

## DETERMINATION OF POTENTIAL BIOTECHNOLOGICAL TARGETS OF BRILLIANT BLUE BY USING COMBINATION OF *IN SILICO* TARGET FISHING AND MOLECULAR DOCKING TECHNIQUES

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### ABSTRACT

Brilliant Blue (C<sub>47</sub>H<sub>49</sub>N<sub>3</sub>O<sub>7</sub>S<sub>2</sub>; E133) is one of the most widely used synthetic food colorants. Although generally regarded as safe under current regulations, concerns persist regarding its synthetic origin and biologically active functional groups. In this study, potential human molecular targets of Brilliant Blue were investigated using *in silico* approaches. The three-dimensional structure of Brilliant Blue was obtained from PubChem (CID: 136664753). Target fishing was performed using the TargetNet web server, followed by molecular docking analyses with the Schrödinger Maestro Suite (v13.9). Sixteen potential human targets were identified, including receptors, transcription factors, metabolic enzymes, and immune-related proteins such as 5-hydroxytryptamine receptor 1E, caspase-9, cytochrome P450 2C9, DNA (cytosine-5)-methyltransferase 1, hepatocyte nuclear factor 4-alpha, nuclear receptor ROR-alpha, and Toll-like receptor 9. Docking analyses confirmed these interactions, with binding affinities ranging from -4.447 to -7.604 kcal/mol. These findings provide insight into the potential molecular interactions of Brilliant Blue and contribute to its toxicological and biotechnological evaluation.

**Keywords:** E133; food colorant; *in silico* target prediction tools; molecular docking; target fishing

## IN SILICO HEDEF TARAMA VE MOLEKÜLER KENETLEME TEKNİKLERİNİN KOMBİNASYONU KULLANILARAK BRİLLİANT BLUE'UN POTANSİYEL BİYOTEKNOLOJİK HEDEFLERİNİN BELİRLENMESİ

### ÖZET

Brilliant Blue (C<sub>47</sub>H<sub>49</sub>N<sub>3</sub>O<sub>7</sub>S<sub>2</sub>; E133), en yaygın kullanılan sentetik gıda boyalarından biridir. Mevcut düzenlemeler kapsamında genel olarak güvenli kabul edilmesine rağmen, sentetik kökeni ve moleküler yapısında bulunan biyolojik olarak aktif fonksiyonel gruplar nedeniyle çeşitli endişeler bulunmaktadır. Bu çalışmada, Brilliant Blue'un potansiyel insan moleküler hedefleri *in silico* yaklaşımlar kullanılarak araştırılmıştır. Brilliant Blue'un üç boyutlu moleküler yapısı PubChem veritabanından (CID: 136664753) elde edilmiştir. Olası protein hedeflerinin belirlenmesi amacıyla TargetNet web sunucusu kullanılarak hedef avlama (target fishing) analizi gerçekleştirilmiş, ardından öngörülen hedefler Schrödinger Maestro Suite (v13.9) kullanılarak moleküler kenetlenme (docking) analizleri ile değerlendirilmiştir. Çalışma sonucunda, 5-hidroksitriptamin reseptörü 1E, kaspaz-9, sitokrom P450 2C9, DNA (sitozin-5)-metiltransferaz 1, hepatosit nükleer faktör 4-alfa, nükleer reseptör ROR-alfa ve Toll-benzeri reseptör 9 gibi reseptörler, transkripsiyon faktörleri, metabolik enzimler ve bağışıklıkla ilişkili proteinleri içeren toplam 16 potansiyel insan hedefi tanımlanmıştır. Moleküler kenetlenme analizleri, -4.447 ile -7.604 kcal/mol arasında değişen bağlanma afiniteleri ile bu etkileşimleri doğrulamıştır. Elde edilen bulgular, Brilliant Blue'un potansiyel moleküler etkileşimlerine ışık tutmakta ve bu maddenin toksikolojik ve biyoteknolojik değerlendirilmesine katkı sağlamaktadır.

**Anahtar Kelimeler:** E133; gıda boyası; *in silico* hedef tahmin araçları; moleküler kenetleme; hedef tarama.

### 1. INTRODUCTION

Since ancient times, colorants have been frequently utilized in the food and beverage industry to improve the visual richness of products and increase their consumer appeal. Today, these colorants serve more than purely aesthetic functions; they are essential for flavor perception, product identity, and meeting the demands of contemporary consumers (Ghorpade et al., 1995; Saleh et al., 2016; Rovina et al., 2017; Fiorito et al., 2023). The association of specific colors with specific flavors is a well-established phenomenon in consumer psychology. This relationship emerges as an important factor influencing consumers' choices and preferences. In this context, it can be stated that food dyes shape the perception of food products not only through visual cues but also through sensory and psychological interactions.

Colourants in the food industry are divided into two main groups: natural and synthetic dyes (Renita et al., 2023). While natural colourants are obtained from plants, microbial sources, and minerals, synthetic colourants are produced through various chemical formulations (Renita et al., 2023). In addition to their low cost, superior coloring efficiency, and extended shelf life, synthetic colorants exhibit high stability against environmental factors such as pH, light exposure, and oxygen. Moreover, their economic advantage makes them a more attractive option for large-scale production compared to natural colorants (Martins et al., 2017; Silva et al., 2022; Renita et al., 2023).

Brilliant Blue, a well-known representative of the synthetic dye class among triphenylmethane dyes, is an important colorant widely used in the food, pharmaceutical, and cosmetic industries (Jasińska et al., 2015). This dye, also recognized by names such as E133, Blue No. 1, and Acid Blue 9, has the chemical name *disodium 2-[[4-[ethyl-[(3-sulfonatophenyl)methyl]amino]phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azanyumidene]cyclohexadiene-1-ylidene]methyl]benzenesulfonate* (Matsufuji et al., 1998). It is commonly employed in the food industry, particularly for achieving intense blue shades (Tanaka et al., 2008; Saxena and Sharma, 2015; Chen et al., 2016; de Lima Barizão et al., 2020; Wu et al., 2020; Oztürk et al., 2023). In the form of a water-

soluble disodium salt, Brilliant Blue not only provides a strong blue color on its own, but, in combination with other dyes, it can also create intermediate shades such as green or purple. This coloring agent is frequently used in the production of various bakery products, ice creams, confectionery, and beverages to enhance visual appeal; it also has various applications in pharmaceutical and cosmetic products (Guo et al., 2013; Ferreira et al., 2016; Ahmed et al., 2021).

On the other hand, long-term and excessive consumption of Brilliant Blue causes significant health problems. It is believed to induce serious adverse effects, such as dose-dependent methemoglobinemia, neurotoxicity, and oxidative stress at the cellular level (Amchova et al., 2015; Bužga et al., 2022; Silva et al., 2022). Furthermore, scientific studies have demonstrated potential associations between excessive consumption of food products containing Brilliant Blue and behavioral disorders, allergic reactions, and toxic effects, particularly during childhood and early adulthood (Table 1).

This raises concerns regarding the safety of Brilliant Blue use in food products and underscores the need for comprehensive toxicological research to establish safe usage limits. Regulatory authorities, particularly the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA), encourage rigorous investigation of the potential health risks associated with colorants such as Brilliant Blue to protect public health and to improve understanding of food additive safety. Special attention should be given to vulnerable populations, including children and individuals with allergies or chronic diseases, as the effects of these substances are critical in shaping food safety policies (Amchova et al., 2015; Silva et al., 2022). In this context, toxicological studies aim to elucidate the molecular mechanisms of compounds such as Brilliant Blue and to provide more detailed information on potential risks.

**Table 1.** Health effects of Brilliant Blue and related diseases

Diseases	Health Effects	References
Asthma	Exacerbation of asthma symptoms	Li et al. 2020
Allergic reactions	Possible allergic reactions such as skin rashes, itching, swelling, and respiratory difficulties	Weimann et al. 2010; Gičević et al. 2020
Hyperactivity	Behavioral changes and attention deficit	Bateman et al. 2004; Cheng et al. 2021
Toxic and genotoxic effect	Genetic mutations and DNA damage in cells	Koç and Pandir, 2018
Neurological effects	Attention deficit and memory problems	Wang et al. 2015; Le et al. 2022
Cancer risk	Carcinogenetic effects	Curry et al. 2013
Liver and kidney problems	Liver overload and kidney damage	Ganesh et al. 2021

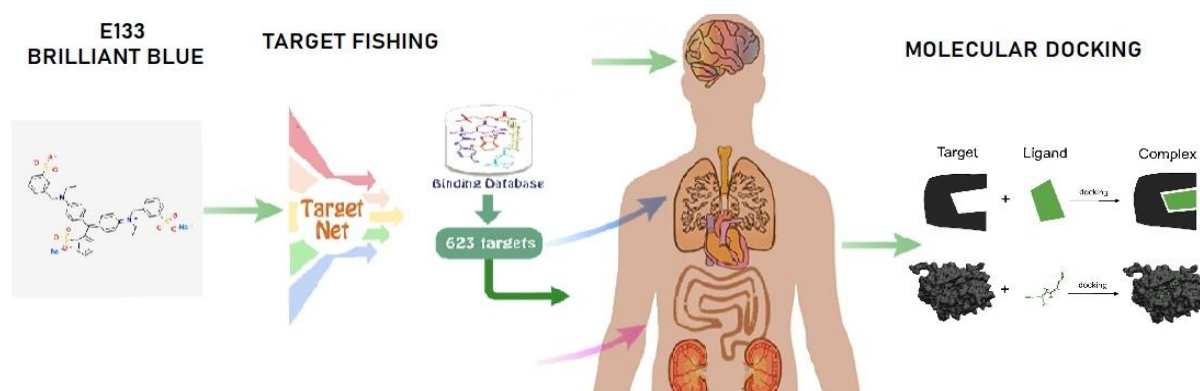
Today, studies aimed at determining potential toxicity are generally based on animal testing. However, such tests require the use of thousands of animals (Allen and Waters, 2013). This leads to substantial budget requirements as well as ethical concerns. Moreover, the complexity of biological systems and the ability of molecules to affect multiple biochemical pathways further complicate the design of experimental processes (Leist et al., 2008; Andersen and Krewski, 2009; Ford, 2017). As a result, this not only hampers the understanding of potential toxic effects but also limits the reliability of the experimental data obtained.

Computational toxicology (*in silico*) approaches are gaining increasing importance in overcoming the challenges encountered in toxicity studies and in generating preliminary data that can contribute to preclinical research. These computer-assisted methods offer the possibility of assessing potential toxicity profiles of chemical compounds more rapidly and at a lower cost by modeling their interactions with biological systems at the molecular level.

Techniques such as molecular target fishing and molecular docking, particularly for food colorants such as Brilliant Blue, assist in predicting the effects of these compounds on target proteins and biomolecules, thereby supporting the determination of safe dosage ranges (Kavlock et al., 2008; Raies and Bajic, 2016).

Target fishing is an important *in silico* approach based on chemical structure that aims to predict potential biological targets. This method accelerates the identification of potential targets by analyzing the structural analogues and biological activities of query molecules in existing databases. Target discovery is especially effectively applied in new drug development processes. Additionally, the target fishing approach plays a crucial role in the target discovery process by facilitating the evaluation of interactions between ligands and their potential biological targets (Wang and Xie, 2014; Galati et al., 2021).

Molecular docking is a computational method that simulates where a ligand (compound) binds to a target protein and how it interacts with that protein (Tao et al., 2019). It is used to select the ligands that are most likely to interact effectively with the potential targets identified by this method. Molecular docking determines which targets molecules can bind to with greater stability by predicting binding sites and binding energies. When target fishing and molecular docking are used together, the potential effects of synthetic compounds such as Brilliant Blue on human targets can be effectively demonstrated (Figure 1). These approaches enable a more precise assessment of potential risks for individuals and represent a significant advancement in food safety evaluation (Tao et al., 2019; Kamerlin et al., 2020; Tortosa et al., 2020).



**Figure 1.** Study summary chart

In this context, the present study was carried out to evaluate the potential effects of Brilliant Blue FCF on human biological targets using *in silico* tools. This approach allows for the development of more focused and effective experimental designs in future studies investigating the toxicity of Brilliant Blue. In addition, it contributes to a deeper and more comprehensive understanding of the potential biological effects of Brilliant Blue and enables a more accurate assessment of toxicological risks. The prediction results generated by TargetNet provided a comprehensive dataset for evaluating the possible biological effects of Brilliant Blue on the human body, which was further supported by molecular docking studies. Moreover, by examining the stability and selectivity of Brilliant Blue within biological systems, a deeper insight into its potential toxicity and biological activity profiles was obtained. In conclusion, this study represents an important step toward evaluating the safety and toxicological profile of food dyes by elucidating the possible interactions of Brilliant Blue with its biological targets. In this context, the findings obtained are expected to contribute to the development of safer applications in both the food industry and public health.

## 2. MATERIALS AND METHODS

### 2.1. Determination of the 3D Structure of Brilliant Blue: PubChem Database

The three-dimensional molecular structure of Brilliant Blue (E133) was obtained from the PubChem database (CID: 136664753), an open-access chemical database that provides comprehensive information on chemical compounds. This database offers detailed data on the structural and biological properties of various compounds.

### 2.2. Selection of In Silico Target Tools for Library Design

TargetNet (<http://targetnet.scbdd.com/calcnet/index>) was used as one of the *in silico* target identification tools to construct the potential target library. TargetNet was selected due to its ability to identify multiple protein targets associated with a given molecule. Designed to predict and explain the interactions of small molecules with a large number of biological targets, this user-friendly, free, and open-access web server offers significant advantages for molecular target identification studies (Gómez et al., 2024).

This platform generates a wide range of QSAR (Quantitative Structure–Activity Relationship) models to predict the activity of 623 human proteins based on existing chemogenomic data. In this study, the SDF file of Brilliant Blue (CID: 136664753), downloaded from the PubChem database, was uploaded to the TargetNet server. Using the default settings, overlapping protein targets identified by TargetNet were allowed. The resulting data were evaluated as predefined targets for subsequent molecular docking analyses.

### 2.3. Molecular Docking

To further investigate the interactions of potential Brilliant Blue targets identified using the TargetNet database, molecular docking studies were performed using the Schrödinger Maestro Suite (version 13.9). Molecular docking is a computational method used to model how a given ligand (in this case, Brilliant Blue) interacts with the active site of a target receptor (Erdoğan et al., 2021; Gómez et al., 2024).

The first step in the molecular docking process involves obtaining the molecular structures of the target proteins identified through target identification tools from the Protein Data Bank (PDB) and PubChem databases. In this step, crystal structures are optimized by cleaning binding sites and enhancing docking sensitivity (Berman et al., 2000; Madhavi Sastry et al., 2013; Erdoğan et al., 2021).

Protein optimization begins with protein preparation, which is critical for improving the precision and accuracy of docking simulations. Using the Protein Preparation Wizard tool, bond networks were optimized, missing atoms in the protein crystal structures were reconstructed, and overall structural stability was enhanced. The protein structures were subsequently stabilized through energy minimization procedures (Madhavi Sastry et al., 2013; Erdoğan et al., 2021).

Following protein optimization, ligand optimization was performed as the second step. The LigPrep computational tool, included in the Schrödinger software package, was used to prepare the ligands for subsequent docking simulations. LigPrep plays a crucial role in assigning correct stereochemistry, protonation states, and tautomeric forms, as well as optimizing ligand

geometry to ensure suitability for docking analyses (Shelley et al., 2007; Madhavi Sastry et al., 2013; Erdoğan et al., 2021).

After ligand preparation, the third stage involved identifying potential binding sites of the optimized ligands on the target proteins. For this purpose, the Protein Grid Generation method was employed. This process involved generating a three-dimensional grid representing the physical and chemical properties of the protein binding sites. The generated grid was then used by an algorithm to evaluate ligand–protein interactions and to predict, with high accuracy, the binding modes and positions of the ligands. Finally, the ligands were docked into the target proteins using the Glide tool. Interactions between the ligands and the binding sites of each protein were evaluated and docking scores were obtained. These scores serve as quantitative indicators of ligand binding affinity and interaction strength within the protein binding sites (Madhavi Sastry et al., 2013; Erdoğan et al., 2021).

### 3. RESULTS AND DISCUSSION

Among the computational approaches commonly used in drug discovery and development, molecular docking and target hunting have proven valuable tools for identifying biological targets of chemical compounds and their binding interactions. These methods, which are generally used to explain the mechanisms of therapeutic action, have become increasingly popular due to their potential to determine unwanted interactions and toxicity of chemical substances. In this study, potential biological targets and protein interactions of Brilliant Blue, a synthetic food dye, were investigated using Target.net and molecular docking techniques.

#### 3.1. Target Fishing

Target detection tool Target.Net was used to investigate the potential effects of Brilliant Blue on human health. Its extensive database and ability to make highly accurate target predictions, supported by machine learning, were instrumental in its selection. Target.Net provides a comprehensive analysis of which proteins in biological systems molecules such as Brilliant Blue can interact with by taking advantage of chemical structure similarities.

As a result of *in silico* screening studies conducted in the TargetNet database, 16 biological target proteins with which the Brilliant Blue FCF molecule may potentially interact in the human body have been identified (Table 2). These potential targets provide important clues about the molecule's biological activities and toxicological profile. In particular, these targets that the molecule is predicted to interact with consist of proteins involved in basic physiological functions such as signal transduction, apoptosis regulation, cellular stress responses and immune modulation. Brilliant Blue's interaction with these proteins has been analyzed in depth to better understand the possible biological effects of the compound.

**Table 2.** 16 biological targets that Brilliant Blue could potentially interact with in the human body, obtained from the Target.Net database

Uniprot-ID	Protein	Prob
P51452	Dual specificity protein phosphatase 3	0.805
P28566	5-hydroxytryptamine receptor 1E	0.195
Q00G26	Perilipin-5	0.151

P31941	DNA dC->dU-editing enzyme APOBEC-3A	0.031
P41235	Hepatocyte nuclear factor 4-alpha	0.001
P55211	Caspase-9	0.574
Q16548	Bcl-2-related protein A1	0.148
P35398	Nuclear receptor ROR-alpha	0.017
P24468	COUP transcription factor 2	0.139
Q9HC97	G-protein coupled receptor 35	0.032
O60240	Perilipin-1	0.031
P11712	Cytochrome P450 2C9	0.016
O60755	Galanin receptor type 3	0.095
P26358	DNA (cytosine-5)-methyltransferase 1	0.001
Q9NR96	Toll-like receptor 9	0.004
P06241	Tyrosine-protein kinase Fyn	0.028

Interactions of Brilliant Blue with targets identified through the TargetNet database provided important information regarding the potential biological effects and toxicity profile of this dye. Its possible interaction with the 5-HT1E receptor, which is directly involved in serotonergic signaling associated with mood and cognitive functions, suggests that Brilliant Blue may exert effects on the human central nervous system (Lanfumej and Hamon, 2004).

Interactions with caspase-9 and Bcl-2 family-related protein A1, which are responsible for the regulation of programmed cell death, suggest that Brilliant Blue may potentially influence the cellular life cycle and apoptotic processes. This finding indicates that the effects of Brilliant Blue present in food products on cellular health should be investigated under conditions of long-term exposure (Kale et al., 2018).

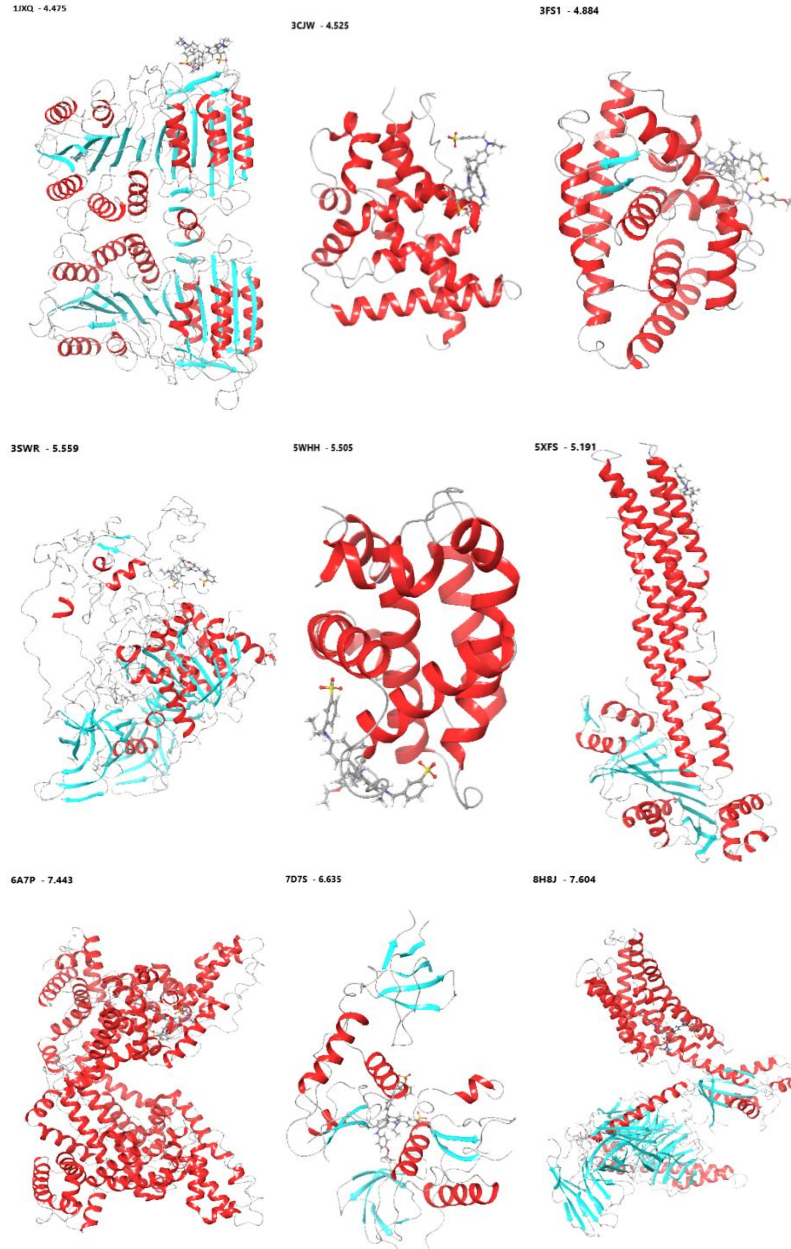
Cytochrome P450 2C9 enzymes, which play a critical role in the biotransformation of numerous chemical compounds, particularly in drug metabolism, were identified as potential targets of Brilliant Blue in hepatic metabolism (Zhou et al., 2009). The interaction of Brilliant Blue with this enzyme underscores the need to evaluate its potential effects on liver enzyme activity, as well as its implications for drug-drug interactions and metabolic processes.

Hepatocyte nuclear factor 4-alpha (HNF4 $\alpha$ ) and nuclear receptor ROR-alpha are key transcription factors involved in the maintenance of metabolic homeostasis and the regulation of cellular differentiation (Gonzalez, 2008). The interaction of Brilliant Blue with these transcription factors suggests that it may have the potential to influence several fundamental metabolic processes, including lipid metabolism and intracellular energy regulation. This emphasizes the need to assess the metabolic effects of the dye in relation to its possible systemic effects and long-term consumption.

Its possible interaction with Toll-like receptor 9 (TLR9) provides important evidence that Brilliant Blue may exert effects on the immune system. TLR9 is a key receptor involved in the regulation of immune responses, and the interaction of Brilliant Blue with this receptor may indicate immunomodulatory potential (Cornelie et al., 2004; Sandholm and Selander, 2014).

### 3.2. Molecular Docking

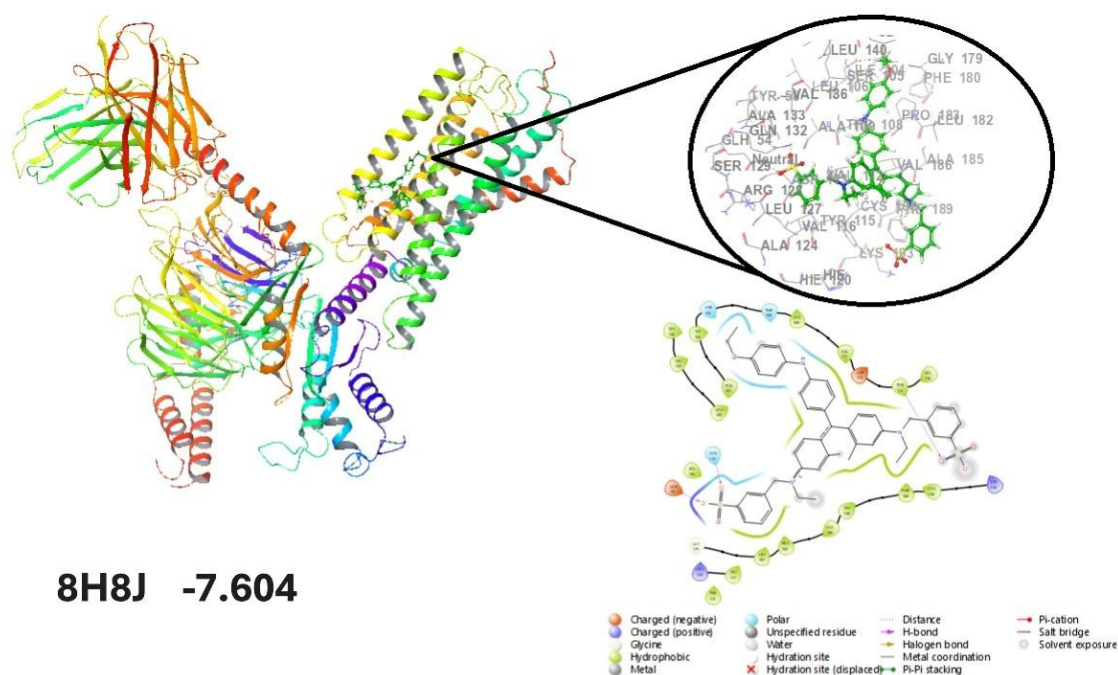
Molecular docking analyses have detailed the interactions between Brilliant Blue and potential target proteins and confirmed the binding affinities of these interactions. As a result of the docking studies, the binding affinities of Brilliant Blue with the target proteins ranged from -4.447 to -7.604 kcal/mol (Figure 2).



**Figure 2.** Molecular docking results (visualized in 2D) of potential human protein targets of Brilliant Blue identified using the TargetNet database.

These binding affinity values indicate that Brilliant Blue interacts strongly with certain targets and may influence specific biological functions. Binding affinity reflects the stability of the complex formed between a ligand and a protein; lower (more negative) binding affinity values indicate stronger and more stable interactions. The high binding affinity of Brilliant Blue for specific targets provides valuable insight into its potential effects on cellular processes. In the molecular docking analyses, the strongest binding affinity was observed with the 8H8J protein, exhibiting a docking score of  $-7.604$  kcal/mol (Figure 3). The high negative value suggests that this interaction may be associated with significant biological activity capable of affecting critical cellular processes. To further elucidate these findings, the structural properties of the proteins interacting with Brilliant Blue were evaluated using grid dimensions, which are critical docking parameters influencing ligand access to protein binding sites and interaction potential.

Accordingly, the relevant grid parameters for each protein analyzed in this study are presented in Table 3.



**Figure 3.** Molecular Docking of Brilliant Blue with 8H8J Protein.

**Table 3.** Docking parameters and glide results for each target protein

Protein name	PDB ID	Glide score	Grid size	vdW scaling factor
Caspase-9	1JXQ	-4,475	20×20×20	0.80
COUP-TFII	3CJW	-4,525	20×20×20	0.80
HNF4a	3FS1	-4,884	20×20×20	0.80
DNMT1	3SWR	-5,559	20×20×20	0.80
Bcl-2-related protein A1	5WHH	-5,505	20×20×20	0.80
PE8-PPE15	5XFS	-5,191	20×20×20	0.80
Human serum albumin (HSA)	6A7P	-7,443	20×20×20	0.80
HIV-1 SF2 Nef	7D7S	-6,635	20×20×20	0.80
Lodoxamide-bound GPR35	8H8J	-7,604	20×20×20	0.80

Molecular docking results revealed that Brilliant Blue exhibited significant binding affinity toward several biological targets, particularly 5-hydroxytryptamine receptor 1E, caspase-9, DNA (cytosine-5)-methyltransferase 1, and Toll-like receptor 9. The high binding affinity of Brilliant Blue for these targets suggests that it may exert biological activity through interactions with these proteins. For instance, Brilliant Blue demonstrated a high binding affinity for the 5-hydroxytryptamine receptor 1E, which is involved in central nervous system disorders such as anxiety and depression, suggesting potential effects on the serotonergic system. This interaction indicates that the possible neurological effects of long-term Brilliant Blue consumption should be evaluated. Caspase-9 is a protein that plays a key role in programmed cell death, and the high binding affinity observed for this protein suggests the potential for interference with cellular apoptotic processes. This finding indicates that long-term consumption of Brilliant Blue may adversely affect cellular health and integrity.

The interaction between Brilliant Blue and DNA (cytosine-5)-methyltransferase 1, an enzyme responsible for the regulation of gene expression, suggests a possible influence on epigenetic mechanisms involved in gene regulation. This finding indicates that the effects of Brilliant Blue

on genetic and epigenetic processes should be further investigated (Legler, 2010; Stevens et al., 2017).

The high binding affinity of Brilliant Blue for Toll-like receptor 9 (TLR9), a receptor involved in the regulation of immune responses, provides important insight into the potential immunomodulatory effects of this dye on the immune system (Vollmer, 2006; Chen and Yu, 2016).

The Glide scores obtained from molecular docking analyses revealed critical information regarding the potential mechanisms by which Brilliant Blue interacts with biological targets identified through the TargetNet database, thereby shedding light on the toxicological and biological profile of the dye. The strong binding affinity of the molecule for certain proteins indicates that Brilliant Blue may pose potential risks, particularly with long-term consumption, and therefore warrants further investigation. The findings of this study contribute to the evaluation of potential health risks associated with Brilliant Blue, which is widely used in the food industry, and to a clearer understanding of its effects on human health. This study highlights the importance of *in silico* approaches such as TargetNet and molecular docking and demonstrates that Brilliant Blue may influence fundamental biological mechanisms, including immune responses, apoptosis regulation, neurological pathways, and other biological processes through interactions with specific target proteins. These interactions underscore the need for further physiological, biochemical, and toxicological studies to elucidate the long-term effects of Brilliant Blue on human health.

#### 4. CONCLUSION

This study demonstrates the significant potential of *in silico* methodologies, particularly target hunting and molecular docking techniques, in identifying the biological targets of food additives such as Brilliant Blue FCF (E133). Our comprehensive analyses and literature review indicate that this compound has the potential to interact with multiple biological pathways. These findings raise critical questions regarding the safety and long-term effects of Brilliant Blue consumption and highlight the need for further research to better understand its mechanisms of action and potential health effects.

The use of computational tools such as TargetNet and the Schrödinger Maestro Suite is of great importance for the assessment of binding affinities and molecular interactions and contributes to a more comprehensive understanding of the toxicological profile of Brilliant Blue. The sixteen potential human targets identified in this study, together with the evaluated binding affinities, enable a thorough investigation of the safety profile of this widely used food colorant. Furthermore, our results emphasize the value of web-based *in silico* approaches in predicting the toxicological and potential therapeutic roles of food additives, facilitating risk assessment processes and potentially reducing dependence on animal experimentation.

In this study and the accompanying literature review, we evaluated the potential interactions of Brilliant Blue with target proteins identified through the TargetNet database based on molecular docking results. Owing to its large molecular size and anionic aromatic rings, Brilliant Blue has the potential to establish significant interactions with polar or positively charged amino acid residues, as well as with hydrophobic residues within protein binding pockets. Moreover, analysis of the binding poses and docking scores of Brilliant Blue revealed that it exhibits distinct interaction motifs across different target proteins (Ferreira et al., 2016). Within the caspase-9 binding site, Brilliant Blue appears to interact preferentially with hydrophobic

pockets, whereas in the 5-HT1E receptor binding pocket, it positions itself within aromatic cavities that recognize ligands, forming  $\pi$ - $\pi$  stacking interactions between aromatic surfaces. In addition, due to its anionic structure, the molecule is likely to form electrostatic interactions with positively charged residues such as lysine and arginine (Kale et al., 2018; Galati et al., 2021). It has also been reported that Brilliant Blue localizes near the DNA-binding surface of DNMT1, exhibiting potential hydrogen bonding and polar interactions with arginine, asparagine, and lysine residues, while interacting more superficially with the TLR9 binding site through predominantly hydrophobic surface contacts (Chen and Yu, 2016; Tortosa et al., 2020). Collectively, these results indicate that the interactions established by Brilliant Blue are not limited to a single mechanism but involve a combination of electrostatic forces, hydrophobic interactions, aromatic stacking, and hydrogen bonding, depending on the chemical properties of the target binding pockets.

Literature surveys indicate that the target proteins identified for Brilliant Blue using computational approaches are largely consistent with those previously reported for triphenylmethane-based dyes. Existing studies primarily focus on cytochrome P450 enzymes, immune response receptors interacting with synthetic dyes, and apoptosis-regulating proteins (Galati et al., 2021; Tortosa et al., 2020), and our findings agree with this body of literature. However, previous studies have not addressed the interaction of Brilliant Blue with nuclear receptors such as HNF4 $\alpha$  and ROR $\alpha$ . In this regard, the present study provides a novel perspective by suggesting potential effects of Brilliant Blue on transcriptional regulation and metabolic processes (Gonzalez, 2008; Kallen and Schlaeppli, 2014; Chen and Yu, 2016; Ferreira et al., 2016; Kale et al., 2018).

In the future, the expanded application of computational techniques in toxicological research may enable regulatory and supervisory authorities in the food industry to improve decision-making processes related to additive use, ultimately contributing to enhanced public health, food safety, and consumer protection. Consequently, this study not only elucidates the molecular interaction profile of Brilliant Blue but also encourages the broader adoption of *in silico* methodologies in food safety research. Future studies should focus on evaluating the clinical relevance of the preliminary findings obtained through computational approaches and on experimentally validating these interactions. In this context, we plan to confirm the interactions of Brilliant Blue with the identified target proteins through binding kinetics analyses, *in vitro* biochemical assays, enzyme inhibition studies, and cell culture models, thereby further advancing our understanding of the safe use of synthetic food additives.

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