Identification of Combination Drug Interaction With Selected Targets of Globlastoma Multiforme Based on Drug Properties and Complex Energy of Docked Complex: a New Approach in Docking Study

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Abstract

Combination drug treatments are frequently used in many diseases, including cancers and AIDS. The main aims of these treatments are to reduce toxicity and improve drug efficacy by inhibiting the activity of a specific target or groups of targets in a cell. The net effect of combination drug efficacy is mainly quantified and interpreted by various terminologies like synergy, additive, agonism, and antagonism based on drugs dose ratio. In single or combination drug treatment, drug action occurs on cells due to the intermolecular level interaction of drugs with protein/DNA/RNA/Enzyme. This type of inter-molecular level interaction can be detected with the help of docking software. However, in the case of combination drug treatments, it is very challenging to identify the multiple drug interaction on each protein with docking score and binding energy. So the proposed work does not follow the classical approach of docking study to predicting the result based on docking score and binding energy. Here we consider drug properties and complex energy of the multiple drug binded complexes for predicting the result. Based on this study, we identified the intermolecular level interaction of synergistic effect showed drugs Crizotinib and Temozolomide in C-MET, C-ROS1, and ALK targets of Glioblastoma Multiforme(GBM) and identified the most stable drug complexes by MD simulation.

Introduction

Glioma is a type of brain tumor developed by glial cells or Neuroglial cells. WHO has categorized gliomas into grade I to IV, in which Grade IV is known as Glioblastoma or Glioblastoma Multiforme(GBM). GBM is considered the most malignant brain tumor[1]. Genetic disorders and ionization factors are the main risk factors of GBM[2]. It is usually found in the frontal, temporal, parietal, and occipital lobe, often located in the cerebellum, and is rare in the spinal cord. In the adult, more than 60% of brain tumors come under the category of GBM. The maximum survival rate of GBM is 14.6 months, thus why new drug development is critical in GBM[1, 2, 3]. The primary treatment of GBM includes surgery, radiotherapy, and chemotherapy[4, 5]. However, drug resistance has frequently developed in GBM patients during chemotherapy, so the prediction of drug response of GBM patients is a significant challenge. The main reason of drug resistance in GBM patients is the co-activation of several functionally linked receptor tyrosine kinases (RTKs), including C-MET (hepatocyte growth factor receptor),C-ROS1 oncogene (ROS1: RTK class orphan), and Anaplastic Lymphoma Kinase (ALK).Thus, the signal redundancy occurs in GBM patients, and that causes drug resistance and poor prognosis effect in treatment. So, target-based treatment is essential in GBM [6, 7]. The standard chemotherapy drug used in the treatment of GBM is Crizotinib. It is a multi-targeted chemotherapy drug used in NSCLC and GBM[8, 9, 10, 11]. However, due to the co-activation of C-MET, C-ROS1, and ALK targets, the prognosis effect of Crizotinib is often unpredictable in GBM patients. To overcome the poor prognosis effect, Crizotinib usually combines with other chemotherapy drugs and has been used as a combination drug in GBM[6, 12, 13, 14]. The main aims of such combination drugs are to achieve synergistic therapeutic effect, dose and toxicity reduction, and minimize or delay drug resistance induction and produce better results against diseases [15, 16]. The drug effecacy of these combination drugs usually be identified by cytotoxic effect [17] based on drug dose ratio, and which is quantified and interpreted by various terminonology like synergy, additive ,agosim and antagonism[18]. In a cell, the cytotoxic effect occurs due to the result of intermolecular interaction between drugs with macromolecules (DNA/RNA/PROTEINS/ENZYMES)[19]. So understanding the intermolecular interaction of drugs with macromolecule studies is most important. The intermolecular interactions of drug and macromolecule can be identified with docking Softwares [20]. However, in the case of combinations drugs, it will be a very challenging process. Because when we apply a combination drug in a particular disease, the combined drug will bind on various sites of single or multiple targets based on its drug properties. Moreover, the desired effect produced by the drug molecules in a diseased pathway always depends on the energy attained the protein structure after when the drug molecules binded on the protein structure. Usually, in docking studies, the potency or effectiveness of drug molecules in a target protein is evaluated by docking score or binding energies [20]. But in the case combination drug docking, we cannot predict the result based on docking score and binding energy. Because here the binding of two drugs on a particular target always depend on its overall energy of the docked complex. When one molecule bind on a particular protein surface, the complex attained a least energy. In that energy level, a second molecule can bind on the protein surface. So if we consider a docked complex with good docking score and binding energy, sometimes the overall energy of the docked complex may be very high. In such situations, the docked complex may not be stable, and the drug cannot produce the desired drug effect in diseases [21]. So in the proposed work, we focused on the drug properties and least complex energy of the docked complex rather than considering its docking score and binding energy.

In the current study, we try to determine the inter-molecular level interaction of combination drug Crizotinib and Temozolomide in C-MET, C-ROS1, ALK targets, which are considered the primary drug resistance factors in GBM. The dose level analysis studies of these targets proteins with the combination drug Crizotinib and Temozolomide showed a synergistic effect in GBM [6]. But the intermolecular level interaction study of these combination drug with C-MET, C-ROS1, and ALK were not reported so far. The molecular-level interaction of Crizotinib with C-MET, C-ROS1, and ALK target proteins has already been reported by X-Ray Crystallographic studies[22, 23] and not in the case of Temozolomide drug. But the insilico studies of individual interaction of both the drugs in targets proteins (C-MET, C-ROS1, and ALK) have also been reported based on drug properties and complex energy. In this study, the result was predicted based on drug properties and least energy of the docked complex rather than using docking score and binding energy of the drugs [21]. From the result, the best docked complex was selected on the basis of MD simulation in pico second. The reason for choosing pico second is that, it is not a study of single compound, which is a study of combination drug docked complex. So if we run MD simulation in nano second for each multiple drug binded complex, it will take more memory and time and also waiting months to year completing all these work. That's why we preferred pico second in MD run.

Results

Docking of Crizotinib and Temozolomide drug with C-MET, C-ROS1, and ALK protein by various Docking methods(forward and reverse docking of drug with proteins).

1. a). Docking of Crizotinib and Temozolomide drug with C-MET protein (Forward drug docking).

From the docking of Crizotinib drug with C-Met protein by various docking methods, a total of 353 drug poses had generated. In which we selected the best-docked complex structure with good druggable binding sites and complex energy were, PDB(P6), RC(S2,P4), DIRECT(P2). In Motif based, we could not identify the druggable binding sites. Based on these three different docking complexes, we again performed protein preparation and also identified the active site for each Crizotinib bounded protein complexes. Then Temozolomide drug docked on each of these binding sites of Crizotinib bounded complexes by four different docking method. In this docking, a total of 77 drug poses of Temozolomide drug were generated and docked on PDB, RC, and DIRECT method of Crizotinib bounded C-MET protein structure .

b). Docking of Temozolomide and Crizotinib drug with C-MET protein(Reverse drug docking).

Docking of Temozolomide drug with C-MET protein, a total of 134 drug poses had generated and docked with C-MET protein by four different active site identification method. From this docking we selected best complexes of each of the method with drug pose number were PDB(P4), RC (S1, P4), DIRECT(P4) and MOTIF(P1). Then we docked second drug Crizotinib on each of the protein complexes followed by protein preparation and active site identification method. From this docking, 507 drug poses of Crizotinib drug were generated and docked on each Temozolomide drug bounded complexes of C-MET protein via four different docking methods.

2. a). Docking of Crizotinib and Temozolomide with C-ROS1 protein (Forward drug docking)

Docking of Crizotinib drug with the protein C-ROS1, 259 drug poses were generated and docked by four docking methods From this docking we selected least energy and good druggable binding site complexes were PDB(P6), RC(S4, P4), DIRECT(P3), and MOTIF(P1). Based on this complex, we performed a second docking with Temozolomide drug followed by protein preparation and active site identification. In this docking, 375 Temozolomide drug poses were generated and docked on each of the Crizotinib bounded complexes of C-ROS1 protein by four different docking methods.

2. b). Docking of Temozolomide and Crizotinib with C-MET protein(Reverse drug docking)

Docking of Temozolomide drug with C-ROS1 protein, a total of 167 drug poses were generated docked in the protein structure by four different docking methods. From this docking, we selected good drug pose with the least energy complexes of Temozolomide drug with C-ROS1 protein structure were PDB (P4), RC (S1 (P4)), DIRECT (P6), and MOTIF (P1). Based on this Temozolomide bounded protein complexes, we performed the second docking of Crizotinib drug followed by protein preparation and four active site identification method in Temozolomide bounded protein complexes. From this docking a total of 238 drug poses of Crizotinib were generated and docked on the Temozolomide drug bounded C-ROS1 protein complex by four different docking method.

3. a). Docking of Crizotinib and Temozolomide drug with ALK protein(Forward drug docking).

In this docking, a total of 388 drug poses of Crizotinib drug were generated and docked on the

ALK protein by four different active site identification method. In this docking, we selected good drug pose with least energy complexes were, PDB(P10), RC(S1(P8)), DIRECT(P1) and MOTIF(P9). Based on these four Crizotinib bounded protein complexes, the second drug Temozolomide, docked on each of the Crizotinib bounded protein complexes by four different active site identification methods followed by protein preparation and active site identification method. In the second drug docking, a total of 79 drug poses of Temozolomide drug were generated and docked on PDB(P10), RC(S1(P8)), DIRECT(P1) and MOTIF(P9) by four different active site identification method docking.

3. b). Docking of Temozolomide and Crizotinib with ALK protein (Reverse drug docking)

Docking of Temozolomide drug with ALK protein, 99 drug poses were generated and docked by various docking methods. In this docking, we selected the least energy Temozolomide bounded complexes of ALK protein from four different methods were PDB (P10), RC (S1 (P8)), DIRECT (P1), and MOTIF (P9). Again we docked the Crizotinib drug on the Temozolomide bounded protein structures by four different docking methods followed by protein preparation and active site identification method. In this docking, a total of 1066 poses of Crizotinib drug were generated and docked on the active site of PDB (P10), RC (S1 (P8)), DIRECT (P1), and MOTIF (P9) complexes of ALK protein by four different docking methods. All the drug docking details, docking method, binding site, the total drug poses generated, drug pose docked on the binding sites, and druggable binding sites are mentioned in the supplementary file S1. This supplementary file S1 also included the docking score, binding energy, complex energy of the docked complex, and the primary hydrogen bond interaction showed amino acid residues at each single and multiple drug-protein complex's binding sites. Based on the drug property and least energy, we selected the least energy showed combination drug bounded complexes of forward and reverse docking from each docking , which are shown in the Table1.

MD SIMULATION analysis result

In the MD simulation analysis study of least energy docked complexes of combination drug Crizotinib and Temozolomide with C-MET, C-ROS1 and ALK protein complexes, first, we consider the combination drug complexes were 2WGJ(PDB(P6)<-RC(S3, P4)) ,2WGJ(DIRECT(P2)<-RC(S1, P5)) and (2WGJ(RC(S2, P4)<-(RC.(S3, P7)). But these complexes show instability in hydrogen bonding during MD simulation. So we considered the next least energy showed complex 2WGJ(PDB(P6)<-PDB (P1)) This complex showed strong hydrogen bond interaction on the binding site of the protein during the MD simulation run. In this complex, the pose number, six of the Crizotinib and pose number one of the Temozolomide drug were showed strong hydrogen bond interaction with

the amino acid residues PRO1158 and MET1160 (PDB(P6)), ASN1167(PDB(P1)) at the binding site of 2WGJ protein. The least complex energy of this docked complex was -11252.7717.

Next we consider the least energy showed complex was the PDB(P6)<-PDB(P4) of 3ZBF protein. This combination drug complex attained the least energy(-11629.573) via when the drug pose number six of the Crizotinib and pose number four of the Temozolomide drugs were bounded on the amino acids were GLU2027, MET2029, ASP2033(Crizotinib), ARG2083, ASP2102,GLY1954(Temozolomide). After the MD simulation run, we could identify that both the drug binded on the active site were strong.

Based on the least energy we considered the combination complex (RC(S1, P8)<-PDB(P2)) of 2XP2 protein. The least energy attained this combination drug complex via when the drug pose number eight of the Crizotinib drug binded on the first site(RC(S1, P8)) of the protein 2XP2(ALK) via RC method and pose number two of the Temozolomide drug binded on the Crizotinib bounded docked complex of 2XP2(RC(S1,P8)) by PDB method (PDB(P2)). The least energy of this docked complex was -11809.0509. After MD simulation, we could understand that the critical amino acid residues MET1199, GLU 1197 interacted with the Crizotinib drug, and the Temozolomide drug interacted with LYS1150 amino acid residues of the 2XP2 protein were strong. Apart from this interaction, some other interactions were identified, which are listed in the Structural analysis Table 3. The results of MD simulation analysis are listed in Table 2. Based on the standard dynamic cascade run, we analyze the protein complex's trajectory by using the analyze trajectory tool of Discovery studio to calculate the RMSD and RMSF of this docked complex.

In the MD simulation run, we consider ligand-free protein, Crizotinib bounded protein complex, and Crizotinib and Temozolomide bounded protein structure of C-MET, C-ROS1, and ALK for understand the structural and conformational changes occurred in the protein structure when multiple drugs binded on the proteins. In the MD simulation , we got the average potential energy to C-MET, C-ROS1, and ALK free protein structure were -65303,-94171, and -78997, 2WGJ (PDB (P6), 3ZBF(PDB(P6)), and 2XP2(RC(S1, P8)) were -65903.3,-94428.5, and -78562.8. In the combination drug complexes, 2WGJ(PDB(P6)<-PDB(P1)),3ZBF(PDB(P6)<-(PDB(P4)) and 2XP2(RC(S1, P8)<-PDB(P2)), we got the average potential energy of each of them were -66150.5,-94690 and -78575.1 respectively.

RMSD, RMSF Value analysis C-MET,C-ROS1 and ALK protein

The Analyze trajectory tool generates 100 conformations for each protein structure based on a 224ps time interval. Based on this we generated RMSD and RMSF graph of combination drug complexes of C-MET,C-ROS1 and ALK complexes, which is mentioned in the Figure1 and 2. From this conformations, we can understand structural deviations [24] and the optimized state of the free protein structure and also when a single and multiple ligands bounded on its active site of the proteins. An RMSD value of an optimized protein structure should be less than 1.5A^o, which is considered a good structure. From the analysis study of 2WGJ free protein structure, Crizotinib bounded protein structure, and Crizotinib and Temozolomide protein structure, we could identify each protein structure went through how much structural deviations. From the graph of RMSD values of 2WGJ protein structure, all the three structures showed different RMSD values until 130ps; however, all the values went through below the allowed range. From the time 130ps, the free protein structure of 2WGJ and Crizotinib bounded 2WGJ protein docked complexes (2WGJ (PDB (P6)) went through the values between 1 and 1.2A^o. However, the RMSD of Crizotinib and Temozolomide bounded protein complex showed the value between 1 and .8 until 212ps. After that, all the three protein structures showed an optimized stage throughout the remaining time. The average RMSD value of free protein structure, Crizotinib bounded protein and Crizotinib and Temozolomide bounded protein structure were 0.966915, 1.05556 and .813927, respectively.

In the case of 3ZBF protein structure, the RMSD value of ligand-free protein, Crizotinib bounded protein structure, and Crizotinib and Temozolomide protein structures were gone below 1.5A^o till the time 104ps. After that, the ligand-free protein structure and Crizotinib bounded protein structure values came in between 1.2 and .8A^o. But the RMSD values of Crizotinib and Temozolomide bounded protein complex showed in between 1.4 and 1.6 till the time104 and then after the RMSD value of this complex moved through 1.4A^o until 224ps. The Average RMSD values of ligand-free protein, Crizotinib bounded protein, and Crizotinib and Temozolomide bounded protein structures of 3ZBF were 0.996956, 1.12523, and 1.19434.

From the RMSD graph of ALK protein structures, we could identify that the RMSD values of Crizotinib and Temozolomide bounded protein complex showed variations. Sometimes the values of this complex reached above 1.5A^o; however, at certain times, it maintained the optimized conformation. Since the time 146ps, ligand-free protein, Crizotinib bounded protein structure, and Crizotinib and Temozolomide bounded protein structures were reached the RMSD value 1.2A^o. And also all the structures maintained an optimized state from this region, and RMSD values came in between 1.2 and 1.4Ao till they reached 224ps. The average RMSD values of ligand-free protein, Crizotinib bounded protein structure, and Crizotinib and Temozolomide bounded protein structures were 1.22515, 1.22941, and 1.34772 respectively.

RMSF value is usually used to measure the ratio of overall fluctuations that occurred in the protein structure [25, 26]. The fluctuations occurred in a protein structure by the movements in the amino acid residues when the drug molecule bounded on the active site. Usually, the terminal and loop region residues have more chances to fluctuations. The acceptable range of overall fluctuations in a protein structure should be less than 2.25A^o (RMSF). When we looked at the ligand-free protein structure(2WGJ), Crizotinib bounded protein(2WGJ(PDB(P6)) and Crizotinib and Temozolomide bounded protein structure (2WGJ(PDB(P6)<-PDB(P1)) of 2WGJ, we could understand that the amino acid residues between VAL1092 and GLY1102, there is a higher fluctuation occurred in this region and the RMSF value is above 2.25A^o. However, in the case of the combination of Crizotinib and Temozolomide bounded protein structure, the value is below 2A^o. In the amino acid residues between SER1152 and HIS1162, there is only minimal fluctuation occurred in this region by the drug Crizotinib bounded on the amino acid residue PRO1158, MET1160 and there is a lower fluctuation occurred in the downward direction when the drug Temozolomide drug bounded on the amino acid residues ASN1167.

Look on the RMSF graph of 3ZBF (PDB(P6)<-PDB(P4)); there are no higher fluctuations occurred in the amino acid residues when compared to ligand bounded Crizotinib complex(3ZBF(PDB(P6)). We could also identify that the terminal residues of the left corner of free protein have higher fluctuation above 2.25A^{o}. Also, we can see that right corner of the terminal residues of ligand-free protein (3ZBF) and Crizotinib bounded protein structure, there is a higher fluctuation in amino acid residues, and RMSF values in this region also had gone above 2.25A^{o}. But the overall fluctuations of combination drug Crizotinib and Temozolomide bounded protein complex (3ZBF (PDB (P6) <-PDB (P4)) is less than 2.25A^{o}. Also, when we look at the amino acid residues between ILE2024 and LEU2034, there is a downward fluctuation occurred. This is the region where the first drug Crizotinib bounded on the amino acid residues GLU2027, MET2029, and ASP2033. The second drug Temozolomide drug binding site, ARG2083, ASP2102, and GLY1954, we could see fewer fluctuations in this region.

In the case of ligand free protein, Crizotinib bounded protein structure and Crizotinib and Temozolomide bounded protein structure 2XP2 (RC (S1, P8) <-PDB (P2)) of 2XP2, all these three structures, RMSF fluctuation ratio is below 2.25A^{o}, and there is a minor fluctuation occurred in three structures. When comparing the Crizotinib bounded protein complex(2XP2(RC(S1, P8)) and Crizotinib and Temozolomide protein complexes of 2XP2(2XP2(RC(S1, P8)<-PDB(P2)), the drug Crizotinib bounded key amino acid residues GLU1197, MET1199, and Temozolomide region LY1150, there is no more fluctuations occurred in both the structure when compared to the ligand-free protein structure of 2XP2. The overall fluctuation rate of Crizotinib and Temozolomide with 2XP2 protein structure (2XP2 (2XP2 (RC (S1, P8)<-PDB(P2))) was significantly less compared to Crizotinib bounded protein structure.

Structural analysis studies of the best-docked combination drug complexes

In the combination docking of Crizotinib and Temozolomide with C-MET,C-ROS1 and ALK proteins, we identified 2WGJ(PDB(P6)<-PDB(P1)), 3ZBF(PDB(P6)<-PDB(P4)) and 2XP2(RC(S1, P8)<-PDB(P2)) are most stable complexes by MD simulation. In the binding site of 2WGJ(PDB(P6)<-PDB(P1)) docked complex, a total of 30 noncovalent bond interactions formed by Crizotinib and Temozolomide drugs. In the first binding site of this complex, 22 noncovalent bond interactions were formed between the drug Crizotinib and the amino acid residues. Which include hydrogen bond, hydrophobic and electrostatic interactions. The hydrogen bond interaction includes 3 conventional hydrogen bonds(ASP1164,PRO1158(2 times)) and the 3 carbon-hydrogen bond(ILE1084,ASP1164,MET1160). The electrostatic interaction which includes one attractive charge(ASP1164). And the main hydrophobic interactions were 7 alkyl interaction (ALA1221,ALA1226(2 times),LEU115,LEU1140,VAL1092,LEU1157) and 8 Pi-alkyl interaction(TYR1230(2 times,ILE1084(2 times), ALA1108,MET1211(3 times). The second drug Temozolomide formed a total of seven noncovalent bond interactions at the protein binding site (PDB (P2), which includes two conventional hydrogen bonds (ASN1167 (2times)), one carbon-hydrogen bond interaction (ILE1084). The electrostatic interaction includes one Pi-anion (ASP1164) interaction. The hydrophobic interaction includes one amide–pi stacked interaction formed by the amino acid residue TYR1230, ASP1231 with Temozolomide drug, and one Pi- alkyl interaction formed by ARG1208 and Temozolomide drug. Apart from drugs with amino acid noncovalent bond interactions, we also identified one drug-drug interaction with Crizotinib and Temozolomide at protein binding sites (5394:H17 -:11626560:F3-Hydrogen bond).

From the combination drug complex 3ZBF(PDB(P6)<-PDB(P4)), a total of 23 drug interactions had formed by Crizotinib and Temozolomide drug on the active site of 3ZBF protein. The main noncovalent bond interactions formed were hydrogen bonds, hydrophobic, and electrostatic interactions. In which Crizotinib drug generated 16 interactions with the amino acid residues of the protein at the binding site. Which included six hydrogen bonds (3 convention hydrogen bonds (ASP2033, GLU2027, GLU2027) and three carbon-hydrogen bond interactions (ASP2033, MET2029 (2 times)). The main hydrophobic interaction with amino acid residues and Crizotinib drug were five alkyl interactions(LEU2086, LEU2010, LEU2026, VAL1959, LYS1980) and four Pi-alkyl interactions(LEU1951, ALA1978, LEU2086(2times)). The electrostatic interaction formed with amino acid residue ASP2033 and the Crizotinib drug. The second drug Temozolomide formed seven interactions with the amino acids at the binding site. Which includes three conventional hydrogen bonds (ARG2083, GLY1954, ASP2102), three carbon-hydrogen bond interactions(ARG2083, ASP2079(2times)) and one Pi-alkyl interaction(ARG2083).

In the combination docking complex, 2XP2 (RC(S1, P8)<-PDB(P2), we could identify 26 noncovalent bond interactions, which includes hydrogen bond and hydrophobic interactions. In this, Crizotinib drug formed (2XP2 (RC (S1, P8)) 20 interactions, which includes two conventional hydrogen bond interactions (MET1199, GLU1197), one carbon-hydrogen bond interaction (MET1199). Hydrophobic interactions found on the binding site with Crizotinib were four alkyl interactions (LEU1196, LEU1256, VAL1130, LEU1196) and 4 Pi-Alkyle interactions (LEU1122, ALA1148, LEU1256 (2times)). Apart from this interaction, another type of interaction found at the binding site was Salt Bridge; Attractive Charge hydrogen bond interaction (GLU1210). Apart from this interaction, we could also find out 8 drug –drug interaction at the binding site, which include alkyle(11626560:C12-5394,11626560:C25-5394), pi-alkyle(11626560:C13:5394, 11626560:C11:5394(2times), 11626560:C25:5394) and Pi-Pi stacked(11626560-5394(2 times)). The second drug Temozolomide drug formed 6 non covalent bond interaction at the binding site of Crizotinib docked complex structure 2XP2 (RC (S1, P8)). Which include one conventional hydrogen bond interaction (LYS1150) and two carbon-hydrogen bond interactions (HIS1124, ASP1270). The hydrophobic interactions included one alkyl interaction (LYS1150) and two Pi-alkyl interactions (ALA1126 (2times)). The details of intermolecular interaction of Crizotinib and Temozolomide on the binding site of C-MET (2WGJ), C-ROS1 and ALK protein are shown in the Table 2. The 2D and 3D images of drug interactions of Crizotinib and Temozolomide drug with C-MET, C-ROS1, and ALK protein are mentioned in the Figure 3.

Discussion

Combination drugs are usually used in many diseases, including cancer and AIDS. The main aim of such types of combinations drugs is to reduce toxicity, decrease or delay drug resistance, and improve drug efficacy, thus increasing survival rate. The drug efficacy of these combination drugs is usually determined by the drug dose ratio, and the efficacy of the drugs is interpreted using various terminologies like additive, agonism, synergism, and antagonism [18] . Efficacy of a drug usually occurs due to the result of intermolecular interaction between drug and DNA/RNA/Protein/Enzyme [19]. So the intermolecular interaction studies of combination drugs are significant. The intermolecular interaction of a single drug with protein can be identified by docking software [20]. But in the

case of combination drug treatment, multiple drug interactions will occur on the binding site of the proteins. So finding out such type of multiple drug interaction are very important in structural biology studies. However, identifying multiple drug interactions with protein is very challenging in docking studies, which are not reported so far now. In the current study, we try to find out the intermolecular interaction of the combination drug Crizotinib and Temozolomide with C-MET, C-ROS1, and ALK targets of GBM. To identify the intermolecular interaction these drugs with proteins, we performed a forward and reverse drug docking with C-MET, C-ROS1, and ALK proteins. From this combination drug docking of 2WGJ, 3ZBF, and 2XP2 proteins, a total of 19 combination drug docked complexes of 2WGJ protein, 30 combination drug docked complexes of 3ZBF protein, and 32 combination drug docked complexes of 2XP2 protein were selected based on drug properties and CE. From the result, we could identify that in the single drug docked complexes, Temozolomide drug with C-MET, C-ROS1 and ALK protein were showed the least energy and which are already reported [21]. But in the case of combination docking complexes, (forward and reverse docking of drug with proteins), we could understand the combination drug docked complexes of Crizotinib and Temozolomide with protein 2WGJ, 3ZBF, and 2XP2 were showed the least energy. The details are mentioned in the **Table 1**. From this Table, we selected the most stable combination drug complexes of 2WGJ(PDB(P6)<-PDB(P1)), 3ZBF(PDB(P6)<-PDB(P4) and 2XP2(RC(S1, P8)<-PDB(P2)) by MD simulation run, which are mentioned in the **Table 2.** In the Combination drug complex 2WGJ(PDB(P6)<-PDB(P1), the drug pose number six of the Crizotinib drug and pose number one of Temozolomide drugs were interacts with the key amino acid residues PRO1158, MET1160 (Crizotinib), ASN1167 on the active site of the 2WGJ protein by the PDB method. The amino acid residues, PRO1158,MET1160 are already reported as ATP binding sites of C-MET protein [22] . And also the studies already reported that the Asparagine amino acid promotes cancer cell proliferation [27] . So the binding of Temozolomide drug in ASN1167 amino acid residue will lead to the inhibition of protein activity. So we can say that the binding of combination drug Crizotinib and Temozolomide drug action will strongly inhibit the activity of tyrosine kinase protein 2WGJ function in GBM cancer.

In docked combination drug complex 3ZBF(PDB(P6)<-PDB(P4), the drug pose number six of Crizotinib and drug pose number four of Temozolomide drug were bound on the protein active site by PDB method. The first drug Crizotinib binded on the amino acid residue GLU2027, MET2029, and ASP2033 and Temozolomide binded on the ARG2083, ASP2102, and GLY1954 residues. Here also both the drug binding on the ATP and methylation site of the protein [23,28,29,30,31] . Docking of Crizotinib and Temozolomide drug with 2XP2 protein, we got the least energy complex was 2XP2 (RC (S1, P8)<-PDB(P2)). In this docked complex, pose number eight of Crizotinib binded at first sight of the protein by RC method and pose number two of temozolomide drug binded on the second site of protein by PDB method. In the first active site, Crizotinib drug binded on the amino acid residues GLU1197, MET1199, and Temozolomide binded on the amino acid residue LYS1150 showed a strong hydrogen bond interaction at the binding site. Amino acid residues GLU1197, MET1199 were reported as ATP binding sites of 2XP2 protein and also reported that the binding action of Crizotinib drug on these amino acid residues [22]. The Temozolomide drug action on Lysine amino acid residue was also reported [28,29]. Based on the intermolecular interaction of Crizotinib and Temozolomide drug on 2WGJ,3ZBf, and 2XP2 protein, we can say that the combined effect of Crizotinib and Temozolomide binding will give more potent inhibition in these proteins rather than Crizotinib alone action in C-MET, C-ROS1, and ALK protein.

Methods

The software used for the docking study was Discovery Studio 2018(v18.1.100.18065), Server: DCBDELL-DSSERVER1. The online resources used for the study were Uniprot, PDB, NCBI, PDBsum, and PubChem databases. We downloaded the 3D structure of target proteins 3ZBF(C-ROS1), 2WGJ(C-MET), 2XP2 (ALK) from PDB[32] and the details of X-ray crystallographic structures of these protein complex) and their hydrogen bond interaction with Crizotinib drug are shown in **Table4**.

Here, all the protein structures have only one chain. Moreover, the specific functional region found at MET and GLU amino acid residues in 2XP2 and 3ZBF protein structures, but in 2WGJ protein, reported at MET and PRO amino acid residues.

The small molecules downloaded from PubChem databases for the docking studies [33] and the 2D images and Chemical properties of both Crizotinib and Temozolomide are shown in T**able5**.

Docking

For docking studies, we used the Libdock tool of Biovia Discovery Studio2018 ((Ref: Discovery studio user manual)[34] . The reason is that Libdock will generate hundreds of pose conformations for a single molecule, and also it shows good scoring accuracy in drug poses [35,36] . The pipeline used for the docking study is shown in **Figure4.**

Before docking, we prepared the ligand and protein molecules. In protein preparation, the ligand molecule was removed from all the protein structures. Then protein preparation is performed with an automatic preparation tool of BIOVIA Discovery studio(Ref; Discovery studio user manual). Then the drug molecules Crizotinib and Temozolomide were prepared and minimized by ligand preparation and minimization tool of BIOVIA Discovery Studio 2018. This tool minimized the ligand using 2000 steps by smart minimizer algorithm and set RMS gradient at 0.01. CHARMm force fields were used in both the tools [37] . After the preprocessing of ligands and proteins, we identified the protein's active site for docking by the following methods.

PDB Site record: Here active site of the protein was identified by the already reported binding region from the PDB file.

Receptor Cavity (RC): In this method, the protein's binding site is derived from large cavities of the protein structure.

Direct/Site-specific method: Here, the active site is selected based on prior knowledge about key amino acid residues. In C-MET, C-ROS1, and ALK proteins, we selected the amino acid residues were (PRO1158, MET1160), (GLU2027 MET2029) and (GLU1197, MET1199) respectively.

Motif-based method: Here, we selected the DFG motif of each RTKs protein along with key amino acid residues. Because the DFG motif of RTKs protein has significant role in activating the kinase protein activity [38]. In C-MET, C-ROS1, and ALK proteins, we selected key amino acid residues and DFG motif were (PRO1158, MET1160, and the DFG motif were ASP1222, PHE1223, GLY1224), (GLU2027, MET2029, and the DFG motif were ASP2102, PHE2103, GLY2104) and (GLU1197, MET 1199, and the DFG motifs were ASP1270, PHE1271, GLY1272) respectively.

After the active site identification, we performed two types of docking in each protein's structure by libdock tool.

1. Docking of Crizotinib and Temozolomide drugs with C-MET, C-ROS1, and ALK proteins (forward docking of drugs with proteins).
2. Docking of Temozolomide and Crizotinib drugs with C-MET, C-ROS1, and ALK proteins (reverse docking of drugs of drug with proteins).

In forward and reverse docking of drugs with proteins, we selected the best-docked complex based on drug properties and complex energy (CE). The Crizotinib drug properties are that, which an inhibitor of RTKs proteins. Which mainly bind on the protein's ATP binding region and thus by inhibiting the protein from phosphorylation. Crizotinib action on C-MET, C-ROS1, and ALK have already been reported [22,23] . Temozolomide drug is an Alkylating agent. It mainly binds on the methylation site of the protein [28,29,30,31]. The main aim of forward and reverse docking of combination drug studies was to understand the inter molecular mechanism of Crizotinib and Temozolomide in C-MET, C-ROS1, and ALK proteins. In docking of Crizotinib and Temozolomide with C-MET, C-ROS1, and ALK proteins, we first docked the Crizotinib drug on C-MET protein by each active site identification method. After the first docking, we selected the top ten Crizotinib bounded docked complexes from each docking method. Then we calculate Binding energy and Complex Energy. In the Discovery studio tool, calculate the Binding and CE of a docked complex via the following equation..

Energy binding = Energy complex − Energy ligand −Energy receptor.

Complex Energy = Energy binding + Energy ligand + Energy receptor.

From the result obtained from the analysis study, we selected the least energy showed Crizotinib bounded docked complexes from each docking method. Then we performed a second protein preparation for the least energy docked complexes of each of the docking methods of C-MET protein. Furthermore, again we find out the active site for each of the Crizotinib bounded C-MET protein complexes. Then Temozolomide drug docked on the active site of each of the Crizotinib bounded docked complexes of C-MET protein. Then calculate binding energy and CE of the Temozolomide docked complexes. Here the Complex energy of the docked complex is the sum of the total least energy attained by a docked complex after when the drug molecules bind on its protein active site. Here we got the CE is the sum of the least energy of Crizotinib, and Temozolomide bounded protein structure. Then we filter the least energy docked complexes from Crizotinib and Temozolomide drug bounded docked complexes from each docking method based on drug properties.

In the reverse Docking of Temozolomide and Crizotinib with C-MET protein, we repeat the same procedure applied in the docking of Crizotinib and Temozolomide drug with C-MET protein. Finally we performed MD simulation analysis based on the least energy showed combination drug complexes for identifying stability and flexibility of best combination drug complexes [39].

MD simulation Analysis: Before the MD simulation analysis, we changed the force field of each protein structure from CHARMm to CHARMm36 by the tool, applied force field in DS (*Ref*: user manual of Simulation protocol in Discovery studio Tool). Then we add water molecules, chloride, and sulfur for each protein structure by solvation tool of DS. By the solvation, the protein structure can interact naturally with the solvent[39] . Here all the protein structures were solvated by using explicit periodic boundary condition.

Standard Dynamic Cascade Tool: After applying the forcefield and solvation to each protein structure, we performed MD simulation analysis using two tool panels of Discovery studio2018. One is a standard dynamic cascade tool, and another is Trajectory analysis tool. The standard Dynamic cascade tool is used for helping to simulate the biological conditions for protein structures and protein docked complexes. Before running this tool, we need to set the parameter for simulation. The main parameters set for MD simulation in the tool were Minimization, Heating, Equilibration, and Production. In the minimization part, there are two minimization parts. In the initial part, we set the steepest descent algorithm at 1000 steps, and in the second part, we used the adopted basis NR algorithm at 2000 steps. During heating, the whole system's initial temperature was set from 50 to 300 K in 4 ps (picosecond) and the simulation time was not controlled, and velocity frequency was set at 50. Here the result was saved at 2ps. In the equilibration of the system setup, the temperature was adjusted to 300 K, and here also adjusted velocity frequency was set to 50, and the simulation time was set to 20 ps. In the production, time was set to 200 ps for a run in 300 K with typed NPT. In the standard Dynamic Cascade tool, all the remaining parameters were set as by default value. In the standard Dynamic cascade tool, the total time allocated for heating, equilibration, and production was 224 ps. Here in all the combination drug docked complexes, we performed the MD simulation run based on picosecond. The main reason for performing picosecond is that it is a study of combination drug docked complex. So if we run the combination drug complexes in nanoseconds, it will require more memory and run time. For considering these things, in the present study, we set an equilibration time as 224 picoseconds in all the protein structures. In MD simulation, there is 3 type of MD simulation run we performed on each protein structure which include free protein structure, single drug bounde complex, and combination drug bounded complexes of C-MET,C-ROS1 and ALK. After the MD simulation run, we performed trajectory analysis study for each complex to understand the conformational stability and flexibility of the single and combination docked complex by using trajectory analysis tool of discovery studio2018 (Ref., the user manual of MD simulation tutorial of Discovery studio2018). In trajectory analysis, the nosolvent file of each docked complex structure was used as a reference molecule. In the parameter boxes RMSD, RMSF, and properties were selected, and also set atom group, atom group to fit parameter for each protein structure was set as Backbone. The remaining parameters were set as by default value.

Abbreviations

WHO: World Health Organization, **GBM**: Glioblastoma multiforme, **NSCLC:** non-small-**cell lung carcinoma, RTKs**: Receptor tyrosine kinases, **CE**: Complex Energy, **RC**: Receptor Cavity, **DS**: Discovery Studio.

Declarations

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Author's contributions

SY performed all the research activities, VC and ASN monitored the research work and SY, SK and VC participated in preparing and drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not applicable

Availability of data and material

All necessary data generated or analyzed during this study are included in this article. Any additional data could be available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1

Details of combination drug position, docking method, docking score, binding energy, complex energy and hydrogen bond interaction showed amino acid residues at the binding site of protein with the both the drug molecules

Sl:No	Protein Name	Name of the drug docked with Position number &Type of docking		Docking score	Binding energy	Complex Energy	Interacting Amino acid on the Druggable Binding site
		First position Crizotinib	Temozolomide Second Position				
Docking of Crizotinib and Temozolomide with 2WGJ (C-MET) proein							
I	2WGJ	PDB(P6)		139.105	-44.1433	-11179.9569	PRO1158, MET1160
			PDB(P1)	76.6468	-22.3612	-11252.7717	ASN1167
			RC(S3,P4)	55.9085	-3.5582	-11257.2432	ARG1166, ARG1170
II	2WGJ	RC(S2,P4)		137.169	-15.3749	-11155.5712	PRO1158 MET1160 ASP1164
			RC(S3,P7)	55.1107	-31.9594	-11255.6352	ARG1166 ARG1170
III	2WGJ	DIRECT(P2)		106.855	114.7614	-11028.5560	ASP1164 PRO1158
			RC(S1,P5)	66.021	-17.4212	-11256.9253	ARG1227, GLU1127
Docking of Temozolomide with Crizotinib in 2WGJ(C-ROS1) protein							
I	2WGJ	PDB(P4)		74.4989	-16.3492	-11289.7872	MET1160, PRO1158
			RC(S1,P5)	120.453	222.3001	-10942.6807	GLU1127, ARG1203, LYS1244
II	2WGJ	RC(S1,P4)		67.5009	-25.2079	-11302.4869	ARG1227
			PDB(P3)	139.545	-44.8256	-11201.0974	PRO1158 MET1160 ASP1164
			RC (S1,P9)	130.605	-47.1786	-11208.0049	PRO1158 ASP1164
			DIRECT (P5)	102.995	46.5792	-11101.4491	HIS1174, HIS1162
III	2WGJ	DIRECT(P4)		68.0813	-27.8584	-11305.0830	MET1160 PRO1158
			PDB(P2)	96.639	324.7148	-10338.8302	HIS1174, HIS1162
			DIRECT(P3)	75.5657	-7.2221	-11174.3462	SER1141
IV	2WGJ	MOTIF(P1)		74.7354	-32.2530	-11309.5066	ARG1208 ASP1222
			PDB(P7)	85.338	72.5417	-11075.2379	ASP1164, PRO1158
			RC	120.453	222.3001	-10927.2889	GLU1127,

			(S1,P5)				MET1229, ARG1203, LYS1244
Docking of Crizotinib and Temozolomide with 3ZBF(C-ROS1) protein							
I.	3ZBF	PDB(P6)		113.349	-106.9428	-11509.9762	GLU2027 MET2029 ASP2033
			PDB(P4)	61.7993	-49.6139	-11629.5730	ARG2083 ASP2102 GLY1954
			RC(S1,P2)	61.06	-18.5902	-11599.3423	ARG2083 ARG2084, ARG2116
			MOTIF(P)2	67.5562	-29.2580	-11607.5555	HIS2006
II.	3ZBF	RC(S4,P4)		92.346	-62.0108	-11400.4896	GLU2027 MET2029
			PDB(P4)	61.7993	-49.6139	-11629.5730	ARG2083 GLY1954 ASP2102
			RC(S1,P10)	60.4143	-43.1446	-11629.6842	ASP2102
			MOTIF(P1)	69.9753	-23.1428	-11614.8793	HIS2006
III.	3ZBF	DIRECT(P3)		98.9302	-20.1590	-11473.5427	GLU2027 MET2029
			PDB(P3)	60.372	-42.3390	-11621.3128	ARG2083, GLY1954, ASP2102
			RC(S6,P7)	73.5836	-28.9263	-11612.0884	ARG2184
			MOTIF(P1)	70.9263	-33.3147	-11616.3849	HIS2006
IV.	3ZBF	MOTIF(P1)		114.545	-112.0438	-11514.2552	GLU2027 MET2029 ASP2033
			PDB(P8)	61.366	-48.3916	-11640.1262	ASP2102
			RC(S10,P1)	77.6734	-9.5586	-11602.9634	HIS2006, MET2001
			MOTIF(P1)	71.168	-31.2576	-11628.5371	HIS2006
Docking of Temozolomide and Crizotinib with 3ZBF (C-ROS1) protein							
I.	3ZBF	PDB(P4)		58.5464	-40.5732	-11659.6814	MET2029
			PDB(P2)	108.487	-79.7002	-11614.1762	ARG2083, LEU1951, ASN2084
			RC(S1,P1)	90.6908	-37.436	-11540.7690	GLY2119
			MOTI(P3)	98.2243	6.6730	-11476.9066	ASP2079
II	3ZBF	RC(S4,P4)		54.3362	-55.3656	-11670.6518	ASP2102
			PDB(P3)	110.33	157.4418	-11359.8124	GLU2027
			RC(S1,P2)	106.201	-5.33333	-11491.7644	ASP2102

			DIRECT(P4)	115.213	-3.1848	-11536.9461	GLU1961, LEU1951
III	3ZBF	DIRECT(P5)		56.4637	-12.0296	-11627.0789	GLU2027, MET2029
			MOTIF(P5)	99.653	29.2318	-11419.0213	ASP2102, ALA1955, ASN2084
IV	3ZBF	MOTIF(P1)		71.1408	-29.3576	-11648.4805	HIS2006
			PDB(P3)	114.039	-88.7423	-11494.9503	GLU2027, ASP2033
			RC(S4,P2)	90.7746	-8.7221	-11508.6183	GLU2027, MET2029
			MOTIF(P7)	109.688	-61.2600	-11520.8136	GLU2027, MET2029
Docking of Crizotinib and Temozolomide with 2xp2(ALK) protein							
I	2XP2	PDB(P10)		113.349	-106.9428	-11509.9762	GLU2027 MET2029 ASP2033
			PDB(P8)	64.8012	-31.3674	-11774.8799	LYS1150 ,ASP1270
			RC(S2,P11)	44.583	-22.7267	-11766.2218	ARG1253, ASN1254, MET1273
			MOTIF(P1)	50.9253	-34.9974	-11776.4960	LYS1150
II	2XP2	RC(S1,P8)		108.615	-189.7012	-11666.7948	MET1199
			PDB(P2)	56.5496	-37.6981	-11809.0509	LYS1150
			RC(S1,P6)	41.7402	-22.1709	-11793.1981	LYS1150, ARG1275
			MOTIF(P2)	51.7183	-30.2246	-11800.8150	LYS1150
III	2XP2	DIRECT(P1)		118.508	-193.8604	-11648.2048	SER1206,ASP1203,GLU1197,MET1199
			PDB(P6)	65.5961	-58.1317	-11801.7198	ARG1253
			RC(S1,P6)	43.8847	-19.6634	-11767.1323	ARG1253 ,ASN1254, MET1273 ,ASP1276
			MOTIF(P2)	60.3589	-22.0080	-11765.7266	LYS1150, GLY1128, HIS1124
IV	2XP2	MOTIF(P9)		107.35	-176.1679	-11656.9972	GLU1197 MET1199 ASP1203
			PDB(P4)	65.0735	-45.8136	-11793.3255	LYS1150, ASP1270

			RC(S1,P3)	53.5578	-40.9016	-11787.3768	LYS1150, ARG1275, ASP1270
			MOTIF(P2)	50.7219	-38.0815	-11784.2476	LYS1150, ASP1270
Docking of Temozolomide and Crizotinib drug with 2XP2(ALK) protein							
I	2XP2	PDB(P3)		61.6198	-3.4005	-11693.1778	MET1199, GLU1197
			PDB(P8)	93.7589	-125.3281	-11680.4621	ARG1253
			RC(S1,P1)	110.84	-205.2797	-11770.7336	ASP1160, ASN1254
			MOTIF(P4)	104.422	-170.6199	-11715.7515	ASP1160, ASP1249, ASN1254
II	2XP2	RC(S1,P2)		60.1738	-44.4725	-11734.7565	ARG1275, GLU1167
			PDB(P8)	111.801	-137.5860	-11634.5873	GLU1197 MET1199
			RC(S1,P1)	117.539	-172.9525	-11681.3448	GLU1197, SER1206, ASP1203
			MOTIF(P6)	110.072	-171.0224	-11658.4859	GLU1197
III		DIRECT(P3)		61.0851	-25.9938	-11715.3869	MET1199
			PDB(P4)	97.8268	-13.8383	-11498.8871	LEU1122
			RC(S1,P1)	111.041	-197.5889	-11776.449	ASN1254
			MOTIF(P1)	110.036	-210.253	-11786.4155	ASP1160, ASN1254
IV		MOTIF(P2)		72.7795	-27.8563	-11717	LYS1150, ASP1249, ASP1270
			PDB(P1)	110.036	-210.2526	-11786.4155	ASP1160, ASN1254
			RC(S2,P5)	58.5502	-3.4804	-11612.9795	PRO1398
			MOTIF(P1)	116.52	-205.2280	-11710.0064	SER1206, ASP1203, GLU1197

Table 2

Details of best-docked complexes of single and multiple drug binded on the protein C-MET, C-ROS1 and ALK proteins with docking score, binding energy, least complex energy and main hydrogen bond interaction showed amino acid residues on the binding sites selected via MD simulation.

PROTEIN NAME& DRUG NAME	TYPE OF DOCKING OF DRUG WITH POSITION NUMBER		DOCKING SCORE	BINDING ENERGY (kcal/mol)	COMPLEX ENERGY(kcal/mol)	INTERACTIN RESIDUES
2WGJ+CRIZOTINIB	2WGJ(PDB(P6))	FIRST	139.105	-44.1433	-11179.9569(Crizotinib(PDB(P6)) with 2WGJ protein)	PRO1158,M (Crizotinib)
2WGJ+TEMOZOLOMIDE	2WGJ(PDB(P1))	SECOND	76.66468	-22.3612	-11252.7717 Total energy attained after when the drug pose number six of the Temozolomide drug bounded on the Crizotinib drug bounded docked complex via PDB method)	PRO1158,
2WGJ(CRIZOTINIB+ TEMOZOLOMIDE)	2WGJ(PDB(P6) <- PDB(P1))	FIRST, SECOND	215.81468(sum of docking score of first and second drug)	-66.5045 (sum of binding energy of first and second drug)		MET1160(C and ASN1 (Temozolom
3ZBF+CRIZOTINIB	3ZBF(PDB(P6))		113.349	-106.9428	–11509.9762(Crizotinib (PDB(P6)) with 3ZBF protein)	GLU2027, MET2029, ASP2033
3ZBF+TEMOZOLOMIDE	3ZBF(PDB(P4)		61.7993	-49.6139	-11629.5730(Total energy attained after when the drug pose number four of the Temozolomide drug bounded on the Crizotinib drug bounded docked complex via PDB method)	GLU2027,
3ZBF(CRIZOTINIB+TEMOZOLOMIDE)	3ZBF(PDB(P6)<-PDB(P4))		175.1483 (sum of docking score of first and second drug)	-156.5567 (sum of binding energy of first and second drug)		MET92029, ASP2033 ar ARG2083, ASP2102 GLY1954 (Temozolom
2XP2+CRIZOTINIB	2XP2(RC(S1,P8))		108.615	-189.7012	-11666.7948	GLU1197, MET1199
2XP2+TEMOZOLOMIDE	2XP2(PDB(P2))		56.5496	-37.6981	-11809.0509	GLU1197,
2XP2(CRIZOTINIB+TEMOZOLOMIDE)	2XP2(RC(S1,P8)<-PDB(P2))		165.1646 (sum of docking score of first and second drug)	-227.3993 (sum of binding energy of first and second drug)	(Total energy attained after when the drug pose number two of the Temozolomide drug bounded on the first site of the Crizotinib drug bounded docked complex via RC method)	MET1199 (Crizotinib) LYS1150 (Temozolom

Table 3

Structural analysis details of best docked complexes of combination drug Crizotinib and Temozolomide bounded protein complexes of C-MET,C-ROS1 and ALK proteins

Protein Name& Drug Name	Interaction details of docked drug with drugID, Docked site details (First site Crizotinib and second site Temozolomide)	The interaction details at the Binding sites	Distance	Type of interaction	Category
2WGJ(PDB(P6)<-PDB(P1)) (Crzotinib(P6)-Temozolomide(P1)	**Crizotinib(11626560)-PDB(P6)**	:11626560:H31 - A:ASP1164:OD1 :11626560:H33 - A:PRO1158:O :11626560:H34 - A:PRO1158:O	2.77861 1.86868 2.2336	Conventional Hydrogen Bond	Hydrogen Bond
		:11626560:H36 - A:ILE1084:O :11626560:H44 - A:ASP1164:OD1 :11626560:H46 - A:MET1160:O	2.0732 2.14764 2.50719	Carbon Hydrogen Bond	Hydrogen Bond
		:5394:H17 - :11626560:F3	2.40487	Drug-drug Interaction(C-H bond)	Hydrogen Bond
		:11626560:N6 - A:ASP1164:OD2	5.18385	Attractive Charge	Electrostatic
		A:TYR1230 - :11626560	4.24196	Pi-Pi Stacked	Hydrophobic
\		A:ALA1221 - :11626560:Cl2 A:ALA1226 - :11626560:Cl2 A:ALA1226 - :11626560:C25 :11626560:Cl2 - A:LEU1140 :11626560:Cl2 - A:LEU1157 :11626560:C25 - A:VAL1092 :11626560:C25 - A:LEU1157	3.53917 3.24234 4.4322 4.97198 5.29497 4.20848 5.18592	Alkyl	Hydrophobic
		A:TYR1230 - :11626560:Cl1 A:TYR1230 - :11626560:C25 :11626560 - A:ILE1084 :11626560 - A:ALA1108 :11626560 - A:MET1160 :11626560 - A:MET1211 :11626560 - A:MET1211 :11626560 - A:ALA1221	4.4462 4.5456 4.18544 3.50683 4.94339 4.6591 4.64448 4.86609	Pi-Alkyl	Hydrophobic
	Temozolomide(5394)-PDB(P1)	A:ASN1167:HD21 - :5394:O2 A:ASN1167:HD21 - :5394:N6	2.49038 2.01845	Conventional Hydrogen Bond	Hydrogen Bond
		:5394:H20 - A:ILE1084:O	2.40487 2.44445	Carbon Hydrogen Bond	Hydrogen Bond
		A:ASP1164:OD2 - :5394	4.44306	Pi-Anion	Electrostatic
		A:TYR1230:C,O;ASP1231:N - :5394	4.27058	Amide-Pi Stacked	Hydrophobic

		:5394 - A:ARG1208	4.61472	Pi-Alkyl	Hydrophobic
3ZBF PDB(P6)<-PDB(P4)	**Crizotinib(11626560)-PDB(P6)**	:11626560:H31 - A:ASP2033:OD1 :11626560:H33 - A:GLU2027:O :11626560:H34 - A:GLU2027:O	2.18685 2.38508 2.03485	Conventional Hydrogen Bond	Hydrogen Bond
		:11626560:H41 - A:ASP2033:OD1 :11626560:H46 - A:MET2029:O :11626560:H48 - A:MET2029:O	2.8236 2.18373 2.57775	Carbon Hydrogen Bond	Hydrogen Bond
		:11626560:N6 - A:ASP2033:OD2		Attractive Charge	Electrostatic
		:11626560:Cl1 - A:LEU2086 :11626560:Cl2 - A:LEU2010 :11626560:Cl2 - A:LEU2026 :11626560:C25 - A:VAL1959 :11626560:C25 - A:LYS1980	4.92316 5.02729 4.82276 4.09395 4.55288	Alkyl	Hydrophobic
		:11626560 - A:LEU1951 :11626560 - A:ALA1978 :11626560 - A:LEU2086 :11626560 - A:LEU2086	4.54312 3.64113 4.50735 4.04759	Pi-Alkyl	Hydrophobic
	Temozolomidel5394) **PDB(P4)**	A:ARG2083:HH11 - :5394:O1 :5394:H15 - A:GLY1954:O :5394:H16 - A:ASP2102:OD2	2.81257 2.76637 2.15023	Conventional Hydrogen Bond	Hydrogen Bond
		A:ARG2083:HD2 - :5394:O1 :5394:H19 - A:ASP2079:OD2 :5394:H20 - A:ASP2079:OD2	2.05535 3.03654 3.0832	Carbon Hydrogen Bond	Hydrogen Bond
		:5394 - A:ARG2083	4.86186	Pi-Alkyl	Hydrophobic
2XP2 (RC(S1,P8)<-PDB(P2)	**Crizotinib(RC(S1,P8)**	:11626560:H33 - A:MET1199:O :11626560:H35 - A:GLU1197:O	2.74946 2.16462	Conventional Hydrogen Bond	Hydrogen Bond
		:11626560:H48 - A:MET1199:O	2.37879	Carbon Hydrogen Bond	Hydrogen Bond
		:11626560:Cl2 - A:LEU1196 :11626560:Cl2 - A:LEU1256 ::11626560:C25 - A:VAL1130 :11626560:C25 - A:LEU1196	4.5645 4.54928 4.1025 5.15443	Alkyl	Hydrophobic

		:11626560 - A:LEU1122	5.43873	Pi-Alkyle	Hydrophobic
		:11626560 - A:ALA1148	4.62228		
		:11626560 - A:LEU1256	4.3131		
		:11626560 - A:LEU1256	4.08598		
		:			
		:11626560:H32 - A:GLU1210:OE2	2.00555	Salt Bridge;Attractive Charge	Hydrogen Bond;Electrostatic
		11626560:Cl2 - :5394:C13	4.12456	Alkyle	Hydrophobic
		:11626560:C25 - :5394:C13	3.36074		
		11626560 - :5394:C13	3.80447	Pi-Alkyl	Hydrophobic
		:5394 - :11626560:Cl1	3.9617		
		:5394 - :11626560:Cl1	4.0158		
		:5394 - :11626560:C25	4.86966		
		:11626560 - :5394	3.91743	Pi-Pi Stacked	Hydrophobic
		:5394 - :11626560	5.28544		
	Temozolomide PDB(P2)	A:LYS1150:HZ1 - :5394:O1	1.99751	Conventional Hydrogen Bond	Hydrogen Bond
		:5394:H17 - A:HIS1124:O	2.53002	Carbon Hydrogen Bond	Hydrogen Bond
		:5394:H19 - A:ASP1270:OD2	2.53636		
		:5394:C13 - A:LYS1150	4.99937	Alkyl	Hydrophobic
		:5394 - A:ALA1126	5.14915	Pi-Alkyl	Hydrophobic
		:5394 - A:ALA1126	4.2119		

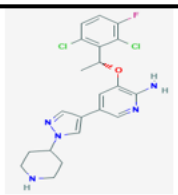
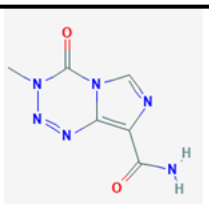
Table 4

Details of Crizotinib binded protein and their main hydrogen bond interaction residues

PROTEIN	RESOLUTION	CHAIN	SEQUENCE LENGTH	KEY RESIDUE
2XP2	1.9 Å	A	327	MET1199,GLU1197
2WGJ	2 Å	A	306	MET1160,PRO 1158
3ZBF	2.2 Å	A	322	MET2029,GLU2027

Table 5

list of ligands with CAS number which used for the study

SI.NO:	Ligand Name	CAS number	Molecular formula	MW	Log P	H-Bond donors	H-Bond acceptors	Chemical Structure
1	**Crizotinib** (3-[(1*R*)-1-(2,6-dichloro-3-fluorophenyl)ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl)pyridin-2-amine)	877399-52-5	$C_{21}H_{22}Cl_2FN_5O$	450.3 g/mol	3.7	2	6	
2	**Temozolomide** (3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide)	85622-93-1	$C_6H_6N_6O_2$	194.15 g/mol	-1.1	1	5	

Figures

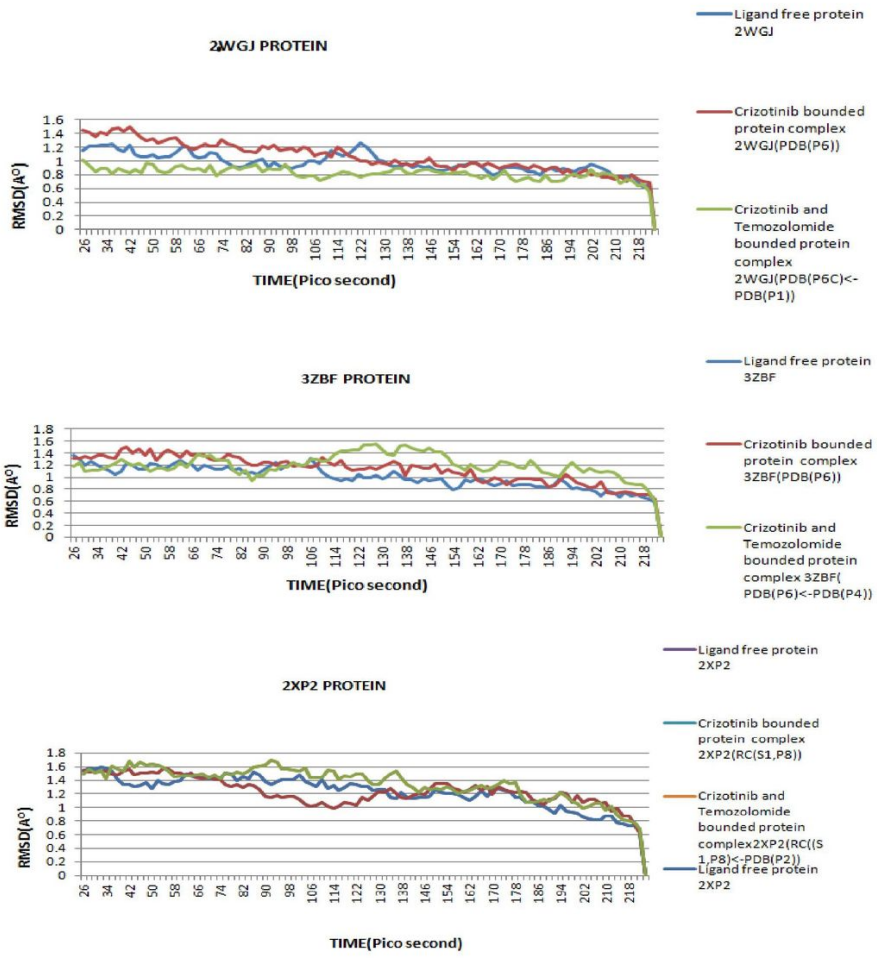


Figure 1

RMSD graph of single and combination drug complexes of C-MET, C-ROS1 and ALK protein

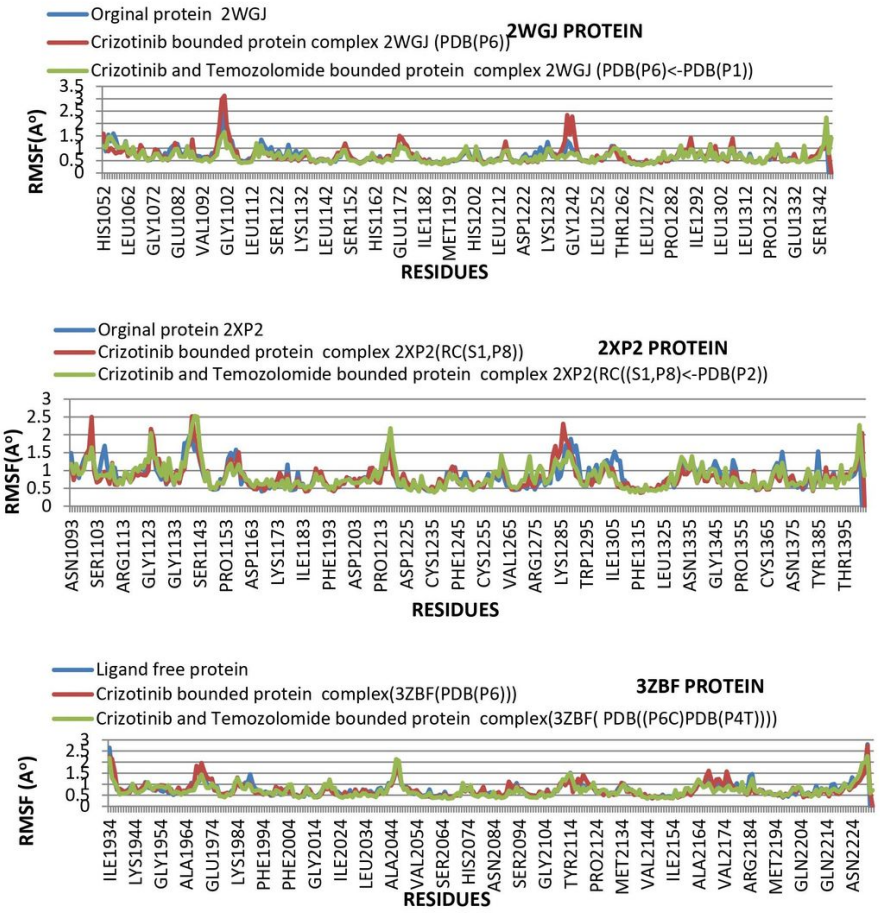


Figure 2

RMSF graph of single and combination drug complexes of C-MET, C-ROS1 and ALK protein

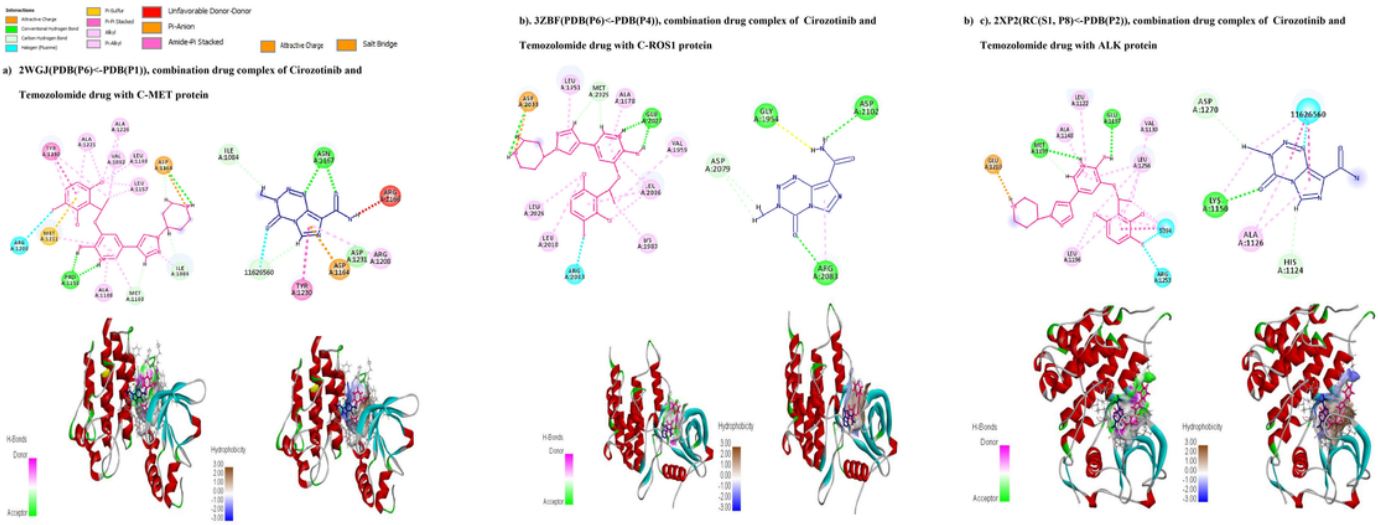


Figure 3

(a, b, c): 2D and 3D structures of combination drug complexes of Crizotinib and Temozolomide bounded with C-MET, C-ROS1 and ALK proteins.

Each color in the square box indicates the type of interactions obtained between the drugs and amino acid residues at each protein's binding site, shown in the 2D diagram. The 3D diagram shows the hydrogen bond and hydrophobic interaction of Crizotinib and Temozolomide drug at the binding sites of each protein (Magenta color indicates Crizotinib drug, and dark blue indicate Temozolomide drug).

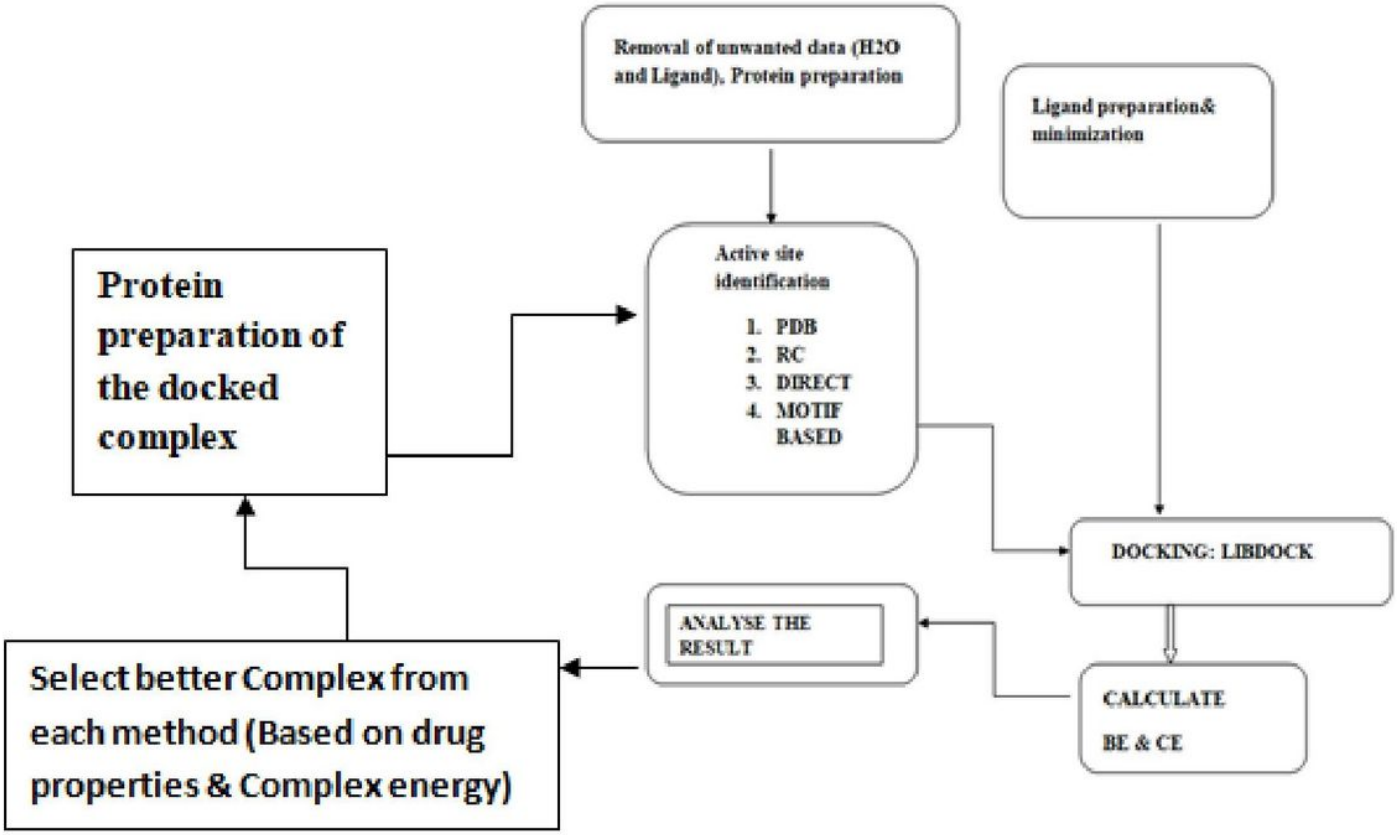


Figure 4

The pipeline used in the docking study of combination drug CRizotinib and Temozolomide with

C-MET,C-ROS1 and ALK proteins.

Supplementary Files

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- supplementaryfile.docx