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RESEARCH ARTICLE

Structure-based virtual screening for novel inhibitors of Japanese encephalitis virus NS3 helicase/nucleoside triphosphatase

Agnieszka Kaczor & Dariusz Matosiuk

Department of Synthesis and Chemical Technology of Medicinal Substances, Faculty of Pharmacy, Medical University of Lublin, Lublin, Poland

Correspondence: Agnieszka Kaczor, Department of Synthesis and Chemical Technology of Medicinal Substances, Faculty of Pharmacy, Medical University of Lublin, 6 Staszica Str., 20-081 Lublin, Poland. Tel.: +48 815 357 365; fax: +48 815 357 355; e-mail: agnieszka.kaczor@am.lublin.pl

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Japanese encephalitis; helicase/nucleoside triphosphatase; virtual screening.

Abstract

Japanese encephalitis (JE) is a significant cause of human morbidity and mortality throughout Asia and Africa. Vaccines have reduced the incidence of JE in some countries, but no specific antiviral therapy is currently available. The NS3 protein of Japanese encephalitis virus (JEV) is a multifunctional protein combining protease, helicase and nucleoside 5′-triphosphatase (NTPase) activities. The crystal structure of the catalytic domain of this protein has recently been solved using a roentgenographic method. This enabled structure-based virtual screening for novel inhibitors of JEV NS3 helicase/NTPase. The aim of the present research was to identify novel potent medicinal substances for the treatment of JE. In the first step of studies, the natural ligand ATP and two known JEV NS3 helicase/NTPase inhibitors were docked to their molecular target. The refined structure of the enzyme was used to construct a pharmacophore model for JEV NS3 helicase/NTPase inhibitors. The freely available ZINC database of lead-like compounds was then screened for novel inhibitors. About 1 161 000 compounds have been screened and 15 derivatives of the highest scores have been selected. These compounds were docked to the JEV NS3 helicase/NTPase to examine their binding mode and verify screening results by consensus scoring procedure.

Introduction

Japanese encephalitis (JE) is the primary cause of viral encephalitis in Asia, with 30 000–50 000 cases reported annually, mainly in children (Diagana *et al.*, 2007). Acute encephalitis develops in about 1–20 cases per 1000 infections, leading to death in 25% of cases and producing serious neurological lesions in 30% (Diagana *et al.*, 2007; Jackson *et al.*, 2007). Infections with Japanese encephalitis virus (JEV) are most often asymptomatic. Only one in 300 cases produce clinical features (Solomon, 1997). The first signs of infection appear after an incubation period of between 6 and 14 days (Diagana *et al.*, 2007). The disease usually begins with a high fever, chills, muscle pain and meningitis-type headaches accompanied by vomiting. The initial clinical features in children usually involve gastrointestinal symptoms (nausea, vomiting and abdominal pains). These nonspecific symptoms can continue for 2–4 days. After this period, the patient's condition deteriorates rapidly. Eighty-five percent of subjects suffer from convulsions (Kumar *et al.*, 1990). The meningeal syndrome predominates, causing painful neck stiffness. Additionally, motor paralyses including hemiplegia and tetraplegia may also occur. In about 30% of patients, tremor, rigidity, abnormal movements and other signs of extrapyramidal involvement are present (Kumar *et al.*, 1994). Recovery usually leaves serious behavioral and neurological sequelae such as persistently altered sensorium, extrapyramidal syndrome, epileptic seizures and severe mental retardation in children (Diagana *et al.*, 2007).

JE is a mosquito-borne arboviral infection caused by *Flavivirus* transmitted by anthropophilic rice field-breeding mosquitoes of the *Culex* species (mainly the *Culex tritaeniorhynchus* group). Vaccines have reduced the incidence of JE in some countries, but no specific antiviral therapy is currently available. Sampath & Padmanabhan (2009) pointed out the following molecular targets for the flavivirus drug discovery: envelope glycoprotein, NS3 protease, NS3 helicase, NS5 methyltransferase and NS5 RNA-dependent RNA polymerase (Fig. 1).

The NS3 protein (nonstructural protein 3) of JEV is a multifunctional protein combining protease, helicase, and

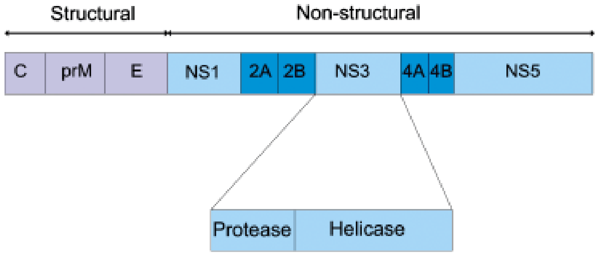


Fig. 1. Schematic representation of the flaviviral polyprotein. C, capsid; prM, precursor membrane; E, envelope; NS1–NS5, nonstructural proteins.

nucleoside 5′-triphosphatase (NTPase) activities (Sampath & Padmanabhan, 2009). In particular, NS3 helicase/NTPase seems to be a promising antiviral drug target, as its enzymatic activity is essential for viral genome replication, transcription and translation (Yamashita *et al.*, 2008).

Recently, the crystal structure of the catalytic domain of JEV NS3 helicase/NTPase has been solved using a roentgenographic method with a resolution of 1.8 Å (Yamashita *et al.*, 2008). JEV helicase, composed of three domains, displays an asymmetric distribution of charges on its surface, and contains a tunnel large enough to accommodate single-stranded RNA. Each of the motifs I (Walker A motif), II (Walker B motif) and VI contribute to the NTP-binding pocket. From mutation analysis (Yamashita *et al.*, 2008), it was possible to conclude that all of the residues in the Walker A motif (Gly199, Lys200 and Thr201), in addition to the polar residues within the NTP-binding pocket (Gln457, Arg461 and Arg464), and also Arg458 in the outside of the pocket in the motif IV, were significant for NTPase and helicase activities and virus replication. In particular, Lys200 was crucial and could not be exchanged for other amino acid residues without sacrificing activity.

The availability of JEV NS3 helicase/NTPase crystal structure, as well as the mutation analysis of the residues constituting the NTP-binding pocket, enabled structure-based virtual screening for novel inhibitors of JEV NS3 helicase/NTPase. Virtual screening with application of a protein of experimentally determined structure as a target has become an established method for lead discovery and for enhancing efficiency in lead optimization (Jain, 2004). It offers the possibility to go beyond the pool of existing active compounds and thus find novel chemotypes (Cavasotto & Orry, 2007). Moreover, it makes it possible to evaluate the potency of millions of compounds in a relatively short period of time.

The aim of this work was to identify novel potent medicinal substances for the treatment of JE upon application of structure-based virtual screening of the freely available ZINC database of lead-like compounds (Irwin & Shoichet, 2005), verification of screening results in the docking procedure and, finally, refinement of the results using the consensus scoring technique (Feher, 2006).

Materials and methods

Modeling of ATP and compounds 1–22

The energy and geometry of ATP and compounds **1**–**22** were first optimized with the *ab initio* method in Hartree–Fock approximation with application of 6–31G* basis set of SPARTAN08. The obtained structures were next subjected to conformational analysis with GA Conformational Search of SYBYL8.0 (with simulation of water as a solvent) and finally, the lowest-energy conformers were optimized as in the first step. The GA Conformational Search of SYBYL8.0 was selected for conformational analysis as it produces good results in a relatively short time.

SPARTAN08 calculations were performed on the graphical station HP xw 4400, Intel COREDUO 2 6300, 1.86 GHz, 2 Gb RAM, WINDOWS XP Professional. SYBYL7.3 calculations were carried out on the graphical station 2xXeon2000, 3 GHz, 1 Gb RAM, FEDORA CORE 4.

Molecular docking of ATP and compounds 1–2 and 8–22

Docking was performed with the flexible docking method of Surflex (Jain, 2003) incorporated in SYBYL8.0. Surflex is a fully automatic flexible molecular docking algorithm, which combines the scoring function from the Hammerhead docking system with a search engine relying on a surface-based molecular similarity method used for rapid generation of suitable putative poses for molecular fragments (Jain, 2003). JEV NS3 helicase/NTPase crystal structure (PDB file 2Z83) obtained by Yamashita *et al.* (2008) was used for the docking procedure. In the case of ATP and inhibitors **1**–**2**, the side chain conformations of residues constituting the binding pocket in obtained ligand–enzyme complex were optimized with YASARA STRUCTURE upon application of the Yamber3 force field (Krieger & Vriend, 2002). This allowed optimization of the conformations of the residues constituting the binding pocket and made it possible to obtain the final enzyme structure used for virtual screening. Docking of identified hits **8**–**22** was not refined in the procedure of molecular dynamics. Automatically obtained results of library docking were treated as a relative measure of potency and used for consensus scoring.

YASARA STRUCTURE calculations were performed on the graphical station HP xw 4400, Intel COREDUO 2 6300, 1.86 GHz, 2 Gb RAM, WINDOWS XP Professional.

PYMOL (DeLano, 2002), VEGA (Pedretti *et al.*, 2004), CHIMERA (Pettersen *et al.*, 2004), SPDBV (Guex & Peitsch, 1997) and YASARA STRUCTURE (Krieger & Vriend, 2002) were used for visualization of results. All graphics were produced with PYMOL (DeLano, 2002).

Structure-based virtual screening and consensus scoring

The structure of JEV NS3 helicase/NTPase refined in the procedure of docking of ATP and **1**–**2**, followed by molecular dynamics simulation of ligand–enzyme complexes, was utilized to generate a structure-based pharmacopohore model upon application of Interaction Generation module of DISCOVERY STUDIO 2.1. All the crucial residues identified in mutagenesis studies (Yamashita *et al*., 2008), i.e. Gly199, Lys200, Thr201, Glu286, Gln457, Arg458, Arg461 and Arg464, were identified as the binding site residues. The obtained pharmacophore model was tested in the screening (with the application of Screen Library module of DISCOVERY STUDIO 2.1) of a database of 10 000 ZINC drug-like compounds, which additionally contained known inhibitors **1**–**2**, noncompetitive inhibitors **3**–**4** and compounds **5**–**7** with the confirmed lack of activity toward JEV NS3 helicase/NTPase. Next, the Screen Library module of DISCOVERY STUDIO 2.1 was applied to screen the ZINC database of about 1 161 000 lead-like compounds. Fifteen hits (**8**–**22**) have been selected and docked with Surflex to the JEV NS3 helicase/NTPase-binding site. The final ranking list was established by the simple consensus scoring procedure. The sum of the total value obtained in the docking with Surflex and the fit value obtained in the Screen Library procedure with DISCOVERY STUDIO 2.1 multiplied by 2 (to obtain equally significant contributions) was used as the final score. For the identified hits, ability to cross blood–brain barrier and lipophilicity (with the Suzuki–Kudo atomic contribution method) were calculated using Preadmet server (preadmet.bmdrc.org).

DISCOVERY STUDIO 2.1 calculations were performed on the graphical station HP xw 4400, Intel COREDUO 2 6300, 1.86 GHz, 2 Gb RAM, WINDOWS XP Professional.

Results

Refinement of JEV NS3 helicase/NTPase structure

In the first step of research, the natural ligand of NS3 helicase/NTPase, ATP, was docked with Surflex incorporated in SYBYL 8.0 to the ATP-binding site. During the docking procedure, a significant problem was the bioactive conformation of ATP. In over 70% of binding poses the obtained ATP conformation was not linear. The value of the dihedral angle determined by C5′ atom of ribose, the neighboring oxygen atom, α phosphorus atom and the bridging oxygen atom varied from -162.25° to 53.63° for the most bent conformers. The dihedral angle determined by C5′-connected ribose oxygen atom, α phosphorus atom, the bridging oxygen and the β phosphorus atom varied from 162.63° to 93.87° for the most bent conformers. It was observed that the lowest energy conformers were characterized by the least linear conformation of ATP. The energy difference between the geometrically extreme structures was 54.25 kcal mol^{-1}, due to the presence of hydrogen bonds stabilizing the ATP molecule. During the molecular dynamics simulation of ATP–enzyme complexes the ATP conformation became more bent. However, the lowest energy conformers did not result in the binding pose, which would be in accordance with the mutagenesis data (Yamashita *et al*., 2008), and therefore the compromise conformer was accepted as the final one.

The obtained mode of interaction of ATP with the enzyme is consistent with the reported mutagenesis analysis (Yamashita *et al*., 2008) and literature data concerning the mechanism of ATP hydrolysis by helicases/NTPases (Frick & Lam, 2006; Yamashita *et al*., 2008).

The binding pocket of JEV NS3 helicase/NTPase is formed by positively charged residues, i.e. Lys200, Arg461 and Arg464 of motifs I, II and VI. The most crucial residue, Lys200, projects into the pocket and recognizes the β-phosphate moiety of ATP. It forms a salt bridge with Asp285 and Glu286, which stabilizes the binding site structure. Arg461 and Arg464 in motif VI constitute an arginine finger and act as sensors recognizing the γ- and α-phosphate of ATP. It was reported that they are critical for conformational switching upon ATP hydrolysis (Ahmadian *et al*., 1997; Niedenzu *et al*., 2001; Caruthers & McKay, 2002; Yamashita *et al*., 2008). As stressed by Yamashita *et al*. (2008), the conserved water molecule necessary for ATP hydrolysis is coordinated by residues Glu286, His288 and Gln457. Thr201 directs the molecule of ATP toward interactions with Lys200 and conserved arginines. His288 was reported as essential for RNA unwinding activity (Utama *et al*., 2000a, b).

The side chain conformations of the JEV NS3 helicase/NTPase binding pocket residues were additionally refined in the docking procedure of known JEV NS3 helicase/NTPase inhibitors, **1**–**2** (Fig. 2), followed by molecular dynamics simulation.

In the case of ring-expanded nucleoside **1** (Fig. 3a), the ligand structure is stabilized by two intramolecular hydrogen bonds: one between the C3′ hydroxylic group of the sugar moiety and a nitrogen atom of the imidazole ring, and the other one between one of the keto groups and the sugar ring oxygen atom. The other keto group of the inhibitor is engaged in the network of hydrogen bond with Arg464 and, through the water molecules, with the main chain NH hydrogen atoms of Gly197 and Ser198. Arg464 also interacts with the imidazole ring nitrogen atom through another water molecule. The imidazole moiety interacts through the next water molecule with Glu286. The amino group of **1**

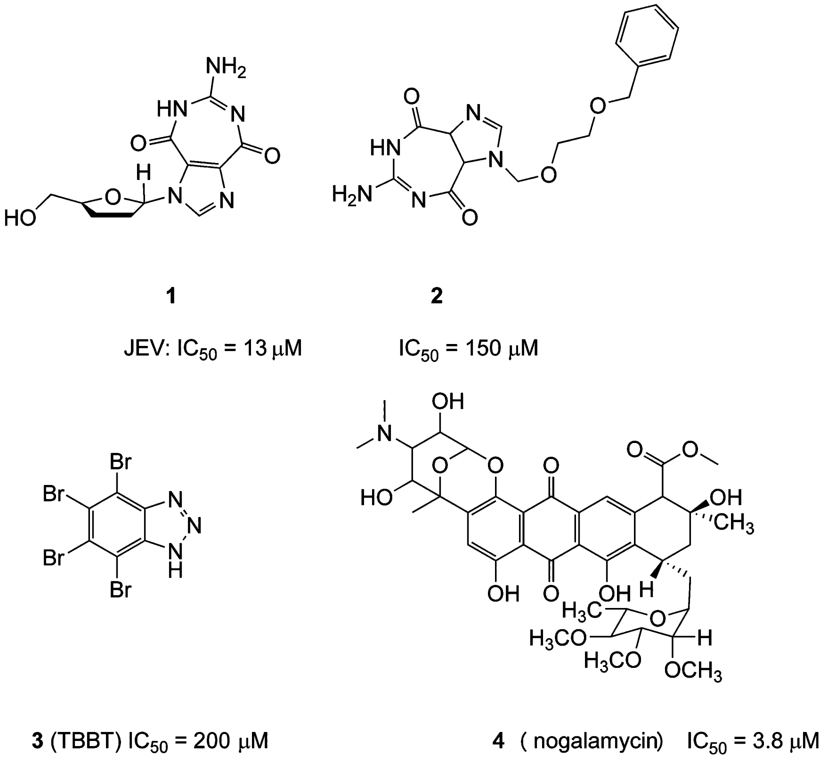

1
JEV: IC_{50} = 13 μM
2
IC_{50} = 150 μM
3 (TBBT) IC_{50} = 200 μM
4 (nogalamycin) IC_{50} = 3.8 μM

Fig. 2. Competitive **1–2** and noncompetitive **3–4** inhibitors of JEV NS3 helicase/NTPase.

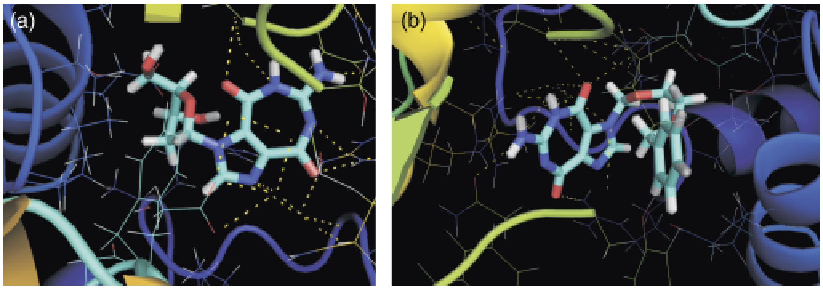


Fig. 3. Ring-expanded nucleosides **1** (a) and **2** (b) in the binding pocket of JEV NS3 helicase/NTPase.

forms a hydrogen bond with the side chain of Asn417. The obtained binding pose of **1** explains its inhibitory activity toward JEV NS3 helicase/NTPase. It interacts with two residues in the JEV NS3 helicase/NTPase binding pocket, which are crucial for ATP binding, namely with Glu286 and Arg464. Glu286 is a conserved glutamic acid residue that probably acts as a catalytic base and accepts a proton from the attacking water molecule during ATP hydrolysis (Frick & Lam, 2006). Arg464, accompanied by Arg461, constitutes an arginine finger. Both arginine residues recognize the γ- and α-phosphate of ATP.

Docking of the ring-expanded nucleoside **2** (Fig. 3b) led to similar observations and conclusions. In the case of this inhibitor, apart from the engagement of Arg464 in the formation of hydrogen bond with the keto moiety of the ligand, Arg202 interacts with the imidazole ring nitrogen atom through a water molecule. Thus Arg202, not mentioned in available literature data, may constitute another key residue of the JEV NS3 helicase/NTPase-binding pocket. Similarly as in the case of **1**, the amino group of **2** forms a hydrogen bond with the side chain of Asn417. The phenyl group of **2** fits well to the hydrophobic part of the pocket and is surrounded by apolar side chains of Val227 and Ile411.

Construction and testing of structure-based pharmacophore model

The final structure of JEV NS3 helicase/NTPase, refined in the docking procedure of ATP and selected inhibitors followed by molecular dynamics simulation, was applied to construct the structure-based pharmacophore model with the Interaction Generation module of DISCOVERY STUDIO 2.1. The pharmacophore model obtained is depicted in Fig. 4. It consists of three hydrogen bond acceptors and 15 hydrogen bond donors, and does not contain any lipophilic moieties. The pharmacophore model was tested in the screening of a database of 10 000 Zinc drug-like compounds, which additionally contained known inhibitors **1**–**2**, noncompetitive inhibitors **3**–**4** (Fig. 2) and compounds **5**–**7** (Fig. 5), with the confirmed lack of activity toward JEV NS3 helicase/NTPase. The Screen Library module of DISCOVERY STUDIO 2.1 was applied. The results are presented in Table 1. The obtained structure-based pharmacophore model for JEV NS3 helicase/NTPase was verified positively as it identified the inhibitors **1**–**2** as hits. The model also proved to be very sensitive for so-called false positives as none of noncompetitive inhibitors **3**–**4** or inactive compounds **5**–**7** was recognized as a potent compound interacting with the ATP-binding site. In this way the noncompetitive mechanism of action for TBBT **3** and nogalamycin **4** was confirmed.

Library screening, docking of the identified hits and consensus scoring

The structure-based pharmacophore model obtained for JEV NS3 helicase/NTPase was applied to screen the ZINC database of about 1 161 000 lead-like compounds. Fifteen hits (**8**–**22**) (cf. Fig. 6) have been selected (Table 1). Compounds **8**–**22** were docked to the ATP-binding site of JEV NS3 helicase/NTPase with Surflex incorporated in SYBYL8.0 (compare Table 2). The screening and docking results were combined in the consensus scoring procedure to give the final ranking list of 15 hits.

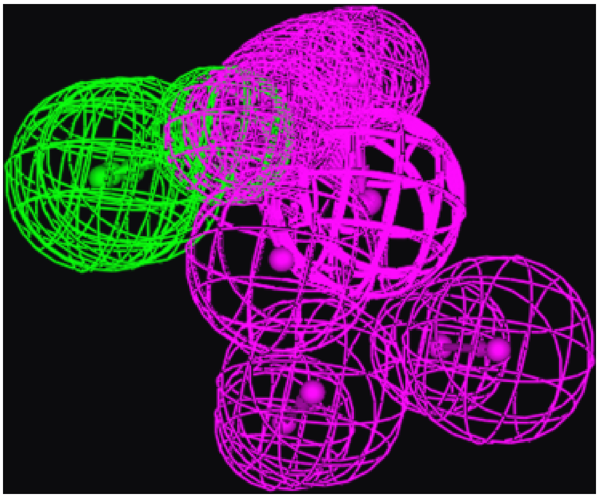


Fig. 4. The structure-based pharmacophore model for JEV NS3 helicase/NTPase inhibitors. Magenta, hydrogen bond donors; green, hydrogen bond acceptors.

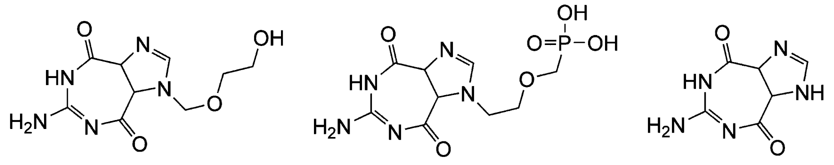
Docking of the most potent hit **8** (ZINC07570349) (Fig. 7a) reveals that the main interactions of this ligand involve the network of hydrogen bonds between Lys200, Glu286 and one of the NH hydrogen atoms of the thiourea moiety of the ligand as well as its hydroxylic group. The phenyl ring of **8** is placed in the hydrophobic cavity formed by Val227, Val228, Phe289 and Ile411.

Docking of the next hit, **9** (ZINC05339577), also revealed engagement of the crucial residues of the JEV NS3 helicase/NTPase with the potential inhibitor (Fig. 7b). In this case two hydroxylic groups of the ligand form hydrogen bonds with Glu286. Additionally, the side chain of Arg202 is engaged in the hydrogen bond with the oxirane moiety of **9**, similarly as in the case of ring-expanded nucleoside **2**. The ketone and hydroxylic groups of **9** interact with the NH hydrogen atoms of the main chains of Thr201 and Lys202.

In the case of **10** (ZINC01590677), which was the first hit in the Screen Library procedure, apart from the already mentioned Arg202 (which forms a bond with the oxygen atom of the ligand) and Thr201 (interacting with the one of NH hydrogen atoms), Glu231 also seems to be engaged, as it forms a hydrogen bond with the other NH hydrogen atoms (Fig. 7c).

The fourth hit, **11** (ZINC11756980) (Fig. 7d), interacts with both Arg202 and Arg464 (through its diazole nitrogen atom and the carbonyl group, respectively). Moreover, its amino group interacts with Asn417 and, through water molecule, with Arg461.

In the case of **12** (ZINC10674215), similarly to **10** and **11**, the side chains of Arg202, Glu231 and Arg464 are engaged in the hydrogen bonds with the ligand hydroxylic and carbonyl group, whereas the next compound identified, **13** (ZINC06668757), interacts through water molecules with the side chain of Arg464 and with the main chains of Gly199 and Lys200. The compound, **14** (ZINC04887000), is also worth mentioning because it possesses a pentose moiety and in this regard is similar to nucleosides. It forms hydrogen bonds with the side chains of Arg202 and Glu286.



5 **6** **7**

Fig. 5. Compounds **5**–**7** with the confirmed lack of anti-JEV activity used for the testing of pharmacophore model.

Table 1. Testing of structure-based pharmacophore model for JEV NS3 helicase/NTPase inhibitors (**1**–**7**) and the results of virtual screening for JEV NS3 helicase/NTPase inhibitors (**8**–**22**)

Compound number	Compound name	Fit value	Absolute energy	Number of hydrogen bond donors	Number of hydrogen bond acceptors	Pharmtype
1	–	0.414	62.165	3	0	3
2	–	2.340	58.376	2	1	3
3	TBBT	Not identified as a hit				
4	Nogalamycin	Not identified as a hit				
5	–	Not identified as a hit				
6	–	Not identified as a hit				
7	–	Not identified as a hit				
10	ZINC01590677	2.669	36.888	2	1	3
16	ZINC05344670	2.638	87.903	3	0	3
14	ZINC04887000	2.595	44.255	2	1	3
11	ZINC11756980	2.444	114.204	2	2	4
12	ZINC10674215	2.335	53.021	3	0	3
8	ZINC07570349	2.334	31.737	3	1	4
9	ZINC05339577	2.322	281.609	3	1	4
13	ZINC06668757	2.249	41.631	3	1	4
18	ZINC08566495	2.219	40.893	3	1	4
15	ZINC00134670	2.174	29.364	1	2	3
21	ZINC07570567	1.984	44.922	3	1	4
17	ZINC07198995	1.856	29.837	3	0	3
20	ZINC07570363	1.830	32.462	3	1	4
19	ZINC01231723	0.993	35.251	3	2	5
22	ZINC04413017	0.447	17.323	3	2	5

The other eight potential inhibitors **15–22** identified interact with the binding pocket of JEV NS3 helicase/NTPase in a similar way to **8–14**. However, they are characterized by significantly lower scores, which indicates a worse fit to the binding site.

It is worth emphasizing that among 15 identified potential inhibitors only one of them, **14**, exhibits partial similarity to the natural ligand, ATP. The others constitute novel chemotypes of JEV NS3 helicase/NTPase inhibitors.

Additionally, lipophilicity and the ability to cross the blood–brain barrier for identified hits were calculated with Preadmet server (preadmet.bmdrc.org). The results are presented in Table 3. Although most compounds do not cross the blood–brain barrier easily, first of all because of low lipophilicity value, compounds **8**, **12**, **18**, **20** and **21** are good candidates for further modification directed toward better ADMET properties of a central nervous system-active drug.

Discussion

A number of potent inhibitors of helicases encoded by herpes simplex virus, severe acute respiratory syndrome coronavirus, hepatitis C virus (HCV), West Nile virus (WNV), human papillomavirus and JEV have been reported recently in the scientific literature (Borowski *et al.*, 2002, 2003; Zhang *et al.*, 2003; Bretner *et al.*, 2004a, b, 2005; Ujjinamatada *et al.*, 2007). Some inhibitors have been demonstrated to decrease viral replication in cell culture and animal models (Frick & Lam, 2006).

Most JEV NS3 helicase/NTPase inhibitors belong to two chemical classes: ring-expanded 'fat' nucleosides and nucleotides **1–2** (Zhang *et al.*, 2003) or benzimidazoles and benzotriazoles **3** (Borowski *et al.*, 2003; Bretner *et al.*, 2005) (Fig. 2). The first class may be treated as close analogs of nucleosides and nucleotides. As these inhibitors are similar to the natural NS3 helicase/NTPase ligand, ATP, they are very likely to compete with ATP for the same binding site. Benzimidazoles and benzotriazoles as well as some naturally occurring compounds such as antibiotic nogalamycin **4** are modulators that interact with the allosteric binding site (Borowski *et al.*, 2002, 2003). The mechanism of their modulating effect remains unclear. However, it may be speculated that the second binding site, which could be occupied by a nucleotide, nucleoside and even by nucleotide base, probably fulfils a regulatory function with respect to the NTPase and/or helicase activities of the enzyme (Borowski *et al.*, 2002).

The research presented provides for the first time potential competitive JEV NS3 helicase/NTPase inhibitors that are structurally distinct from nucleosides and their analogs. The design of medicinal substances constituting prototypes of anti-JEV drugs raises at least three important concerns: first, whether there is a need for anti-JEV therapy if several vaccines against JE are available; secondly, the possibility of

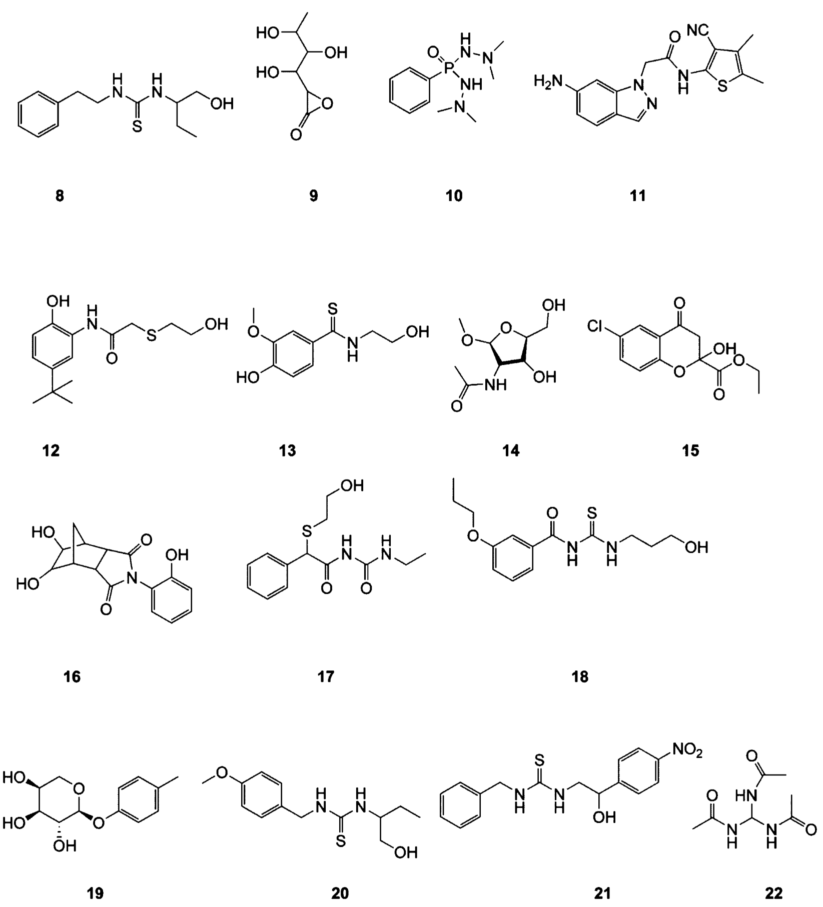


Fig. 6. The hits **8**–**22** identified in virtual screening procedure.

Table 2. The results of docking of identified hits to JEV NS3 helicase/NTPase binding site and consensus scoring

Compound number	Compound name	Total score	Crash	Polar	D score	PMF score	G score	Chem score	C score	Consensus score
10	ZINC01590677	4.68	− 1.1	0.92	− 80.17	− 7.68	− 153.84	− 7.25	3	10.08
16	ZINC05344670	2.96	− 2.43	3.82	− 94.35	− 4.76	− 167.68	− 6.30	4	8.24
14	ZINC04887000	3.38	− 0.51	3.35	− 66.81	− 16.73	− 95.15	− 3.54	2	8.57
11	ZINC11756980	4.71	− 1.90	4.28	− 50.20	29.06	− 167.47	− 8.62	2	9.60
12	ZINC10674215	4.87	− 1.02	5.19	− 77.62	17.34	− 111.27	− 3.64	1	9.54
8	ZINC07570349	6.04	− 2.46	3.01	− 101.79	18.98	− 212.43	− 16.08	4	10.71
9	ZINC05339577	5.62	− 0.72	5.91	− 85.85	− 9.56	− 104.50	− 7.60	3	10.26
13	ZINC06668757	4.07	− 2.06	3.10	− 93.05	30.03	− 136.16	− 10.84	4	8.59
18	ZINC08566495	3.15	− 1.73	1.73	− 90.87	16.11	− 115.33	− 1.73	2	7.59
15	ZINC00134670	4.03	− 0.56	3.16	− 60.73	− 8.09	− 89.40	− 12.15	3	8.38
21	ZINC07570567	1.13	− 1.80	3.97	− 118.85	17.57	− 213.25	− 7.22	3	5.10
17	ZINC07198995	4.21	− 1.51	0.91	− 97.55	10.97	− 180.86	− 5.80	3	7.92
20	ZINC07570363	2.90	− 3.53	2.70	− 120.82	15.77	− 192.00	− 7.21	4	6.56
19	ZINC01231723	4.59	− 1.75	3.39	− 87.64	− 18.16	− 126.91	− 6.51	4	6.58
22	ZINC04413017	4.01	− 1.22	3.57	− 46.29	6.06	− 78.67	− 5.57	2	4.96

laboratory diagnosis before application of anti-JEV drugs; and last but not least, whether the designed compounds are capable of reaching the central nervous system, which will be discussed later.

Indeed, the main pillar of JE control is the use of a live attenuated vaccine for humans, developed about 40 years ago (Igrashi, 2002). Although currently available JE vaccines are relatively safe and effective, the drawback is that multiple

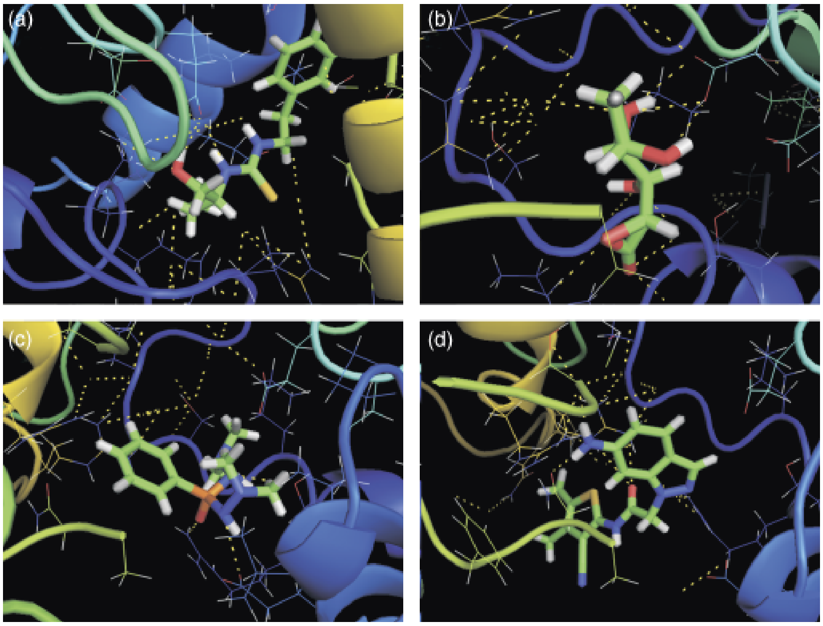


Fig. 7. The identified hits **8** (a), **9** (b), **10** (c) and **11** (d) in the binding pocket of JEV NS3 helicase/NTPase.

Table 3. Ability to cross blood–brain barrier and lipophilicity of identified hits calculated with Preadmet server

Compound number	Compound name	C_{blood}/C_{brain}	SKlogP
8	ZINC07570349	0.87	2.01
9	ZINC05339577	0.27	− 1.74
10	ZINC01590677	0.49	0.01
11	ZINC11756980	0.09	2.38
12	ZINC10674215	0.34	2.72
13	ZINC06668757	0.45	0.68
14	ZINC04887000	0.27	− 1.81
15	ZINC00134670	0.17	1.83
16	ZINC05344670	0.36	− 0.51
17	ZINC07198995	0.08	1.30
18	ZINC08566495	0.30	2.04
19	ZINC01231723	0.08	0.60
20	ZINC07570363	0.32	1.81
21	ZINC07570567	0.20	2.49
22	ZINC04413017	0.20	− 1.99

doses are required. Furthermore, effective delivery of the vaccines to poor communities remains a formidable challenge and compliance and delivery costs have to be considered (Erlanger *et al.*, 2009). Taking the above into consideration, at present when the system of vaccination does not cover the whole population in danger of JEV infection, antiviral therapy remains a useful alternative. Fortunately, reliable laboratory diagnosis of JE is at present available.

The diagnosis of JEV infection should be made within an epidemiological context (Diagana *et al.*, 2007). During epidemic outbreaks a febrile meningeal syndrome should be considered JE above any other diagnostic consideration. The combination of central hyperpneic breathing associated with extrapyramidal symptoms has an 81.3% positive and 41.3% negative predictive value (Diagana *et al.*, 2007). As it is difficult, due to the transiency of viremia, to isolate the virus in blood cells obtained by venipuncture, serology plays an important role in confirming the diagnosis. The enzyme-linked immunosorbent assay method reveals antibodies (IgM) directed against the viral particles in 75% of cases (Diagana *et al.*, 2007).

Although the activity of proposed anti-JEV compounds has not been experimentally verified yet, the reliability of the results is enhanced by the fact that the crystal structure of the catalytic domain has been solved by a roentgenographic method (Yamashita *et al.*, 2008) and was refined by molecular docking of ATP and known inhibitors followed by molecular dynamics simulations. The quality of this refinement depends on how well the binding pose of ATP (as well as of inhibitors **1–2**) was predicted.

Although the position of ATP bound to neither JEV NS3 helicase/NTPase nor to any viral helicase/NTPase has not yet been visualized, the mechanism of its hydrolysis most likely resembles that seen in other helicases (Frick, 2007). The approximate configuration of ATP in the binding site can be seen by comparing a JEV helicase structure with one of a similar helicases crystallized in the presence of a nonhydrolyzable ATP analog. For example, in the crystal structure of the *Escherichia coli* RecQ helicase catalytic core in the complex with the ATP analog ATPγS (PDB code 1OYY) the adenine moiety is packed between Tyr23 and Arg27 side chains and hydrogen bonds are formed between the N6 and N7 atoms of the adenine and Gln30 of RecQ motif 0 (Bernstein *et al*., 2003). The ATPγS triphosphate is bound to RecQDC by Lys53 and several backbone amides in motif I, and through an Mn^{2+} ion, which makes water-mediated contact with Ser54 of motif I and Asp146 of motif II. The obtained binding mode of ATP to JEV NS3 helicase/NTPase corresponds to the position of ATPγS RecQ helicase catalytic core described above. Moreover, it should be stressed that the conformation of ATPγS is slightly bent, similar to the final conformation of ATP. The conformation and binding mode of ATP in the binding pocket of JEV NS3 helicase/NTPase are also consistent with the recently obtained crystal structure of dengue virus 4 NS3 helicase in complex of ADP, PDB file 2JLS (Luo *et al*., 2008). In this crystal structure, the role of conserved lysine (Lys199) and two conserved arginines (Arg460 and Arg463) are clearly visible.

The obtained binding mode of ATP in the JEV NS3 helicase/NTPase as well as the pose of ligands in two crystal structures described above allows speculation about the role of arginine fingers in the process of ATP hydrolysis. Apart from recognition of triphosphate group of ATP, arginine fingers may be responsible for displacement of water out from the binding site. Such a role of arginine fingers was recently demonstrated for the Ras–RasGAP complex in the QM/MM calculations (Heesen *et al*., 2007).

A more detailed analysis of JEV NS3 helicase/NTPase structure may lead to the conclusion that to function as a catalytic base, the pK_a of Glu286 would need to be much higher than that of a typical glutamic acid residue in a protein, as suggested for HCV helicase (Frick, 2007). It was thus proposed that the neighboring aspartic acid residue (Asp285 in JEV NS3 helicase/NTPase) may serve as a catalytic base instead.

Docking of known JEV NS3 helicase/NTPase inhibitors **1**–**2** revealed engagement of crucial binding pocket residues in the interactions with ligands. In particular, the role of Glu286 and Arg464 was clearly depicted. Moreover, docking of **1**–**2** allowed the identification of Arg202 as an additional important residue of the binding pocket, making this arginine a straightforward candidate for mutational studies. The analysis of ATP–enzyme complex allowed speculation about the role of conserved threonine Thr201. Most probably, it directs the ligand properly toward interactions with Lys200 and the conserved arginine residues. A similar role may be assigned to the branched side chains of apolar amino acids (especially Val227 and Ile411), which was demonstrated in the case of **2** and was suggested earlier for ionotropic glutamate receptors (Kaczor *et al*., 2008). Docking of **1**–**2** indicated Asn417 as an additional anchoring point, whereas docking of identified hits **8**–**22** also indicated Glu231 as a potentially important residue for interactions with inhibitors.

Virtual screening procedure made it possible to identify 15 potential inhibitors of JEV NS3 helicase/NTPase. Only one of them, namely the one containing pentose moiety **14**, may be treated as a far analog of nucleosides. This structural diversity may prove beneficial because it increases the likelihood that the new inhibitors will be selective toward human ATPases. This is a significant problem: it is worth emphasizing that ring-expanded nucleosides **1** and **2** also have high affinity to human Suv3 mitochondrial helicase (routinely used to test the selectivity of novel inhibitors of viral helicase/NTPase), which excludes them as drug candidates (Zhang *et al*., 2003).

On the other hand, compounds **1** and **2** were also active toward all the tested viral helicase/NTPases: WNV and HCV. This seems promising, as the research on specific anti-JEV compounds may lead to the development of a drug with broad antiviral spectrum of activity. Indeed, structural analysis of the helicases of dengue virus (DEN), yellow fever virus (YFV) and HCV revealed that these viral helicases have very similar structures composed of three functional domains (Yao *et al*., 1997; Wu *et al*., 2005; Xu *et al*., 2005; Yamashita *et al*., 2008). The amino acid sequences of the NS3 helicase domain of JEV exhibited 65%, 44% and 23% homology to those of DEN, YFV and HCV, respectively (Yamashita *et al*., 2008). The crystal structures of the NS3 helicases of DEN (Xu *et al*., 2005) and YFV (Wu *et al*., 2005) are similar to that of JEV, but slightly different from HCV (Yao *et al*., 1997). Yamashita *et al*. (2008) emphasized that the distance between domains 1 and 2 of HCV helicase is longer than that in most flavivirus NS3 helicases. This leads to the conclusion that the HCV helicase has a larger ATP-binding pocket than other flaviviruses, and that the folding of domain 3 of the HCV helicase is unique, whereas the folding of JEV is very similar to those of other flaviviruses, including DEN and YFV (Yamashita *et al*., 2008). Superposition of JEV, DEN, YFV and HCV helicases further clarified that the HCV helicase has a unique conformation in the NTPase-binding region and domain 3 in comparison with JEV, DEN and YFV helicases (Yamashita *et al*., 2008). In particular, the conformation of motifs I and II of HCV helicase was different from that of JEV, DEN and YFV helicases. The distance between motifs I and II of Cα of HCV and the other flaviviruses was 6.7 and 3.5 Å, respectively (Yamashita *et al*., 2008). There was also a 4.7 Å difference in the distance of Nz of Lys200 in the motif I between JEV and HCV, suggesting that HCV helicase has a wider ATP-binding pocket than other flaviviruses (Yamashita *et al*., 2008). In contrast to the structure of motifs I and II, that of motif VI was well conserved among the flavivirus helicases, including HCV. Although a subtle difference is observed, the ATP-binding residues in JEV, DEN, YFV, and HCV helicases are well conserved, suggesting that flavivirus helicases possess similar mechanisms of ATP hydrolysis, which reflects the lack of specificity of compounds **1** and **2**.

The virtual screening performed allowed the noncompetitive mode of action of **3** and **4** to be confirmed, as they were not identified as hits for the ATP-binding site.

Although the antiviral activity of the identified hits needs to be confirmed in experimental studies, the reliability of the computational results obtained is enhanced by several factors. As mentioned, the refined crystal structure of the catalytic domain of JEV NS3 helicase/NTPase was utilized to construct the pharmacophore model. Moreover, the residues constituting the ATP-binding site were identified in the mutational analysis. Finally, the application of consensus screening procedure improved the hit ranking list.

The consensus scoring procedure has been demonstrated to improve virtual screening results significantly (Feher, 2006). It was reported that consensus scoring usually substantially enhances virtual screening performance, contributing to better enrichments. It also seems to improve, although to a lesser extent, prediction of bound conformations and poses. In particular, the consensus scoring procedure improves prediction of binding energies, which is the greatest problem in virtual screening. Although the obtained binding energy predictions are still inaccurate and further development is required before they can be used for this purpose in routine lead optimization, the consensus scoring procedure is at present the only alternative for improvement of the *in silico* screening procedure.

Wang & Wang (2001) distinguished three main ranking methods of consensus scoring for virtual screening: rank-by-number (all the candidates are ranked according to the average predicted values given by all the scoring functions), rank-by-rank (all the candidates are ranked by the average ranks predicted by all the involved scoring functions) and rank-by-vote (if a candidate is predicted to be on the top, for example 2%, by a certain scoring function, then it gets a 'vote' from that scoring function; the final score of a candidate compound is the number of votes gathered from all the scoring functions, which may range from 0 to the total number of scoring functions). The approach we applied may be treated as a modification of rank-by-number method, as we used a sum of total score by Surflex and a doubled value of fit obtained with the Screen Library module of DISCOVERY STUDIO 2.1.

Although most of the proposed hits are characterized by lipophilicity < 2 (the range for CNS active drugs is from 2 to 4) and do not cross blood–brain barrier easily, it is obvious that they should be treated as prototypes of drugs that require further optimization (especially of their ADMET properties) before reaching the market.

The studies performed allowed 15 potential inhibitors to be selected from the database of 1 161 000 compounds, which constitutes the reasonable alternative for experimental HTS procedure. Moreover, novel structural features of JEV NS3 helicase/NTPase have been identified, including new important residues in the enzyme-binding pocket. The problem of anti-JEV specificity of novel compounds and their selectivity over human ATPases was also addressed. To conclude, the computational project performed may be treated as a guide for experimental work on viral helicases/NTPases and antiviral drug design.

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