druggability potential (Fig. 11). However, virtual screening results for toxicity testing should be validated with further laboratory testing due to a relatively low accuracy score of 54% (Table 8).

Table 8. Toxicity prediction information for ZINC09041947 as identified by Protox 3.0:

Predicted LD50 (mg/kg)	Toxicity Class	Average Similarity (%)	Accuracy (%)
1000	4	44.86	54.26

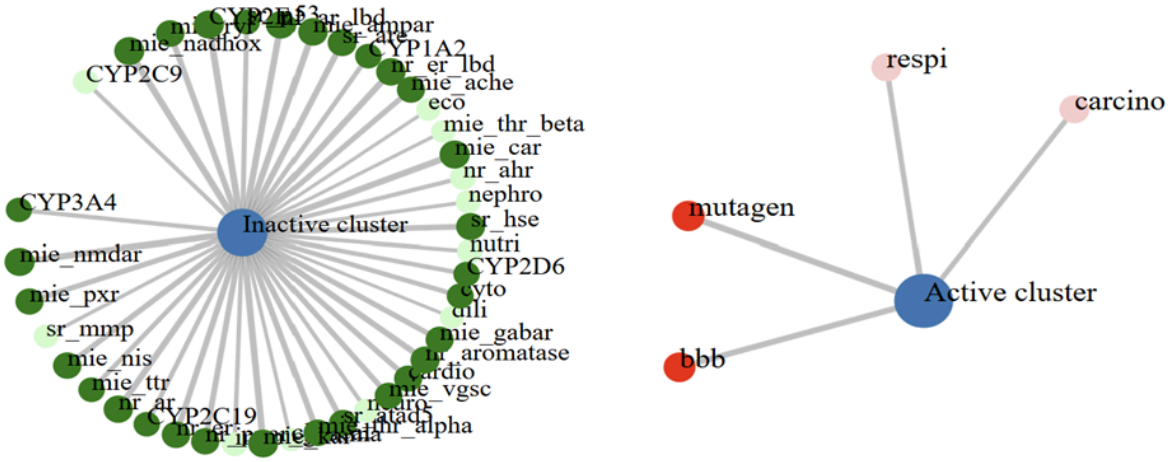

Figure 12. The network charts for the small molecule ZINC09041947 depict the inactive types of toxicity in green (left) and the active types of toxicity in red (right) as identified by Protox 3.0.

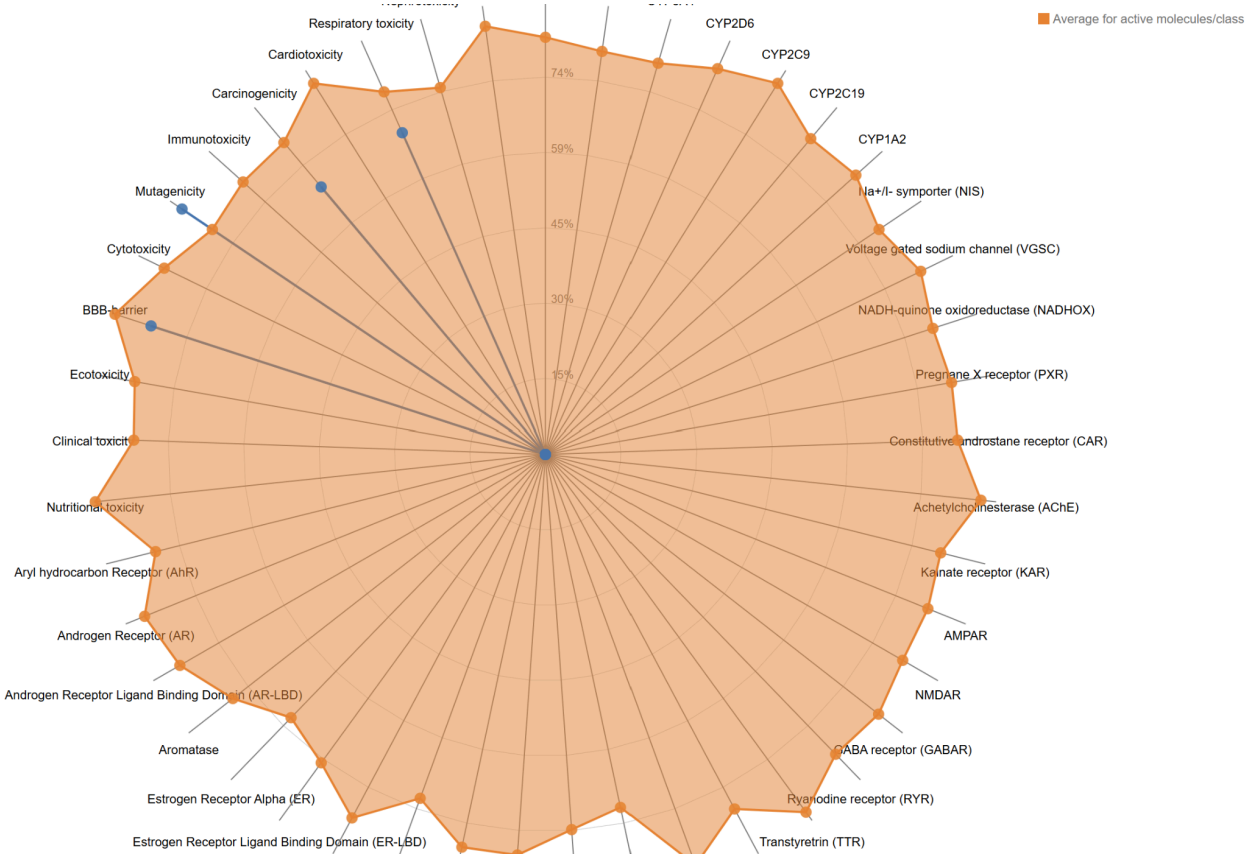

Figure 13. The toxicity radar chart for the small molecule ZINC09041947 compares the toxicity level of ZINC09041947 to the average toxicity levels of similar active molecules as identified by Protox 3.0.

4. Discussion & Conclusions

In this study, we aimed to discover potential modulators of the GITR protein for cancer immunotherapy and autoimmune disease treatment applications. Many GITR-targeted immunotherapy treatments currently being tested utilize mAbs, but none, to our knowledge, investigate the applications of small molecules. Small molecule treatments provide distinct advantages over mAb treatments because they are administered orally, penetrate the tumor microenvironment more efficiently, circulate more quickly, reduce immunogenicity, and have greater accessibility (Adams et al., 2015; Cheng et al., 2025). Consequently, we utilized in-silico screening methods to identify promising small molecule candidates binding to the GITR protein, a crucial first step in the small molecule drug discovery process. First, DoGSiteScorer, FTsite, and Prankweb were used to verify that the GITR protein has suitable binding sites for small molecule compounds. After that, pharmacophore-based virtual screening was performed using

Pocketquery and ZINCPharmer to identify twenty small-molecules with the potential to interact with binding sites on GITR. The drug potential of these small-molecule candidates was then evaluated using free energy calculations determined by Swissdock. The top six small-molecule candidates were then screened using Lipinski's Rule to determine their ADME properties as identified by SwissADME. Lastly, toxicity screening was conducted on the most promising small-molecule compound using Protox 3.0 to verify that it is safe for oral administration. Results indicate that ZINC09041947 is the most promising small-molecule binder to the GITR protein because it has a SwissParam score of -7.9363, adheres to Lipinski's Rule of 5, and has a LD50 value of 1000mg/kg. Moreover, ZINC12921150, ZINC09366332, ZINC09482035, ZINC20808334, ZINC02405966 also exhibit strong binding affinities (SwissParam scores of -7.84 and lower) and follow Lipinski's Rule. Thus, this study identifies six promising small molecule candidates that are worth considering for future testing.

These small molecule candidates that emulate the chemical properties of GITRL have the potential to either activate or to inhibit the GITR costimulatory pathway through the modulation of the GITR protein. These properties are clinically relevant because small molecule agonists of this pathway contribute to increased and enhanced T-eff cell activation and reduced T-reg cell activity, which serves to reinvigorate the immune system to produce a robust antitumor response in cancer patients. Moreover, small molecule antagonists of this pathway contribute to increased T-reg cell activity and reduced T-eff cell activity, which works to temper the immune system to mitigate the impacts of an overzealous immune response in cases of autoimmune disease. Thus, using virtual screening methods to identify potential small molecule modulators of the GITR costimulatory pathway is of therapeutic relevance because these small molecules can eventually be used to treat either cancer or autoimmune disease, which pose serious threats to human health and wellbeing.

One limitation of this experiment is that small-molecule screening was conducted through a completely virtual process because computational methods are restricted in scope and cannot fully validate small molecule binding candidates when used alone. For instance, a fixed crystal protein structure of the GITR-GITRL complex was utilized during computational screening, which may neglect to account for dynamic changes in the proteins during binding. It is known that virtual screening techniques exhibit strong predictive properties, which save scientists a significant amount of time and money in the initial stages of the drug discovery process (Giordano et al., 2022). However, it is important to note that numerical values generated by computational techniques, such as molecular docking scores, provide approximate indicators of each molecule's binding affinities. Thus, computational methods must be supplemented with rigorous laboratory testing in order to confirm the efficacy and safety of small molecule modulators. Potential future steps include physical lab testing with bioassays to verify

ZINC09041947's ability to bind to GITR while determining if it acts as an agonist (cancer treatment applications) or antagonist (autoimmune disease treatment applications).

Furthermore, the structure-activity relationship of the most promising small molecule compounds should be investigated to optimize its efficacy and safety for oral administration. Moreover, each small molecule's potential for serving as bispecific agonists or antagonists for another pathway should be investigated further, since combination therapies and treatments are of increasing relevance (Chan et al., 2022). Many current bispecific treatments are for proteins and mAbs, but the identification of bispecific small molecules is a feasible goal, as demonstrated by a 2023 study that discovered bispecific small molecule inhibitors of PD-L1 and CXCL12 for cancer immunotherapy treatment (Cheng et al., 2023).

In addition to the limitation of utilizing virtual screening with fixed protein structures, another potential concern is that the isolated GITR or GITRL structures may differ from the structure of the GITR-GITRL complex, which may make it challenging for the small molecules identified in this study to bind to GITR in the absence of GITRL. However, a 2021 study on GITR-GITRL complex structure revealed that the GITR protein undergoes minimal conformational changes when binding to GITRL (Wang et al., 2021a), so this factor likely will not impair the ability of promising small molecule candidates to bind to GITR.

Moreover, it is important to note that the GITR-GITRL protein complex is unique because GITRL exhibits trimeric properties and typically binds to three GITR proteins simultaneously. These activated GITR-GITRL complexes then combine to form a branched hexamer network, which plays an essential role in GITR stimulation and contributes to a more robust antitumor response (Wang et al., 2021b). One potential challenge associated with developing small molecule agonists is the difficulty in finding a multivalent binder that is capable of stimulating and clustering GITR proteins together into these hexameric networks while also maintaining the decreased molecular size required by small molecule immunotherapy. This feature may be a strong reason why many GITR agonists being tested and developed utilize multivalent mAbs or multimeric GITRL proteins instead of small molecules (Chan et al., 2022; Davar et al., 2022). Nevertheless, the benefits of small molecules over mAbs mentioned above make the search for GITR-targeted small molecules a worthwhile endeavor. Moreover, small molecule agonists exhibiting these properties have been found for OX40, another costimulatory pathway belonging to the same tumor necrosis factor receptor (TNFR) superfamily (Song et al., 2014). Therefore, finding an effective small molecule agonist for the GITR protein is feasible. In terms of finding small molecule antagonists of the GITR protein to treat autoimmune diseases, the unique clustering properties of the GITR protein may serve as a strength. Specifically, these properties open up the possibility of screening

for small molecules that can prevent the formation of either the hexameric networks or the GITR-GITRL activated trimer, which can temper overzealous autoimmune responses. Moreover, it is important to note that this study focused primarily on small molecule screening for compounds using the interactions displayed by the structure of the activated GITR-GITRL complex as a model. Future studies could also investigate crystal structures depicting interactions between GITR and GITR-specific mAbs currently showing efficacy during testing to potentially find alternate binding pathways that may work better for GITR modulation.

Overall, this study is valuable because it is the first to our knowledge to explore the potential applications of small-molecule immunotherapy on the GITR-mediated costimulatory pathway and sets the groundwork for future research on the development of new treatment alternatives for cancer and autoimmune disease patients.

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Declaration of Academic Integrity

The participating team declares that the paper submitted is comprised of original research and results obtained under the guidance of the instructor. To the team's best knowledge, the paper does not contain research results, published or not, from a person who is not a team member, except for the content listed in the references and the acknowledgment. If there is any misinformation, we are willing to take all the related responsibilities.

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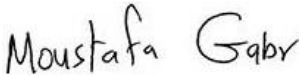

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