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Possible Mechanism of Action of Neurokinin-1 Receptors (NK1R) Antagonists

Özüm Öztürk[1], Esin Aki-Yalcin[1], Tugba Ertan-Bolelli[1], Kayhan Bolelli[1], Andry Nur-Hidayat[2], Ozlem Bingol-Ozakpinar[3], Filiz Ozdemir[4] and Ismail Yalcin[1]

1. Pharmacy Chemistry Department, Faculty of Pharmacy, Ankara University, Ankara 06100, Turkey

2. Bioinformatics Department, Biotechnology Institution, Ankara University, Ankara 06100, Turkey

3. Biochemistry Department, Faculty of Pharmacy, Marmara University, Istanbul 34722, Turkey

4. Analytical Chemistry Department, Faculty of Pharmacy, Marmara University, Istanbul 34722, Turkey

Abstract: Recently, NK1R (Neurokinin-1 receptors) take attention as new and promising target in anticancer drug development area. It has been proved that non-peptide NK1R antagonists L-733,060, aprepitant and L-732,138 inhibited tumor growth in several cancer cell lines. For the development of novel NK1R antagonists as antitumor agents, heterocyclic compounds which were previously synthesized by our team, tested for their cytotoxic activities in several cancer cell lines in this study. Among the tested compounds, a benzothiazole derivative BSN-009 inhibited colon cancer cell lines growth by 57.53% by comparing the activity to the control drug aprepitant. Molecular modeling studies such as molecular docking and pharmacophore generation were performed with known NK1R antagonists and BSN-009 by using Discovery Studio 3.5 in order to explain their binding modes to NK1R. BSN-009 may be a good anticancer drug candidate as a possible NK1R antagonist and is worthy to carry on the anticancer studies.

Key words: Anticancer, aprepitant, benzothiazole, docking, NK1 receptor antagonist, pharmacophore.

1. Introduction

Over the past decade, there has been a major exponential increase in cancer research. Researchers are seeking to identify novel molecular targets for blocking tumor growth. There have been many targets for anticancer research and one of which is NK1R (Neurokinin-1 receptors). Over the last two decades, NK1R (Neurokinin-1 receptor) research has been pursued aggressively to develop drugs that might be useful for a branch of pharmacologic purposes including anticancer, antiviral and antiemetic and dozens of molecules have been entered into various phases of clinical trials. Neurokinin receptors, also known as tachykinin receptors belong to the family of seven transmembrane GPCRs (G-protein coupled receptors) found in the central nervous system and the peripheral nervous system. The endogenous ligand neuropeptide SP (substance P) selectively binds to NK1R at the plasma membrane [1]. SP is an undecapeptide that belongs to the tachykinin peptide family and widely distributed in both the central and the peripheral nervous system of mammals. Activation of NK1R by SP stimulates G-protein mediated signaling pathways that are crucial for regulating cellular excitability and function such as cAMP accumulation, arachidonic acid mobilization and phosphatidylinositol turnover. It has been shown that activation of Akt suppresses apoptosis and stimulation of NK1R by SP induces phosphorylation on Akt or PKB (protein kinase B) activity in human glioblastoma cells. After binding to the NK1R, SP induces mitogenesis and inhibits apoptosis both normal and tumor cells. Hence NK1R antagonists can lead to apoptosis and inhibit tumor cell proliferation. Furthermore, neoangiogenesis, marker of tumor development is associated with NK1R stimulation. SP

Corresponding author: Esin Aki-Yalcin, Ph.D., professor, research fields: synthesis and computational drug design of chemotherapeutically active compounds.

and NK1R are found in intra and peritumor blood vessels of many of the investigated tumors. Endothelial cell proliferation is under control of NK1R/SP system [2]. So, there is direct connection between neovascularization process and NK1R agonists. G-protein coupled receptors regulate the migration of tumor cells just like regulating the recruitment and homing of leucocytes. Agonists of these receptors can induce the tumor cell migration for invasion and metastasis. Hence, NK1R antagonists can inhibit the development of metastasis by blocking the activation of NK1R by SP [3]. The prevention of metastasis is the major goal in cancer treatment [4] that more than 90% of cancer deaths occur from the development of metastasis, not from the primary tumor. It is also known that SP stimulates a rapid cellular shape change (including blebbing) after binding to NK1R which is important for cell movement, cell spreading and cancer cell invasion [5]. NK1R is expressed in malign tissues more than benign tissues and the increased percentage of NK1R expression is found in the most malignant phenotypes. As a result of these, NK1R can be a specific molecular target for the cancer treatment and the NK1R antagonists will be specific against tumor cells as promising agents, by reducing the serious side effects, unlike the other cytotoxic agents [4].

It is shown that NK1R antagonists such as aprepitant, L-733,060 and L-732,138 have antitumor activity against several human cancer cell lines such as melanoma, neuroblastoma, glioma, retinoblastoma, pancreatic, larynx, gastric and colon carcinomas [2, 3]. Currently, there are dozens of compounds that act as NK1R antagonists and many of them such as ezlopitant (CJ-11,974), casopitant, vofopitant (GR-205,171), vestipitant studied in humans so far may have not the same growth inhibitor activity profile in cancer cell lines as found with L-733,060, L-732,138, and aprepitant.

L-733,060 (Fig. 1A) is a potent benzyloxy piperidine derivative of NK1R antagonist and it selectively binds to NK1R. *In vitro* studies show that L-733,060 acts as a growth inhibitor in human neuroblastoma and glioma cell lines [6]. L-732,138 (Fig. 1B) is an *L*-tryptophan derivative of non-peptide NK1R antagonist and its binding way is selective and competitive. L-732,138 shows antitumor activity in several human melanoma cell lines *via* inducing apoptosis as an NK1R antagonist in the *in vitro* studies [7]. Aprepitant (Fig. 1C) is an FDA approved drug that is used for the treatment of acute and delayed CINV (chemotherapy-induced nausea and vomiting) as well as it is also used for the treatment of pain, migraine and psychiatric disorders. It is demonstrated that NK1R antagonist aprepitant indicates growth inhibitory action and induces apoptosis in cancer cell lines [8]. The potency of the antitumor activity of NK1R antagonists from more potent to less potent is shown as L-733,060, aprepitant and L-732,138 respectively [9].

It has been demonstrated that binding sites of peptide antagonists and non-peptide antagonists of NK1R are different than each other. Non-peptide binding site characterization and crucial amino acids for binding studies were performed with CP-96,345 (Fig. 1D) [10].

SP and peptide NK1R antagonists bind to the extracellular terminal region of the receptor, but non-peptide NK1R antagonists bind to intracellular part of the enzyme between transmembrane helices [3, 9]. Ligand binding pocket of an NK1R is a hydrophobic core between the loops of transmembrane TM III-VII. Several residues, such as Gln165 (TM IV), His197 (TM V), His265 (TM VI) and Tyr287 (TM VII) are involved in the binding of many non-peptide antagonists of the NK1R. The other residues that are contributed in non-peptide antagonist binding are Ser169, Glu193, Lys194, Phe264, Phe267, Pro271 and Tyr272 [11].

Over the last decade our group have been working on the drug design studies on the new anticancer active compounds by using both computational techniques and experimental work such as synthesis and activity studies [12-20]. Some of the benzoxazole and benzamide compounds, which were previously synthesized in our laboratory, showed strong inhibitory

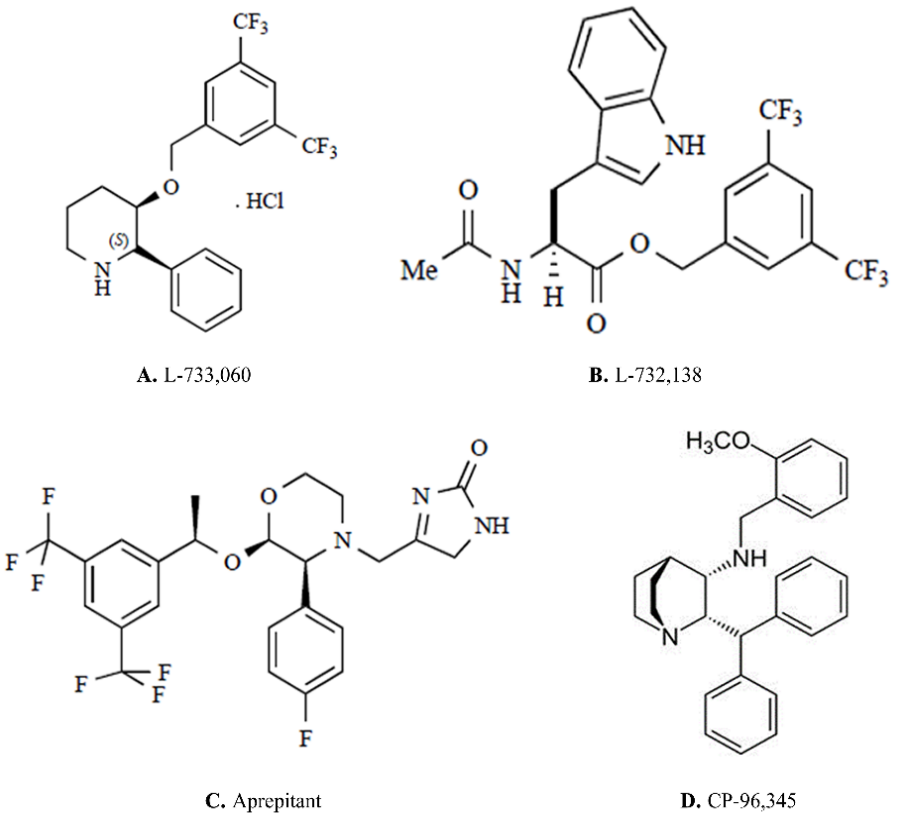

Fig. 1 A. Chemical structure of L-733,060; B. Chemical structure of L-732,138; C. Chemical structure of aprepitant; D. Chemical structure of L-CP-96,345.

activity for human DNA Topoisomerases and Glutathione *S-Transferases* and also anticancer effects observed on various cell cultures [12-20]. These findings encouraged us to search for the new anticancer target NK1R by comparing their activities with the well-known NK1R antagonist aprepitant.

In the field of drug design and discovery, molecular docking has become an increasingly crucial tool to predict the binding mode of a ligand with a protein. Pharmacophore generation is one of the major elements of drug design in the absence of structural data of the target receptor. In this research, we aimed to identify the binding site features and modes of NK1R and the non-peptide antagonists including our synthesized compounds, using molecular modeling studies such as molecular docking and pharmacophore generation working with DS (Discovery Studio) 3.5 software [21].

2. Materials and Methods

2.1 Cytotoxic Assay

The cytotoxic activity of compounds was assayed using the MTT colorimetric protocol and the results are given in Table 1. MTT is cleaved to formazan by the "succinate-tetrazolium reductase" system (EC 1.3.99.1) which belongs to the mitochondrial respiratory chain and is active only in viable cells. Human colon carcinoma cell line HT-29 (ATCC, HTB-38), breast cancer cell line MCF-7 (ATCC, HTB-38), human cervical carcinoma cell line HeLa (ATCC, CCL-2) and mouse embryonic fibroblast cell line NIH3T3 (ATCC, CRL-1658) were used in this study for cytotoxicity experiments. The cells were cultured with DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal bovine serum), 1% *L*-Glutamine and antibiotic solutions (penicillin-streptomycin) [22].

Table 1 Cytotoxic activity of the synthesized compounds.

Cell Line	Inhibition (%)				
	BADA-034	BSN-009	BON-254	BADA-024	Aprepitant
MCF-7	-6.34	8.94	-5.88	-1.8	70.61
HeLa	17.99	-4.35	1.3	-6.2	58.42
HT-29	43.01	57.73	19.2	-	62.06
NIH3T3	10.7	-0.03	0.75	-0.05	8.7

Negative values indicate that compounds have reproductive effects on the cell lines.

Cytotoxicity testing *in vitro* was done by the method of modified Woerdenbag *et al.* [23]. The MTT metabolic assay was carried out in seeded at the density of 1×10^4 cells/well in 96-well flat-bottom cell culture plates with 200 µL of DMEM and 24 h incubation at 37 ºC, 5% CO_2. The following day, media was aspirated and the extracts were dissolved in DMSO and diluted with medium before they were added to the cell cultures at 50 µM concentration. The cells were incubated for 24 h at 37 ºC, 5% CO_2. After the incubation period, 10 µL of the MTT labeling reagent was added to each well. The samples were incubated for 4 h in a humidified atmosphere (e.g., 37 ºC, 5% CO_2) and 100 µL of the solubilization buffer was added into each well. The plate was allowed to stand overnight in the incubator in a humidified atmosphere and the formazan precipitates were then solubilized. The absorbance of the formazan product was measured spectrophotometrically at 570 nm. The degree of inhibition of drug-treated cells is expressed as the percentage of the untreated cell control using the formula:

Growth inhibition (%) = 1 – [100 × (Sample/Control)].

2.2 Molecular Docking

The most straight forward computational approach for finding new leads for therapeutic macromolecular targets are increasing because of rapid availability of three-dimensional (3D) information of proteins. An effective way to predict whether small molecules, which are referred as ligands, bind to a macromolecular target is a molecular docking technique. A primary objective in molecular docking is the ability to evaluate protein-ligand binding energetics as a means for hit identification (virtual screening) and lead optimization (enhance desired drug properties). This method is also successfully used as computational tools in rational drug design.

2.2.1 Preparation of the Enzyme

The homology model of the NK1R with CP-96,345 was developed by Evers and Klebe [24]. For preparation of protein and ligands, DS 3.5 software was used [21]. The target protein was taken, hydrogens were added and their positions were optimized using all atom CHARMm forcefield and the Powell method available in DS 3.5 protocol until the RMSD (root mean square deviation) gradient was < 0.05 kcal⁄mol $Å^2$. The minimized protein was defined as the receptor using the binding site module. The binding site was defined from the cavity finding method which was modified to accommodate all the important interacting residues in non-peptide antagonist binding site of the NK1R. Binding sphere for NK1R (59.689, 13.788, -16.42, 6.60) was selected from the active site using the binding site tools.

2.2.2 Preparation of Ligands

All the ligands were sketched. All atom CHARMm forcefield parameterization was assigned and then minimized using the Adopted Basis set Newton Raphson (ABNR) method as described above. A conformational search of the ligands was carried out using a simulated annealing MD (molecular dynamics) approach. The ligands were heated to a temperature of 700 K and then annealed to 200 K.

2.2.3 Docking

CDOCKER (CHARMm-based DOCKER) has an all-atom CHARMm force field-based docking

algorithm and uses soft-core potentials with an optional grid representation to dock ligands into binding site of the protein. The protein is held rigid while the ligands are allowed to be flexible during refinement. CDOCKER is considered as a viable research tool because it offers all the advantages of full ligand flexibility (including bonds, angles, and dihedrals), the CHARMm 19 family of force fields, the flexibility of the CHARMm engine, and reasonable computation times [25, 26]. CDOCKER has been shown to give highly accurate docked poses [27]. CDOCKER generates random ligand conformations in the active site by using MD (molecular dynamics) calculation. Each conformation is treated with high temperature of MD using a modified version of CHARMm. For the initial stage of MD, a softcore potential is used. Each of the structures from the MD run are then located and fully minimized. Ligand partial atomic charges and atom types default to those of Momany-Rone force field [28] as implemented in CHARMm. The typed ligand is first run through a Powell minimization stage [29]. The positions of the ligands are optimized in the binding site using rigid body rotations followed by simulated annealing. The random conformations are refined by grid-based (GRID 1) simulated annealing and a final grid-based or full force field minimization.

The CDOCKER docked ligands are rescored using a physics based implicit solvation model as the final step. Within the CBE subprotocol step, the docked ligand poses are rank scored in terms of their energies of binding. For this study, top CDOCKER poses of neutral and/or charged ligands are rescored using Molecular Mechanics-Generalized Born with Simple Switching (MM-GBSW) methods in DS, CHARMm, which approximates the binding energy [30-32]. GBSW calculations use approximation with a van der Waals based surface with a smooth dielectric boundary. This model also can be used for molecules with membranes [33]. Bound and unbound ligand receptor energy terms contained within the calculated binding energy including three simulations: free ligand, apoprotein, and protein-ligand complex. Solute entropy contributions are put out of these calculations.

The binding free energies ($\Delta G_{binding}$) of protein–ligand complexes were calculated using Eq. (1):

$$\Delta G_{Binding} = \Delta G_{Complex} - \Delta G_{Ligand} - \Delta G_{Protein} \quad \text{Eq. (1)}$$

In this present study, CDOCKER [26] method was performed by using DS 3.5 [21]. The protein was held rigid while the ligands were allowed to be flexible during refinement. The docking and scoring methodology was first validated by docking of ligand CP-96345. The docked position of CP-96345 overlaps well with the homology model position, with an RMSD of 0.50 Å (Fig. 2A).

Afterwards, molecular docking studies were performed on the other ligands. All docked poses were scored by applying Analyze Ligand Poses subprotocol to analyze receptor-ligand interactions or a set of poses (the results of a docking run) using a variety of methods and binding energies were also calculated by applying Calculate Binding Energy subprotocol in DS 3.5 by using in situ ligand minimization step by using Powell method. The lowest binding energy was taken as the best-docked conformation of the compound for the macromolecule (The docking results were given in Table 2 and Fig. 2A-F).

2.3 Pharmacophore Modeling

A pharmacophore model is described by IUPAC as “an ensemble of steric and electronic features that is necessary to ensure the optimal intermolecular interactions with a specific biological target and to trigger (or block) its biological response”. In DS, a pharmacophore is described as the essential features or chemical substructures and their corresponding 3D locations that are responsible for the similar biological activities of a set of compounds. Typically, a model pharmacophore model includes some of these features: hydrophobic, ring aromatic, hydrogen bond acceptor,

Table 2 Molecular docking results.

Compounds	Binding Energy (kcal/mol)	H bonds	π-π interactions	π-cation interactions	π-σ interactions
CP-96345	-8.1379	Gln165 (2.27 Å)	Phe117	His197, Tyr272 (*via* His197)	-
Aprepitant	-10.8319	Gln165 (2.27 Å), His197 (1.87, 2.37 Å), His 265 (2.37 Å)	His265	His197, Tyr272 (*via* His197), His265	Phe264
L-733,060	-8.50528	His197 (2.00 Å)	Phe268, Tyr287 (*via* Phe268)	-	-
L-732,138	-4.07695	Gln165 (2.43 Å), His197 (1.91 Å), Ile182 (1.93 Å)	Phe268, Tyr287 (*via* Phe268)	His187, His197 (*via* His187)	-
BSN-009	-9.67463	Gln165 (2.39 Å), His197 (1.83 Å), Ile 182 (2.12 Å)	-	His 187, His197, Tyr272 (via His197)	-

hydrogen bond donor, positive ionizable, and negative ionizable.

The Create Pharmacophore Automatically tools allow you to automatically build pharmacophore models from a ligand, receptor, or a receptor-ligand complex. The most widely used geometry- and feature-based pharmacophore elucidation method is Catalyst from Accelrys, which is currently a part of the DS package (some recent applications of the methodology). Catalyst is an integrated set of algorithms for conformation generation (ConFirm), molecular superimposition (HipHop), pharmacophore generation (HypoGen) and database searching (Info).

The calculated conformations are used to align common molecular features and generate a pharmacophore hypothesis. HipHop was used to the conformations generated to align chemically important functional groups common to the molecules in the study set. A pharmacophoric hypothesis then was created from these aligned structures.

In this study, pharmacophore analysis of NK1R antagonists was done by using Common Feature Pharmacophore Generation method in DS 3.5 using the Catalyst HipHop algorithm. Common Feature Pharmacophore Generation protocol can generate predictive pharmacophores with the alignment of common chemically important functional groups of the compounds in the training set [34]. The better a ligand fits a pharmacophore (i.e., the more features that map and the closer they are to the feature centroids), the more active it is predicted to be. To generate the pharmacophore model, the docked poses of the ligands were used. Common Feature Pharmacophore Generation was performed 10 Pharmacophore hypotheses. Hypothesis 9 (Hypo9) was chosen as the best hypothesis. The distances of the best hypothesis were shown in Fig. 2G. The Fit values of all of the compounds were indicated in Table 3. The mapping of aprepitant (fit value 2.87291) to hypo9 and BSN-009 (fit value 2.7592) to hypo9 were given in Figs. 2H and 2I respectively.

3. Results and Discussion

In this study, to describe the antagonist activity of some compounds which were previously synthesized by our team comparing with the standart drug aprepitant [18, 35, 36], MTT assay was applied (Table 1). Among these compounds BADA-034, BSN-009, BON-254, BADA-024 were tested for their cytotoxic activities in MCF-7, HeLa and HT-29 cancer cell lines, and NIH3T3 mouse embryonic fibroblast cell line. BSN-009 [35] was found to be the most active compound at a concentration of 50 μM as a result of MTT assay and inhibited colon cancer cell lines growth about by 57.53%. On the other hand, it has also been found that BSN-009 had no toxic effect on the normal cell line Table 1.

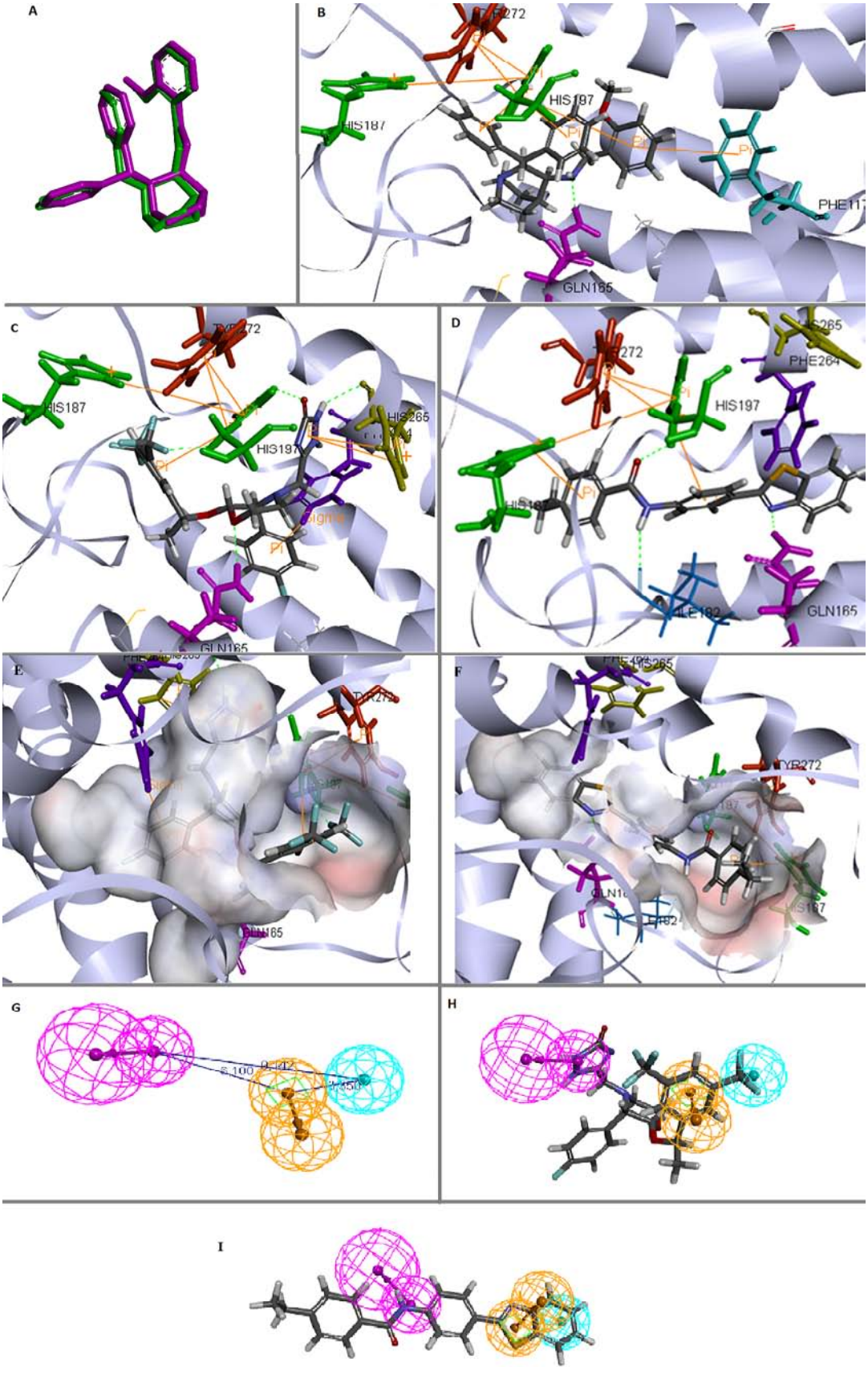
A
B
HIS187
HIS197
PHE117
GLN165
C
HIS187
HIS265
HIS197
GLN165
D
PHE264
HIS197
GLN165
E
F
TYR272
G
H
I

Fig. 2 A. Structural superimposition of the docked CP-96345 (purple) and from the homology model (green) RMSD value: 0.50 Å; B. Docked position of CP-96,345: Exocyclic secondary amine of CP-96,345 has hydrogen bond with Gln165 and phenyl rings have an aromatic interaction with His197; C. Docked position of aprepitant; D. Docked position of BSN-009; E. Docking overlay of aprepitant in atom charged surface area; F. Docking overlay of BSN-009 in atom charged surface area (Hydrogen bonds are represented as dash lines in green and π-π, π-cation, π-σ interactions are represented in orange lines.); G. Distances of pharmacophore model features. The model contains three features: one hydrophobic (cyan), one ring aromatic (orange) and one hydrogen bond donor (pink); H. The mapping of aprepitant (fit value 2.87291), to hypo 9; I. The mapping of BSN-009 (fit value 2.7592), to hypo9.

Table 3 Alignment of pharmacophore model with test set.

Compound	Principal	MaxOmitFeat	FitValue
L-732,138	2	0	2.9509
Aprepitant	2	0	2.87291
L-733,060	2	0	2.87071
CP-96345	2	0	1.99191
Hypothesis 9	RHD Rank: 20.348 DH: 1111 PH: 0000 Max Fit: 3		

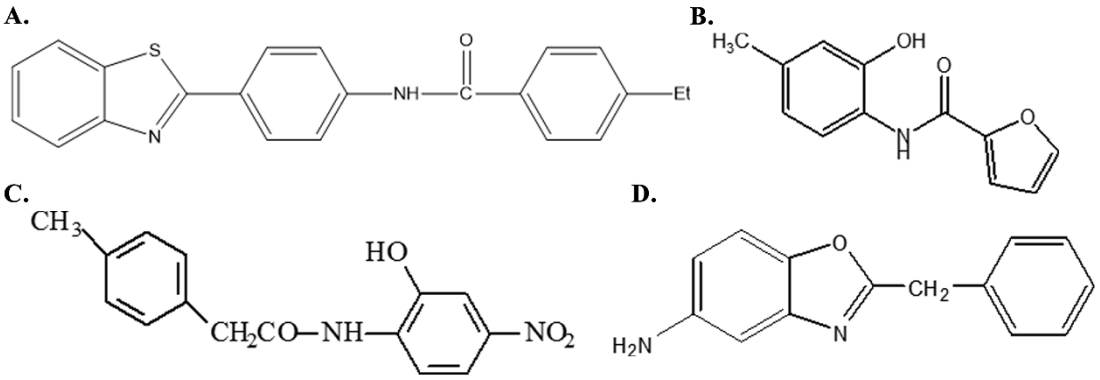

Fig. 3 A. Chemical structure of BSN-009 B. Chemical structure of BADA-034 C. Chemical structure of BADA-024 D. Chemical structure of BON-254.

We used molecular docking and pharmacophore generation methods by using Discovery Studio 3.5 to describe the binding site features of NK1R and generate a model to design novel and more potent NK1R antagonists. It is shown in various studies that Gln165, His197, His265 and Tyr287 are crucial amino acids for non-peptide antagonists to bind to NK1R [11, 24, 37, 38]. Binding mode of CP-96,345 with NK1R was elucidated by Evers and Klebe [24], according to their model CP-96,345 established hydrogen bond with Gln165 and an amino-aromatic interaction with His197 which itself is kept in place by an aromatic-aromatic interaction with Tyr272 and our docking result is coherent with the model (Fig. 2B). After validate the method with CP-96,345, all known antagonists and BSN-009 were docked into the NK1R by using CDOCKER method of DS 3.5 and all of the docking results were shown in Table 2. After the docking procedure, all docked poses were scored by applying Analyze Ligand Poses subprotocol to analyze receptor-ligand interactions or a set of poses (the results of a docking run) using a variety of methods and binding energies were also calculated by applying Calculate Binding Energy subprotocol in DS 3.5 by using *in situ* ligand minimization step by using Powell method. The lowest binding energy was taken as the best-docked conformation of the compound for the macromolecule. The docking results were given in Table 2 and Figs. 2B-2F.

Additionally, the pharmacophore analysis of NK1R antagonists was performed by using docked conformations of the compounds Aprepitant,

CP-96,345, L-733,060 and L-732,138. Common Feature Phamacophore Generation protocol that is provided in DS 3.5 was used to generate the pharmacophore model. Within the generated 10 hypotheses, the hypothesis 9 (Hypo 9) (Figs. 2G-2I) which has been possessed the highest ranking score showing three features containing Hydrogen Bond Donor, Hydrophobic and Ring Aromatic, has been chosen for the as the anticipated pharmacophore model. BSN-009 mapped with the Hypo 9 by using the docked conformation and all the fitted values were shown in Table 3.

As a result of the molecular modeling studies, BSN-009 shows similar binding modes with NK1R as known antagonists L-733,060, aprepitant and L-732,138 (Table 2, Fig. 2D). Oxygen atom of carbonyl group of BSN-009 makes an H bond with His197 and fits also to the Hydrogen Bond Donor feature of Hypo9. The phenyl ring of the benzamide group of BSN-009 has a π-cation interaction with His187 and fits to the Ring Aromatic feature of Hypo 9 (Fig. 2I). As a result of both molecular modeling and the experimental studies, BSN-009 could be a lead compound candidate as a possible NK1R antagonist and it is worthy to carry on with the *in vivo* studies.

4. Conclusions

NK1R becomes a new and promising target for the anticancer drug development. In this research, NK1R antagonist activity of some previously synthesized compounds [18, 35, 36] MTT assay was applied. BSN-009 was found to be the most active compound among the others, and also inhibited colon cancer cell lines growth, comparing to the standard drug aprepitant. Besides, it has also been found that BSN-009 had no toxic effect on the normal cell lines.

Molecular modeling studies were performed on the NK1R to identify the binding modes of non-peptide antagonists to the receptor. It was elucidated that Gln165, His197, His265 and Tyr287 are crucial amino acids in the non-peptide binding site of the receptor. According to molecular docking study BSN-009 has hydrogen bonds with Gln165 (2.39 Å) and His197 (1.83 Å) like other non-peptide antagonists of NK1R (Figure 2B-F). Oxygen atom of the carbonyl group of BSN-009 established a hydrogen bond with His197, also fitted well to Hydrogen Bond Acceptor feature in the pharmacophore model. Phenyl ring of BSN-009 that has a π-cation interaction with His187 also fitted well to Ring Aromatic feature of Hypo9. Binding energy values (kcal/mol) of BSN-009 and aprepitant are close to each other. As a result of the molecular modeling studies and cytotoxic experiments, it can be concluded that BSN-009 may be a good anticancer drug candidate as an NK1R antagonist having no toxicity to the normal cells is worthy to carry on the anticancer *in vivo* studies. This study also provides a model to design novel and more potent antitumor agents as NK1R1 receptor antagonists.

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