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Antidiabetic potential of simple carbamate derivatives: Comparative experimental and computational study

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Abstract: With the increasing global burden of diabetes mellitus type 2, the search for the new drugs, with better pharmacological profile is continued. As a part of this surge, the synthesis, pharmacological *in vitro* and computational evaluation of five, simple carbamate derivatives, against carbohydrate digestive enzyme *α*-glucosidase, is disclosed herein. Results of the experimental and computational assessment indicated that examined carbamates deterred the activity of *α*-glucosidase with acceptable IC_{50} values ranging from 65.34 to 79.89 μM compared to a standard drug acarbose (109.71 μM). Similarly, the studied compounds displayed *in silico* binding affinity for *α*-glucosidase enzyme with significant binding energies. Preliminary toxicity profiles of studied carbamates against three cancerous cell lines indicated their poor activity, suggesting that significant structural modifications have to be made to improve their anticancer efficiency. Results of the present study indicate that the examined carbamates were able to virtually or experimentally interact with an important target of diabetes mellitus type 2. Additionally, a new pharmacophore model is proposed featuring hydrogen bond donating carbamate –NH group, hydrogen bond accepting carbamate $–OCH_3$ group and hydrophobic stabilization of aromatic moieties.

Keywords: diabetes mellitus; Hofmann rearrangement; molecular docking; *α*-glucosidase; molecular dynamics simulation.

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INTRODUCTION

Today, diabetes mellitus (DM) is one of the most important metabolic syndromes causing thousands of deaths worldwide and an efficient therapy to permanently postpone its pathogenesis and/or complications is not yet developed.[1] As a major form, DM type 2 represents for more than 95 % of all diabetic cases and is mainly associated with hyperglycemia and insulin resistance of peripheral tissues.[2] In contrast, DM type 1 is a genetic autoimmune disorder mainly represented for less than 5 % of all diabetic patients and associated with inflammation and pancreatic β-cells dysfunction.[2,3] Other subtypes of DM such as gestational diabetes, mild obesity-related diabetes, mild age-related diabetes and other forms, although occurred in low rates, can also cause physiological problems for affected individuals.[4,5]

Recent advances in synthetic methods, enabled researchers to contrive some new classes of synthetic anti-diabetic drugs which can alleviate the DM complications.[6] A majority of synthetic drugs, developed for the treatment of diabetes mainly target the inhibition of glycol-processing enzymes such as α-glucosidase (α-Glc) and α-amylase, as well as other targets such as sodium glucose co-transporter 2 (SGLT2) and dipeptidyl peptidase 4 (DPP-4) inhibitors. Despite the effectiveness of these artificial inhibitors for postponing the incidence of DM, several shortcomings including gastrointestinal pains, stomach gas, diarrhoea and even vomiting were reported for some of these synthetic drugs.[2,6–8] Therefore, searching for a new class of inhibitors with better pharmacological profile is the main goal of most of research conducted in this area. In that respect, versatile heterocyclic nitrogen containing compounds gained considerable attention as α-Glc inhibitors.[9,10]

The importance of carbamate derivatives in medicinal chemistry is widely studied, and the growing body of evidence indicates that carbamate moiety is a pivotal chemical structural attachment in a variety of approved therapeutic medications.[11] Carbamate moiety is basically generated from an unstable molecule, carbamic acid, by the alteration of amino and carboxyl groups with aryl-alkyl or alkyl-aryl moieties.[12] Studies suggested that carbamate moiety has the potential to interact with the active site of the target proteins forming donor (carbonyl oxygen) and/or acceptor (nitrogen) hydrogen bonds. Moreover, the metabolic stability and good membrane permeability are important features of this structural motif, interesting for medicinal chemistry.[11]

Presently, most of carbamate derivatives are used as potential agrochemical metabolites and intermediates in the organic synthesis of several types of chemical agents.[8,11,13] Carbamates are known to possess a wide range of biological activities including antimicrobial,[14] anti-HIV,[15–17] anticancer[18] and anti-Alzheimer properties.[19] The potential benefits of several carbamate derivatives to deter α-Glc *in vitro*, have been also reported.[8,20]

Over the past decades, computational methods such as docking and molecular dynamics simulations provided an in-depth insight into the biological mode of action of many chemical compounds.[21] In fact, these *in silico* techniques are the upper hands of molecular biologists and medicinal chemists to evaluate the effectiveness of chemical compounds before introducing them into experimental assays. Using these methods for both ligand- and structure-based drug design policies could support researchers to develop several classes of Food and Drug Administration (FDA) approved drugs.[21,22]

Therefore, this study demonstrates an improvement of the previously published procedure for carbamate synthesis,[23] and it briefly sets out to investigate the α-Glc binding mode and inhibitory activity of simple carbamate derivatives **1**-**5**, using both computational and experimental assays (Fig. 1).

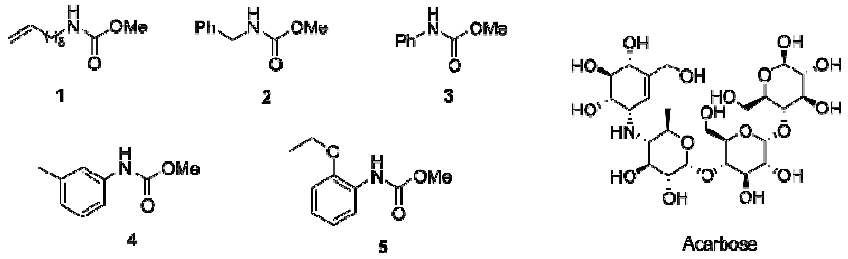


Fig. 1. Structures of the examined carbamates **1**–**5** and acarbose.

EXPERIMENTAL

General information

All reagents were purchased from a commercial vendor and used as supplied, except *N*-bromoacetamide (NBA) which was synthesized according to the literature procedure.[21] All reactions were monitored by thin layer chromatography (TLC). Dry-column flash chromatography was carried out using silica gel (10−18 or 18–32 μm, ICN-Woelm). Melting points were obtained at a heating rate of 4 °C/min, and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance III spectrometer, at 500 MHz for the proton (^{1}H) and at 126 MHz for the carbon (^{13}C) and Varian/Agilent, at 200 MHz for the proton (^{1}H) and at 50 MHz for the carbon (^{13}C). Chemical shifts are given in parts per million (ppm) from tetramethylsilane (TMS) as internal standard in deuterated chloroform ($CDCl_3$). Coupling constants (J) are reported in Hz. All spectra were recorded at 25 °C, unless stated otherwise. High resolution mass spectra (HRMS) were obtained with an ESI-ToF spectrometer. α-Glc from *Saccharomyces cerevisiae*, RPMI 1640 (product number R8755), *p*-nitrophenyl α-D-glucopyranoside (PNP-G), acarbose, fetal bovine serum (FCS), dimethyl sulfoxide (DMSO), hepes, ethidium bromide, sodium dodecyl sulfate (SDS), acridine orange, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate dihydrate and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich. Trypsin and phosphate buffered saline (PBS) were obtained from Institute of Virology, Vaccines and Sera “Torlak”, Belgrade, Serbia.

*General procedure for the synthesis of carbamates **1**–**5***

To a magnetically stirred solution of corresponding carboxamide (3.7 mmol, 1.0 equiv.) in MeOH (5 mL), $LiOH \cdot H_2O$ (5.0 equiv.) and NBA (1.5 equiv.) were added. The mixture was allowed to steer at 65 °C, in dark. Reaction was monitored by TLC, on SiO_2 plates, using the mixture of *n*-hexane/EtOAc = 1:1 and CH_2Cl_2/MeOH = 9:1, as eluent. After 5 min, the mixture was concentrated by rotary evaporator to give a residue which was mixed with 1 M solution of NaOH (20 mL). The mixture was extracted with 2×25 mL of CH_2Cl_2. The organic layers were combined and concentrated by rotary evaporator. The obtained crude product was purified by dry-column flash chromatography (SiO_2; petroleum ether/EtOAc, 8:2 to 1:1). The spectroscopic data for compounds **1**–**5** are given in Supplementary material to this paper and are in accordance with the previously published data.[23]

Enzymatic assay – Anti α*-glucosidase activity*

Anti *α*-Glc activity of compounds **1**–**5** was performed using *α*-glucosidase inhibitory activity test described by McCue *et al*. with some modification.[24] The final concentrations of the extracts in each well were 166.67, 83.33, 41.67, 20.83, 10.42 and 5.21 μM. Acarbose was used as a positive control. The percent of the enzyme inhibition was calculated as Inhibition = = 100(1-(*AS* at 15 min−*AS* at 0 min)/(*AC* at 15 min−*AC* at 0 min)), where *AS* is the absorbance readings of samples, *AC* is the absorbance readings of the control. The experiments were conducted in duplicate and IC_{50} value (estimated concentration of compounds that caused 50 % inhibition of *α*-Glc activity was determined using linear regression analysis. Measurements were done in triplicate.

MTT assay – Cytotoxic activity

The incubation of the cultures was performed according to literature procedure.[25] Target cells HeLa, cervix adenocarcinoma cell line (2000 cells per well), A549, non-small cell lung carcinoma cell line (5000 cells per well), and MDA-MB-453 human breast cancer cell line (3000 cells per well) were seeded into 96-well plate. The final concentrations range was 1––200 μM. The final concentration of DMSO solvent never exceeded 0.5 %, which was non--toxic to the cells. All concentrations were set up in triplicate. The cultures were incubated for 72 h.

Molecular docking simulation

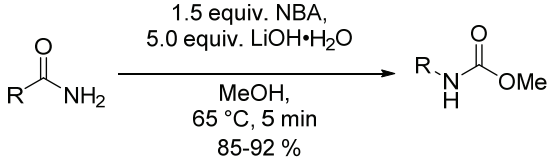
To reveal the type of interaction of synthesized compounds against target receptor, the following procedures have been applied. First, using Biovia Draw academic version, the 2D structure of input ligands were generated and then converted to "*.pdb*" format using OpenBabel and Avogadro tools. Next, polar hydrogens were added to the structure of these ligands and 3D geometries were optimized using Hyperchem software. Crystal structure of human *α*-Glc (ID: 5NN8)[26] was taken up from the Protein Data Bank (PDB). Water molecules and other types of unwanted atoms were deleted from the structure of the obtained receptor and using Modeller 9.21 software, missing residues were fixed. AutoDock tools version 2.4.6 was applied for docking analysis. The coordination of grid box parameters including x = = 19.023, y = 23.328 and z = 23.3467 was fixed up based upon the location of target active site residues. Lamarckian genetic algorithm was applied for docking searches and other parameters of docking simulation were considered based on default options.[27] To enhance the reliability of docking outcomes, the applied docking procedure was repeated three times and no violation was observed for the prepared results.

Molecular dynamics (MD) simulation

In order to determine all dimensions of possible biological interactions between synthesized carbamate derivatives and human α-Glc enzyme, MD simulation using GROMACS 5.1.2 tool was performed using GPU support. All calculations including geometry optimization, minimization, NVT/NPT equilibration were performed for all docked complexes. GROMOS96 43A1 force field was applied for protein–ligand complexes. Enough water molecules were added to the predefined simulation box and sodium and chloride ions were used for neutralization of prepared systems. By setting the Langevin dynamics at 300 K, the whole system temperature was controlled. Default parameters were applied for performing the NVT/ /NPT ensemble simulations.[28] Overall, 50ns MD simulation was performed for all of the prepared ligand–protein complexes and for the evaluation of MD results; the generated MD trajectories were analysed to produce final graphs and interpretation of generated results.

RESULTS AND DISCUSSION

The mild and scalable method for Hofmann rearrangement of carboxamides that we previously published, is suitable for the synthesis of wide variety of carbamates.[23] Nevertheless, the important method drawbacks were relatively high amounts of reagents NBA and $LiOH \cdot H_2O$ and 24–48 h reaction time, especially in the case of complex carboxamides. Even though, these reagents can be considered as easy affordable, economizing in terms of quantity is always favourable, from both financial and ecological point of view. Furthermore, reducing the reaction time is with no doubt one of the ever-present goals in synthetic chemistry. Our attempts to make this procedure greener, cost and time more effective, led to a substantial improvement in the synthesis of complex carbamates.[29] Important changes included reducing the amount of reagents, but even more reaction time, *i.e.*, from 24–48 h to 5 min, by increasing the temperature from 25 to 65 °C. We then decided to extend the improved method on the production of simple carbamates for the purposes of this research. The amounts of reagents sufficient for the reaction completion were found to be comparable with our previously reported synthesis of simple carbamates, albeit all reactions were completed within the 5 min.[23] Thus, carbamates **1**–**5** (Fig. 1) were obtained in high yields starting from readily available carboxamides (Scheme 1). The products of Hofmann rearrangement of NBA itself, were not observed, regardless of the known temperature dependent stability of NBA under basic conditions.



R = CH_2=CH_2(CH_2)$_8$, **1**; $PhCH_2$, **2**; Ph, **3**; 3-MePh, **4**; 2-EtOPh, **5**

Scheme 1. Synthetic procedure for carbamates **1**–**5**.

Previously, the antidiabetic potential of different carbamates, which expressed high *α*-Glc inhibitory activity was disclosed.[8] Encouraged by these results we decided to extend the research toward simpler carbamate derivatives that were part of our library of compounds (Fig. 1). Compounds **1**–**5** were examined for their *α*-Glc inhibitory activity, together with acarbose (Fig. 1), a commercial FDA-approved antidiabetic drug, which was used as a standard for the assays. According to the obtained results, all studied compounds expressed higher *α*-Glc inhibitory activity than acarbose (Table I). These results are consistent with our previously published data on carbamate derivatives and some imidazo[1,2-*a*]pyridines linked to carbamate group,[8,20] underlying the importance of carbamate moiety for *α*-Glc inhibitory activity.

Encouraged by the reported anti-cancer activities of some carbamate derivatives,[28] the compounds **1**–**5** were additionally examined for *in vitro* cytotoxic effect against HeLa, A549 and MDA-MB-453 human tumor cell lines, using MTT assay. The obtained results indicated that compounds **1**, **4** and **5** possess moderate anticancer activity, while compounds **2** and **3** did not show anticancer activity up to the tested concentration of 200 μM. The highest activities were observed for compounds **1** and **5** against A549 and MDA-MB-453 cell lines, respectively (Table I). This was in accordance with our previously published results suggesting that even though there is some anticancer potential in this kind of carbamate derivatives, some significant structural modifications have to be made to improve their anticancer efficiency.[8]

TABLE I. *In vitro* *α*-Glc inhibitory activity, cytotoxic effect and calculated binding affinities of compounds **1**–**5**; results are presented as a mean ± *SD*

Compd.	α-Glc inhibiton (IC_{50} / μM)[a]	Calc. bind. affinity kJ mol^{-1}	MTT assay (IC_{50} / μM)		
			HeLa	A549	MDA-MB-453
1	79.65±1.82	–33.47	132.27±1.91	111.64±29.54	136.53±5.39
2	65.34±1.64	–37.24	>200	>200	>200
3	68.55±0.09	–34.73	>200	>200	>200
4	69.26±2.71	–37.66	159.02±9.18	180.15±9.65	146.99±10.09
5	79.89±1.16	–42.68	167.49±13.23	162.68±6.41	118.96±17.54
Acarbose[a]	109.71±1.19	–41.84	–	–	–

[a]Acarbose was used as a standard drug in *α*-Glc inhibition assay

In order to obtain insight in the binding mode of the examined carbamates and *α*-Glc, molecular docking was performed. According to the docking analysis, the studied compounds established acceptable binding energies with the *α*-Glc active site, being surrounded by catalytic and binding amino acids of the active site (Table I). The crystallographic analysis of *α*-Glc active site, revealed several amino acid residues including Trp376, Asp404, Ile441, Trp516, Asp518, Met519, Arg600, Trp613, Asp616, Phe649 and His674, that play a quintessential

role in the catalytic process.[26] Accordingly, the interactions of inhibitors with these residues can cause conformational change in the α-glucosidase active site, thus preventing the biological function of the enzyme. All of the tested carbamates were stabilised by the conventional hydrogen bonds with some of the α-Glc active site residues (Fig. 2).

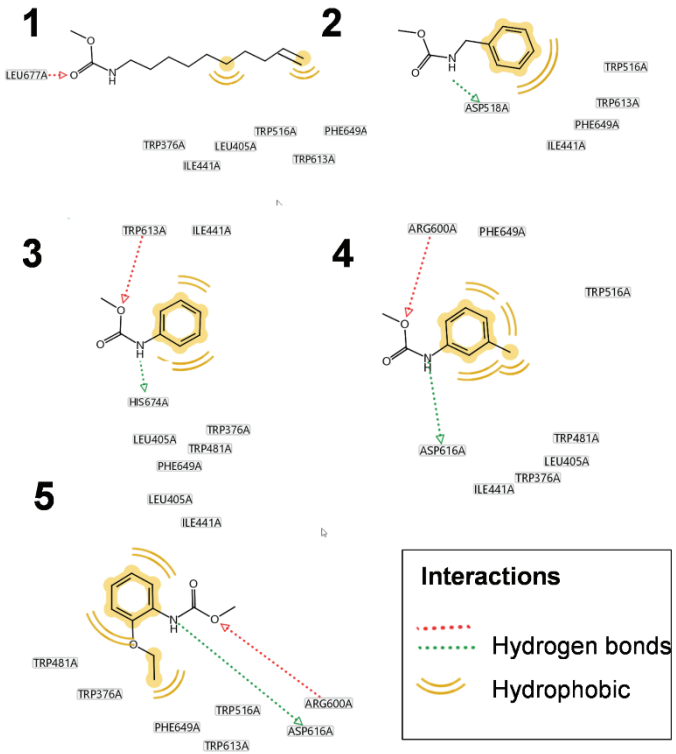


Fig. 2. Graphical presentation of interactions between α-Glc residues and carbamates **1**–**5** in their most stable conformations.

The hydrophobic interactions were also seen to contribute to binding stability. The highest calculated binding affinity was found for the compound **5** (Table I), afforded through the multiple interactions with α-Glc, including conventional hydrogen bonds with Asp616 and Arg600 residues. Compound **5** complexed with glucosidase was found to be more stable than α-Glc complexed with acarbose, a confirmed α-Glc inhibitor. The superimposition of these two molecules in Fig. 3 shows that they are close enough to create hydrogen bonds with the identical critical residues from the α-Glc active catalytic site, Asp616 and Arg600. The most stable conformation of compound **4** in complex with α-Glc was also stabilised by these residues, whereas compounds **1**–**3** maintained

significant stability in complex with *α*-Glc *via* hydrogen bonds with Leu677, Asp518, His674 and Trp613, respectively.

In comparison with **4** and **5**, the other tested carbamates lacked certain chemical features for a favourable mode of interaction in the active site of the *α*-Glc. These features were defined through the pharmacophore model presented in the Fig. 4, which shows that the carbamate –NH group acted as a hydrogen bond donor while the carbamate $–OCH_3$ served as a hydrogen bond acceptor. Furthermore, the aromatic rings additionally stabilised complexes through the hydrophobic interactions.

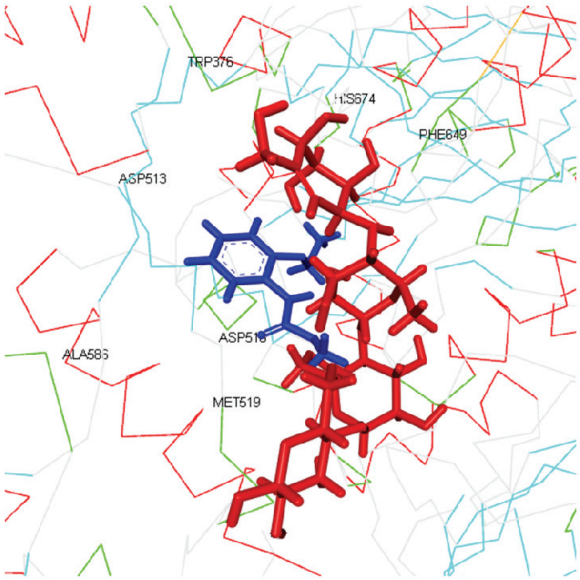


Fig. 3. Compound **5** (blue) and acarbose (red) structures superimposed at the *α*-Glc active site.

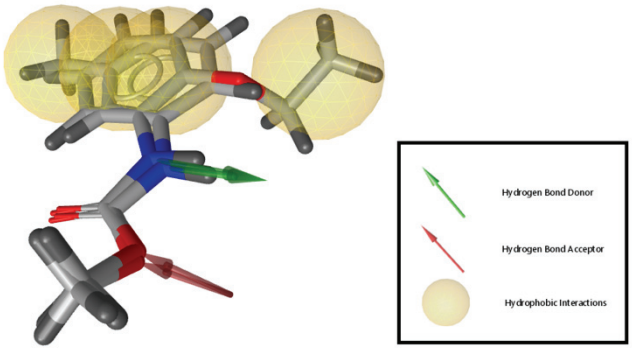


Fig. 4. Chemical features of superimposed carbamates **4** and **5** for favorable binding mode with α-Glc.

MD simulations were conducted in order to compare the structural changes of ligand–enzyme complexes. As depicted in Fig. 5, in comparison to the native, ligand free enzyme, significant structural changes were observed for ligand–protein complexes. Root mean square deviation (*RMSD*) plot of MD simulations indicated that the compounds **3**–**5** obviously decreased the *RMSD* value of protein–ligand complexes in comparison to the native protein. The highest decrease of *RMSD* value was observed for compound **3** while compounds **1** and **2** partially increased the *RMSD* value (Fig. 5A and B).

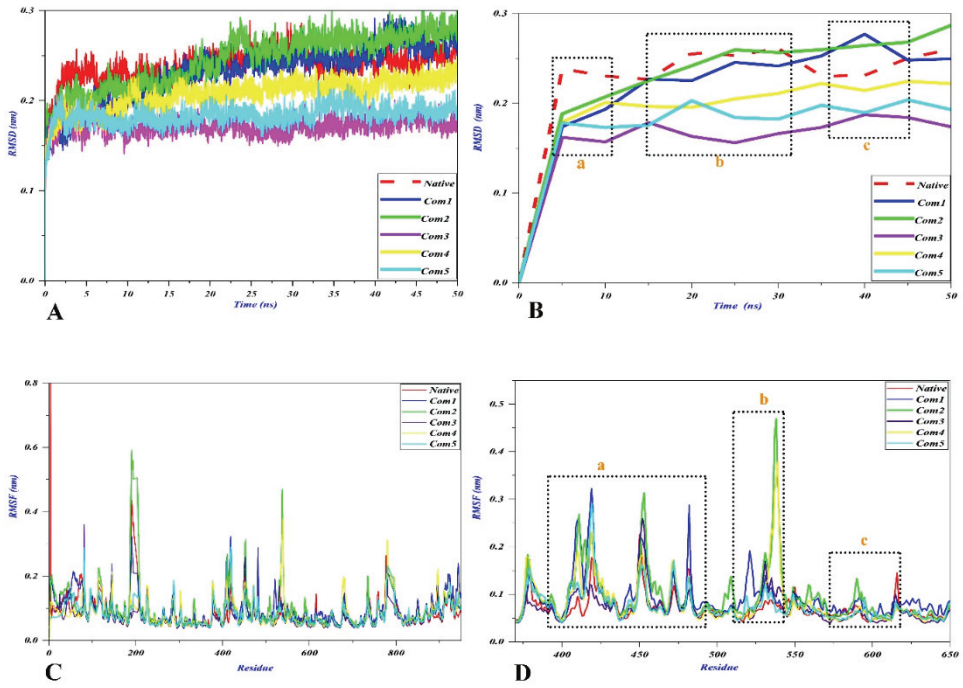


Fig. 5. *RMSD* and *RMSF* plots of studied carbamate derivatives: A) compressed view of *RMSD* curves for ligand–protein complexes vs. native protein; B) close view of *RMSD* curves of studied complexes; C) *RMSF* plot of entire protein residues; D) *RMSF* plot of active site residue.

As illustrated in Fig. 5B, the *RMSD* score has been changed in three regions namely a, b and c. In the first 10 ns of MD simulations, all docked compounds decreased *RMSD* curve with compound **3** causing the major changes for this time-period. Afterwards, between times the series of 15 and 33 ns, *RMSD* curves of each protein–ligand complex fairly changed and it seems that during this period of time the target complexes reached a primary stabilization phase.

After 40 ns (region c, Fig. 5B), the biggest changes of *RMSD* values were observed for the complexes **1** and **2** whereas in this time-period the protein and complexes **3**–**5** showed partial decrease in their *RMSD* curves. Overall, after 10 ns, all of the complexes reached a stabilised condition, though during the MD process several changes were observed for their *RMSD* values. Because *RMSD* plot quantitatively measures the average distances between backbone atoms of a protein (or a group of atoms), it can determine the possible conformational changes of either ligands or proteins, whenever they are bound to other types of macromolecules.[31] Based on the chemical essence of simulated bio-macromolecules, *RMSD* values can possess higher or lower scores. Indeed, the numerical values of *RMSD* plot also demonstrated how ligand–protein complexes may move between folded and unfolded states.[31]

Plots C and D of Fig. 5 indicated the root mean square fluctuation (*RMSF*) values changes for the protein backbone residues and the active site amino acids of *α*-Glc. As shown, all docked compounds violated the conformational pose of active site residues. Accordingly, these results have proven that the interacted carbamate derivatives were able to approach the active site residues and bind to their side chains atoms. Basically, the conformational changes in the side chain of amino acids after the interaction with inhibitor ligands can disrupt the biological activity of target enzyme. The numerical values of *RMSF* plot suggest the total flexibility (or fluctuation) of protein residues during MD simulation. The total fluctuation of protein residues towards backbone atoms or chemical moieties of nearby ligands may predict the tendency of target amino acids for further chemical interactions. *RMSF* per residue plot showed the enhanced active site residue flexibility for all tested compounds (Fig. 5D) compared to the native enzyme which is known to decrease enzyme kinetic stability.[32]

The solvent accessible surface area (*SASA*) plot is another output of MD simulation to determine how protein–ligand complexes may behave during MD process. Any change in *SASA* of proteins determines how much surface of a protein is exposed to the solvent. According to the Fig. 6A–D, carbamates **1**–**4** significantly changed the numerical values of *SASA* plot in comparison to the native protein. In the regions a1–a4, the fingerprinting of several changes can be followed. The compound **2**–*α*-Glc complex, which had the greatest *SASA* value, exemplifies these shifts the most. This suggests that the number of hydrophobic residues that can be solvent-accessible and the interaction with their molecules has increased significantly. In addition, the *SASA* values for the protein complexes containing carbamates **1**–**4** were unstable during MD. The instability was particularly noticeable in areas a1 and a2 (Fig. 6B and C), indicating that the *α*-Glc surface underwent considerable conformational changes while interacting with these carbamates. During MD simulation however, the compound **5** *SASA*

plot remained relatively stable, implying a mode of interaction with the α-Glc active site that prevented significant protein structural changes.

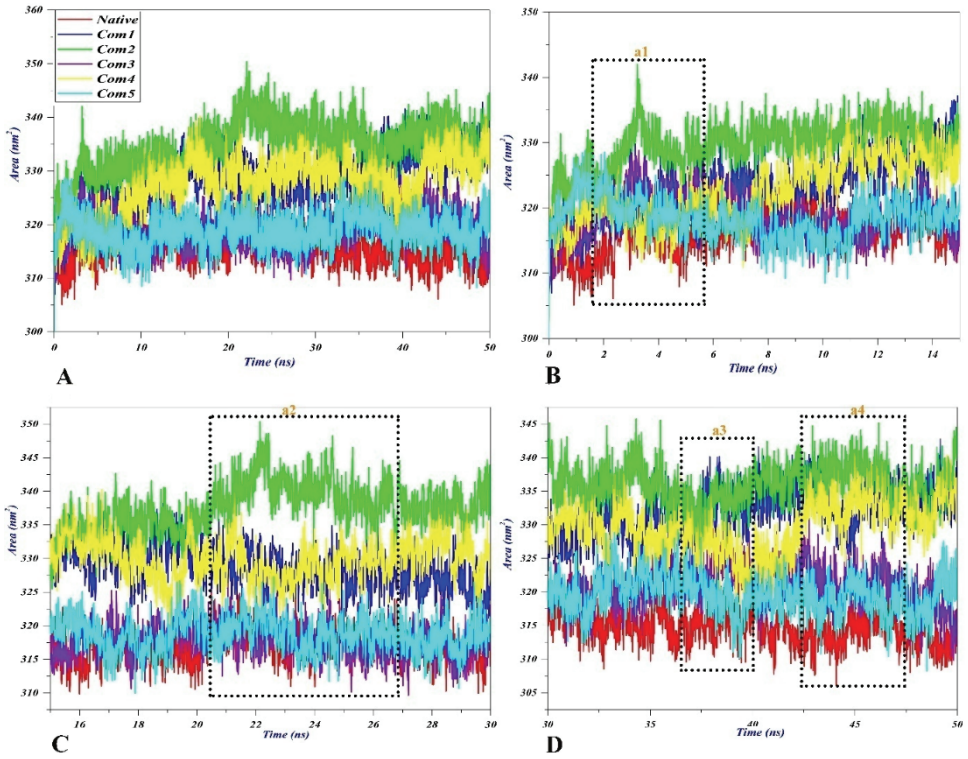


Fig. 6. *SASA* plots of studied complexes during time-period of 50 ns MD simulation: A) the total changes of *SASA* during the time from 0 to 50 ns; B) *SASA* changes between 0 and 15 ns; C) observed changes between 15 and 30 ns; D) changes between 30 and 50 ns.

CONCLUSION

The α-Glc inhibitory activity of five carbamate derivatives was investigated *in vitro* and according to the obtained results, the examined carbamates were able to inhibit the normal action of the enzyme, showing the IC_{50} values lower than the standard drug, acarbose. Computational analysis revealed that all tested compounds exhibited high binding affinities and had a considerable impact on the flexibility of the α-Glc active site residues. However, a favourable binding mode was confirmed only for compounds **4** and **5**, making their pharmacophore model, composed of two hydrophobic, one hydrogen acceptor and one hydrogen donor features, a potential model for the development of novel carbamate derivatives, glucosidase inhibitors with higher glucosidase IC_{50} values.

The preliminary toxicity profiles of studied derivatives against three cancerous cell lines indicated their poor activity, suggesting that some significant structural modifications have to be made to improve their anticancer efficiency.

The results of the present study indicate that the examined derivatives were able to virtually or experimentally interact with an important target of DM type 2. The current results corroborate well with the previously published data in the analysis of the potential of this class of compounds as potential new antidiabetic drugs. In spite of that, the additional examples of simple carbamate derivatives should be examined for efficient SAR analysis.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12085, or from the corresponding author on request.

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И З В О Д

АНТИДИЈАБЕТСКИ ПОТЕНЦИЈАЛ ЈЕДНОСТАВНИХ КАРБАМАТА: КОМПАРАТИВНА ЕКСПЕРИМЕНТАЛНА И РАЧУНАРСКА СТУДИЈА

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Са порастом појаве дијабетеса типа 2 у свету, јавља се потрага за новим лековима са што ефикаснијим фармаколошким профилом. Као део овог истраживања, приказана је синтеза, фармаколошко *in vitro* и рачунарско испитивање пет карбамата једноставне структуре, као инхибитора – глукозидазе, ензима који учествује у дигестивном разлагању шећера. Резултати експерименталног испитивања показали су да испитивани карбамати инхибирају активност – глукозидазе са задовољавајућим IC_{50} вредностима у опсегу од 65,34 до 79,89 μM, а у поређењу са стандардним леком, акарбозом (109,71 μM). Такође, *in silico* методом добијене су значајне енергије везивања за активно место – глукозидазе. У прелиминарном испитивању цитотксичности према три типа канцерозних ћелија, карбамати су показали лошу активност, сугеришући да су потребне значајне структурне промене за побољшање њиховог антиканцерозног дејства. Уопштено говорећи, резултати ове студије показали су да су испитивани карбамати успешно виртуелно и експериментално интераговали са важном метом код дијабетеса типа 2. Такође је предложен и нови фармакофорни модел за -глукозидазу, који укључује карбаматну –NH групу као донора водоничне везе, затим карбаматну $–OCH_3$ групу као акцептора водоничне везе, а такође и стабилизујуће хидрофобне интеракције ароматичних прстенова.

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