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ORIGINAL ARTICLE

SEARCHING FOR NEW ANTIMICROBIAL AGENTS BY TARGETING BACTERIAL NAD METABOLISM: EVALUATION OF FRENTIZOLE DERIVATIVES SELECTED BY MOLECULAR DOCKING

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Summary

Growing evidence of antibiotic-resistant pathogens is a serious medical issue that has to be addressed. Our antimicrobial research is focused on searching for novel small molecules that differ from the most clinically used antibiotics by chemical structure and mechanism. However, this fundamental research is like looking for a needle in a haystack. In addition, *in vitro* methods are time-consuming and expensive to screen large number of compounds in reasonable time. Off-target screening can represent a solution to find novel and effective antimicrobial agents that can eliminate these problems. Accordingly, molecular docking in the family of selected frentizole derivatives predicted their potential to inhibit bacterial nicotinate mononucleotide adenylyltransferase (NadD). This bacterial-essential specific enzyme has an important role in NAD metabolism. Thus, underlying mechanism of antimicrobials derived from frentizole would be interference with this biochemical process. Unfortunately, broth microdilution assay did not display any antimicrobial activity of tested compounds. On the other hand, herein we propose that off-target screening can facilitate searching for new drugs and that NadD could be a relevant target for antimicrobials.

Key words: antimicrobials; molecular docking; NadD inhibitors; frentizole derivatives

Introduction

Increase and spread of resistance of bacterial pathogens to the most clinically used antibiotics lead to search for new antimicrobial targets. One of the promising strategies could be targeting the bacterial central metabolic pathways (1). Our interest is oriented towards inhibition of bacterial nicotinate mononucleotide adenylyltransferase (NadD; E.C. 2.7.7.18) representing key bacterial enzyme in order to developed new and potentially broad-spectrum antibacterial agents.

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NadD is an essential bacterial enzyme conserved in the majority of bacterial species. There are two common NAD biosynthetic pathways – *de novo* and salvage, both of them are dependent on the action of NadD enzyme. NAD cofactor is indispensable for the numbers of redox and even non-redox reactions in cell (2,3). Some studies have identified several inhibitors of NadD revealed by kinetic enzyme evaluation that are potentially applicable as antibacterial pharmacophores (2,4).

The set of small molecules tested in this study was originally designed to modulate the amyloid beta-binding alcohol dehydrogenase (ABAD) activity and to prevent its interaction with amyloid-beta (Aβ). ABAD is a mitochondrial dehydrogenase implicated in pathophysiology of Alzheimer's disease (AD) due to interaction with intracellular Aβ (5–7). According to this hypothesis, it is believed that hampering this interaction might mitigate AD symptoms and impose disease-modifying effect (8). However, the off-target screening of these benzothiazole derivatives to other clinically relevant targets predicted their inhibitory potential against NadD. To pursue the hypothesis, we applied the molecular docking as a powerful tool in the search for other biological systems potentially affected by these small molecules. Moreover, the docking studies can enable considering side effects of designed structures before their synthesis and testing. Another advantage of employing *in silico* simulation prior the synthesis is the possibility to study a large number of protein targets in a relatively short time with low costs.

Since the antimicrobial activity was expected for the tested frentizole derivatives by using the off-target screening, the main goal of the present study was to confirm this assumption by *in vitro* testing and to find new potentially useful antimicrobial agents that could serve as hit for ongoing modulation.

Experimental part

Off-target screening

The process of the off-target screening can be divided into three steps. During the first part, the set of 58 frentizole derivatives selected from the internal "K-database" of small organic molecules (deposited at Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, Hradec Kralove, Czech Republic) was docked into a database containing more than 9 thousands protein receptors (database scPDB v. 2013 (9)). Based on the docking score (predicted binding energy), 47 receptors were preselected for the second phase. The set of frentizole derivatives and their decoys (40 decoys for each inhibitor) were docked into the preselected protein receptors. In the results array, the docking scores of frentizole derivatives were compared to the scores of decoys for each receptor. In these two phases, the semiflexible docking was used (flexible ligand and rigid receptor). The evaluation was carried out by the method of receiver operating characteristics (ROC) curve (10) and quantified by the area under curve (AUC). The receptors with preferred scoring were determined as probable biological targets in the set of frentizole derivatives. Three clinically relevant targets emerged as follows: monoaminooxidase B, Abelson tyrosine kinases 1 and 2, and bacterial nicotinate mononucleotide adenylyltransferase (NadD).

In the last step of the *in silico* study, top-scored frentizole derivatives were preselected for further *in silico* testing. This set was docked into several crystal structures of NadD – PDB IDs: 1K4M (11) (*Escherichia coli* NadD), 2H29 (12) (*Staphylococcus aureus* NadD), 2QTR (13), 3E27 (2), 3MLA and 3MLB (4) (*Bacillus anthracis* NadD). For assessment of selectivity for the bacterial NadD, some human targets (human nicotinamide/nicotinic acid mononucleotide adenyltransferase, NMNAT) were implemented to this part of the study – PDB IDs: 1GZU (14), 1KQN, 1KR2 (15), 1NUP (16). The flexible docking was used with spherical selections of flexible residues around the binding cavity. Based on the docking scores and predicted selectivity, 20 frentizole derivatives were recommended for further *in vitro* testing. The emphasis was put on their high affinity to NadD and selectivity for bacterial over human species.

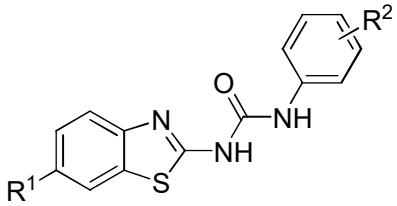
The receptors were prepared by the software MGLTools (17,18), the small molecules were built by the software ChemSketch (v. 12.01), their 3D structures were built by the software OpenBabel (v. 2.3.1) and prepared for docking by the software package MGLTools (17). The docking calculation was performed by the software AutoDock Vina (v. 1.1.2) (19). The suitability of the process was tested by docking the frentizole derivatives as known ABAD inhibitors and their decoys into the receptor PDB ID 1U7T (Crystal Structure of ABAD/HSD10 with a bound Inhibitor, 2.0 Å) (20) and by constructing the ROC curve from the docking scores. The docking simulation ranked the frentizole derivatives ahead the decoys so it the process can be considered with high predicted value and suitable.

Bacterial strains and media

The following panel of four Gram-positive and four Gram-negative bacterial strains was used for *in vitro* antibacterial susceptibility tests: *Staphylococcus aureus* (C1947), Methicillin-resistant *Staphylococcus aureus* (C1923), *Staphylococcus epidermidis* (C1936), Vancomycin-resistant *Enterococcus, Escherichia coli* (A1235), extended spectrum B-lactamases (ESBLs) not producing *Klebsiella pneumoniae* (C1950), ESBLs producing *Klebsiella pneumoniae* (C1914) and multi-resistant *Pseudomonas aeruginosa* (A1245). All aforementioned bacteria used in study were obtained as clinical isolates from patients (University Hospital Hradec Kralove, Czech Republic) and stored at −70 °C in Cryobanks according to the manufacturer's instructions. Bacterial strains were inoculated and cultivated on Mueller-Hinton agar.

Tested compounds

Structures of the small molecules selected for testing are derived from frentizole. They possess several common structural features like benzothiazole and a phenyl moieties, tethered by either urea, thiourea or guanidine linkers (Fig. 1) (5–7). A two-fold serial dilution of the selected compounds was used to quantify the biological activities. All concentrations were prepared by dissolving in DMSO and added into the microplate wells contained Mueller-Hinton broth (buffered to pH 7.0). The final concentration of DMSO was 1%. Due to solubility issues, the standard concentration normally ranging between 500–0.49 μM was shifted to 250–0.25 μM or 125–0.13 μM. The compound‘s solubility at concentration of 125 μM was the minimum required for the evaluation. Having said that, only 11 out of 20 compounds underwent the antimicrobial assay. The final concentration range for each compound is displayed in Table 1.



K692 R^1 = F; R^2 = 3-COOH, 4-OH
K699 R^1 = Cl; R^2 = 3-COOH, 4-OCH_3
K700 R^1 = F; R^2 = 4-OPh
K708 R^1 = F; R^2 = 4-$NHCOCH_3$
K709 R^1 = Cl; R^2 = 4-$NHCOCH_3$
K809 R^1 = OCH_3; R^2 = 4-COOH
K825 R^1 = OCF_3; R^2 = 3-Cl, 4COOH
K826 R^1 = OCF_3; R^2 = 2-OH, 4COOH
K827 R^1 = OCF_3; R^2 = 3-OH, 4COOH
K828 R^1 = OCF_3; R^2 = 3-COOH, 4OH
K833 R^1 = OCF_3; R^2 = 3-OCH_3, 4COOH

Figure 1. General structure of tested frentizole derivatives. Only derivatives containing urea linker are outlined since these were the only included into biological evaluation.

Table 1. Concentration range for the tested compounds. For the sake of clarity, compounds excluded from evaluation are also displayed in the table

Concentration range	Excluded from evaluation due to low solubility profile	125–0.13 μM	250–0.25 μM	500–0.49 μM
Compounds	**K701, K702, K703, K706, K707, K818, K820, K822, K824**	**K700, K709, K809**	**K692, K708, K825**	**K699, K826, K827, K828, K833**

Evaluation of antimicrobial activity

The antibacterial susceptibility against bacteria were determined by a microdilution broth method according to standard M07-A07 (21), the optimized protocol was published previously (22, 23). The bacterial suspensions were controlled densitometrically to reach 1.5×10^8 viable colony forming units (CFU) per 1 mL. The minimal inhibitory concentrations (MIC), defined as 95% inhibition of bacterial growth, were determined after 24h and 48h of incubation at 36 °C ± 1 °C. The minimal bactericidal concentrations (MBC) were determined as the concentration of compound causing a decrease in the number of bacterial colonies by > 99.9 %.

Results

Table 2 displays calculated binding energy for each frentizole derivative into human, *E. coli*, *S. aureus* and *B. anthracis* NadD enzymes. The selected compounds revealed high affinity for bacterial enzymes, especially for *Bacilus anthracis* NadD (the best docking score lower than −13.6 kcal·mol^{-1}) and also high affinity over human NMNAT (the best docking score −10.7 kcal·mol^{-1}).

Figure 2 shows a representative interaction of a frentizole derivative (K833) with *S. aureus* NadD (PDB ID 2H29). The binding energy of this pose was predicted at −12.7 kcal·mol^{-1}. The carboxylate group of K833 creates an ionic interaction with Arg133. There is also a complex web of hydrogen bonds between the carboxylate group and His15, His18, Ser155 and Ser156, another hydrogen bond is formed between ether oxygen from methoxy group and Arg133. The nitrogen atoms from urea moiety enabled hydrogen bond formation with the backbone oxygen of Gly8, and urea oxygen is also anchored to Lys45. The aromatic nitrogen is attached to Lys45 and Ser42.

Table 2. Top-scored binding energies of frentizole derivatives selected for testing (kcal·mol^{-1})

organism	*E. coli*	*S. aureus*	*B. anthracis*				*H. sapiens*			
PDB ID	1K4M	2H29	2QTR	3E27	3MLA	3MLB	1GZU	1KQN	1KR2	1NUQ
K692	−9.8	−11.7	−10.9	−10.9	−12.3	−12.3	−9.5	−9.3	−8.5	−10.0
K699	−9.5	−11.5	−11.3	−11.0	−12.5	−9.9	−8.8	−8.5	−8.7	−10.0
K700	−10.7	−12.6	−12.0	−11.8	−12.8	−13.1	−10.7	−10.6	−11.5	−10.4
K701	−10.8	−12.5	−12.0	−11.6	−12.9	−12.2	−10.0	−10.0	−9.8	−9.9
K702	−9.9	−11.7	−11.4	−10.9	−12.8	−9.9	−10.0	−8.3	−9.2	−9.4
K703	−9.9	−11.5	−10.8	−10.5	−13.1	−12.0	−10.0	−10.3	−8.9	−9.6
K706	−9.2	−11.2	−10.3	−10.9	−12.7	−11.2	−10.1	−9.7	−8.7	−9.6
K707	−9.2	−11.1	−10.9	−11.1	−13.0	−11.9	−10.1	−9.9	−8.8	−9.7
K708	−9.8	−11.7	−10.8	−10.9	−11.9	−11.1	−10.2	−8.1	−8.7	−9.3
K709	−9.9	−11.5	−10.8	−10.7	−12.2	−12.3	−9.7	−10.6	−9.8	−9.3
K809	−9.2	−11.0	−10.8	−10.8	−12.8	−11.1	−9.8	−8.6	−9.1	−9.6
K818	−9.4	−10.9	−11.0	−10.7	−12.8	−11.1	−10.2	−9.0	−8.7	−9.8
K820	−9.7	−11.1	−11.1	−10.5	−12.8	−12.0	−9.3	−8.7	−8.8	−9.3
K822	−10.6	−11.5	−11.0	−11.1	−11.8	−11.5	−9.7	−8.7	−8.3	−9.9
K824	−10.2	−11.5	−11.4	−11.0	−11.5	−11.5	−9.8	−8.3	−8.4	−10.2
K825	−10.9	−12.4	−11.7	−11.5	−13.6	−12.5	−10.3	−10.4	−10.1	−10.6
K826	−10.8	−12.3	−11.9	−11.3	−11.3	−12.3	−10.3	−9.1	−10.0	−10.7
K827	−11.2	−12.3	−11.9	−11.5	−10.9	−12.5	−10.2	−9.0	−9.9	−10.4
K828	−10.4	−11.8	−11.6	−12.0	−12.3	−11.8	−10.1	−9.6	−10.1	−10.3
K833	−11.0	−12.7	−12.0	−11.2	−12.5	−12.5	−10.1	−9.8	−9.2	−10.5

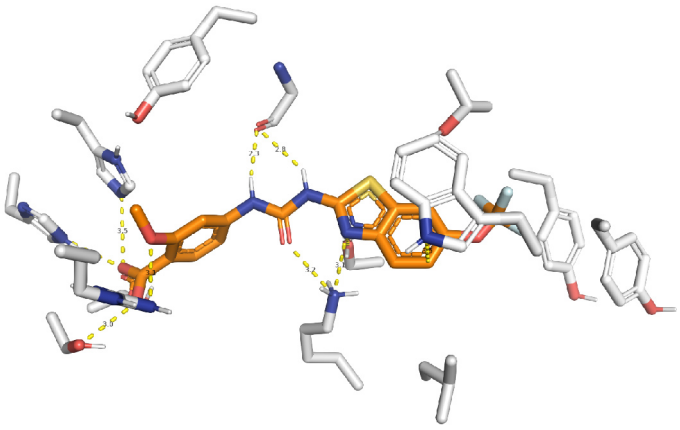


Figure 2. The best scored docking pose of K833 in the *S. aureus* NadD (PDB ID 2H29). An ionic interaction with Arg133, hydrogen interactions with His15, His18, Ser155, Ser156, Arg133, Gly8, Lys45 and Ser42, one sandwich integration with Trp116. The interaction is displayed in dashed lines, distance measured in angstrom (Å).

The benzothiazole system creates π-π stacking with Trp116. Trifluoromethyl group is placed in a cavity formed by Tyr84 and Tyr117.

11 frentizole derivatives underwent antimicrobial activity determination. The obtained MIC and MBC values were compared with benzalkonium bromide (BAC_{14}), as the commonly used standard disinfection. Unfortunately, the antimicrobial effect of all the tested derivatives against the Gram-negative bacteria was not established. Six compounds (K825, K826, K827, K828, K833 and K809) have shown antimicrobial activity against Gram-positive bacteria – *Staphylococcus Aureus*, Methicillin-resistant *Staphylococcus aureus* (except for K828 and K809), *Staphylococcus epidermidis* (except for K828) and Vancomycin-resistant *Enterococcus* (only K833). However, even the best obtained MICs and MBCs have been substantially higher than values corresponding to standard BAC_{14}. Therefore, none of the tested derivatives can be considered as novel promising antimicrobial agent. Obtained MIC a MBC values of tested compounds and standard are summarized in Table 3.

Discussion

The computational methods based on benchmarking with a set of decoys provides robust data. It enables to predict the affinity and sometimes even intrinsic activity of ligand (small molecule, protein) to selected biological targets (enzyme, receptor). In our study, the computational data demonstrated high probability of interaction of frentizole derivatives with bacterial NadD. Molecular docking predicted their affinity to this specific enzyme but it cannot certainly determine the effect. The comparison of the top-scored docking pose of K833 in the *S. aureus* NadD enzyme with known inhibitors in the crystal structure clearly shows possibility to bind to the active site and assumes the inhibition activity (12).

Based on the predicted NadD inhibition by molecular docking, the antimicrobial activity of certain frentizole derivatives was expected. Unfortunately, the evaluation by microdilution broth method did not show any effectiveness against the Gram-negative bacteria and only six of the tested compounds established very low antimicrobial effect against the Gram-positive bacteria in the comparison to standard BAC_{14}. Since the effectiveness is dependent on the action of compound inside the bacterial cell, the critical factor orchestrating *in vitro* failure is probably poor penetration of agents across the bacterial wall with subsequent low concentration of active compound inside the cell. The insufficient transport of agents inside the bacteria, especially across the very impermeable Gram-negative bacterial wall, can explain complete inactivity against those bacteria. Similar conclusion has already been observed in the study of *Sorci et al*. reporting much weaker susceptibility of NadD inhibitors against the Gram-negative bacteria in comparison to the effect delivered against the Gram-positive (2). Possible solution and another step forward understanding the underlying mechanism in the family of frentizole derivatives would be *in vitro* evaluation between NadD enzyme and each compound, thus avoiding cellular testing.

Table 3. Results of antimicrobial activity determination.

	MIC (μmol·l^{-1}); 24h incubation											
	MIC (μmol·l^{-1}); 48h incubation											
	MBC (μmol·l^{-1}); 48h incubation											
Compound	**K692**	**K699**	**K708**	**K825**	**K826**	**K827**	**K828**	**K833**	**K700**	**K709**	**K809**	**BAC$_{14}$**[a]
Staphylococcus aureus	>250	>500	>250	**62,5**	**125**	**125**	**125**	**62,5**	>125	>125	**125**	**0,98**
	>250	>500	>250	**125**	**125**	**125**	**125**	**125**	>125	>125	>125	**1,95**
	>250	>500	>250	**125**	**125**	**125**	**500**	**250**	>125	>125	>125	**1,95**
Methicillin-resist. Staphylococcus aureus	>250	>500	>250	**125**	**250**	**250**	>500	**250**	>125	>125	>125	**7,81**
	>250	>500	>250	**125**	**250**	**250**	>500	**250**	>125	>125	>125	**7,81**
	>250	>500	>250	>250	>500	**250**	>500	**500**	>125	>125	>125	**15,63**
Staphylococcus epidermidis	>250	>500	>250	**125**	**500**	**250**	>500	**250**	>125	>125	**250**	**7,81**
	>250	>500	>250	**250**	**500**	**250**	>500	**250**	>125	>125	>250	**7,81**
	>250	>500	>250	>250	>500	**250**	>500	**500**	>125	>125	>250	**15,63**
Vancomycin-resist. Enterococcus	>250	>500	>250	>250	>500	>500	>500	**500**	>125	>125	>125	**15,63**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**15,63**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**31,25**
Escherichia coli	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**31,25**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**31,25**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**31,25**
Klebsiella pneumoniae ESBL –	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
Klebsiella pneumoniae ESBL +	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**62,5**
Pseudomonas aeruginosa Multirezistent.	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**500**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**500**
	>250	>500	>250	>250	>500	>500	>500	>500	>125	>125	>125	**500**

[a] BAC$_{14}$ is an abbreviation for benzalkonium bromide, chemically *N*-benzyl-*N*,*N*-dimethyl-*N*-tetradecylammonium bromide

Even though our compounds failed as antimicrobial agents, the investigation of NAD metabolism deserves considerable attention in drug development. Similar targets are also studied in Mycobacteria (24) and *Plasmodium falciparum* (25). Broad-spectrum antimicrobial potential could be expected from the agents acting through this mechanism. Therefore, the examination of other potential inhibitors could continue with a goal to optimize chemical structure with emphasis to improve transport ability inside the bacterial cell, without losing the inhibitory activity.

Conclusion

Several compounds from the set of frentizole derivatives chosen by molecular docking as potential NadD inhibitors underwent antimicrobial activity evaluation. Despite the fact that the inhibition of this essential bacterial enzyme is considered as new promising antimicrobial mechanism, any effectiveness of tested compounds against bacteria have not been proved.

Conflict of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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Adherence to Ethical Standards

This article does not contain any studies involving animals performed by any of the authors. This article does not contain any studies involving human participants performed by any of the authors.

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