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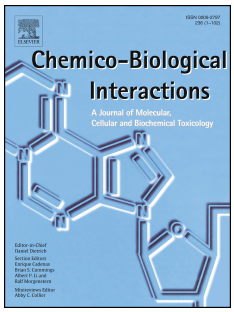

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Benzimidazole-based dual dipeptidyl peptidase-4 and xanthine oxidase inhibitors

Katarina Tomovic[a,*], Budimir S. Ilic[b], Zaklina Smelcerovic[c], Marija Miljkovic[d], Denitsa Yancheva[e], Milan Kojic[d], Anelia Ts. Mavrova[f], Gordana Kocic[g], Andrija Smelcerovic[b,*]

[a] *Department of Pharmacy, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000, Nis, Serbia*

[b] *Department of Chemistry, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000 Nis, Serbia*

[c] *Center for Biomedicinal Science, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000 Nis, Serbia*

[d] *Laboratory for Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444/a, P.O. Box 23, 11010 Belgrade, Serbia*

[e] *Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Science, Acad. G Bonchev Str., Build. 9, 1113 Sofia, Bulgaria*

[f] *University of Chemical Technology and Metallurgy, 8 Kliment Ohridski Blvd., 1756 Sofia, Bulgaria*

[g] *Institute of Biochemistry, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000, Nis, Serbia*

Corresponding authors:

* Andrija Smelcerovic

Department of Chemistry, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000 Nis, Serbia

E-mail address: a.smelcerovic@yahoo.com

* Katarina Tomovic

Department of Pharmacy, Faculty of Medicine, University of Nis, Bulevar Dr Zorana Djindjica 81, 18000, Nis, Serbia

E-mail address: katarinat91@gmail.com

A B S T R A C T

Multiple-targeting compounds might reduce complex polypharmacy of multifactorial diseases, such as diabetes, and contribute to the greater therapeutic success. Targeting reactive oxygen species-producing enzymes, as xanthine oxidase (XO), might suppress progression of diabetes-associated vascular complications. In this study a small series of benzimidazole derivatives (**1**-**9**) was evaluated for inhibitory activity against dipeptidyl peptidase-4 (DPP-4) and XO. One 1,3-disubstituted-benzimidazole-2-imine (**5**) and 1,3-thiazolo[3,2-*a*]benzimidazolone derivative (**8**) were shown as effective dual DPP-4 and XO inhibitors, with IC_{50} values lower than 200 μM, and predicted binding modes with both target enzymes. Both selected dual inhibitors (compounds **5** and **8**) did not show cytotoxicity to a greater extent on Caco-2 cells even at concentration of 250 μM. These structures represent new non-purine scaffolds bearing two therapeutic functionalities, being DPP-4 and XO inhibitors, more favorable in comparison to DPP-4 inhibitors with DPP-4 as a single target due to pleiotropic effects of XO inhibition.

Keywords:

Dipeptidyl peptidase-4

Xanthine oxidase

Benzimidazole

Dual inhibition

Molecular dynamics

Cytotoxicity

Abbreviations

ADA adenosine deaminase

DMEM Dulbecco's modified Eagle's medium

DMSO dimethyl sulfoxide

DPP-4 dipeptidyl peptidase-4

FBS fetal bovine serum

NO nitric oxide

NPT normal pressure temperature

RMSD root mean square deviation

RMSF root mean square fluctuation

ROS reactive oxygen species

SDS sodium dodecyl sulfate

SPC simple point charge

XO xanthine oxidase

1. Introduction

Many diseases such as diabetes and other metabolic disorders are multifactorial and cannot be completelly cured by the specific modulation of only one target. Optimally balanced multitargeting compounds might reduce complex polypharmacy and enhance therapeutic efficacy [1]. Dipeptidyl peptidase-4 (DPP-4) is a multifunctional serine peptidase that cleaves *N*-terminal dipeptides from substrates containing Pro or Ala, at the penultimate position. Besides incretin hormones, glucagon like peptide-1 and glucose-dependent insulinotropic polypeptide, with significant role in regulation of glucose metabolism, numerous other biologically active peptides could be substrates for this protease [2,3]. The journey of DPP-4 inhibitors, gliptins, approved for use in the therapy of type 2 diabetes mellitus, has started from sitagliptin in 2006 to the latest gosogliptin in 2016 [4]. The prevalence of diabetes, chronic metabolic disease with long-term and diverse complications, continues to rise and DPP-4 inhibitors represent a recent effective addition to the treatment, with promising favorable pleiotropic effects through incretin-dependent/independent actions [3,5,6], even with suggested possible novel therapeutic indications [7,8]. Besides the protease activity, DPP-4 (CD26) acts as an adenosine deaminase (ADA) binding protein. DPP-4-ADA complex plays an important role in the regulation of adenosine signaling and T-cell proliferation. Adenosine acts as a homeostatic regulator via multiple receptor-dependent and -independent pathways [9]. Complex of DPP-4 and ADA catalyses deamination of adenosine to inosine which is subsequently converted to hypoxanthine [10]. Costimulatory signal of the interaction of ADA with CD26 on lymphocytes also leads to increased interleukin-6, interferon-γ and tumor necrosis factor-α production, enhancing pro-inflammatory response [11].

Xanthine oxidase (XO) converts hypoxanthine to xanthine and xanthine to uric acid, the end product of purine catabolism in humans [12]. This oxidase has been accepted as a major source of free radicals in oxidative reperfusion injury in postischemic tissue, what is controlled by its substrate availability dependent on degradation of adenine nucleotides. ADA inhibition was shown as effective at suppressing the generation of hypoxanthine and xanthine [13,14]. It was shown in non-diabetic infarcted rats that treatment with DPP-4 inhibitor sitagliptin for 4 weeks increased interstitial adenosine levels, decreased XO substrates and uric acid levels and attenuated oxidative stress. Beneficial effects of sitagliptin were similar to the effects of ADA inhibitor and could be reversed by adenosine A1 receptor antagonist and exogenous hypoxanthine [10]. Endogenous adenosine plays a multifaceted role in protection of the ischemic myocardium, reduction of reactive oxygen species (ROS) release and prevention of endothelial damage [15]. DPP-4 stimulates xanthine dehydrogenase expression, promoting the production of uric acid. It was shown that DPP-4 inhibitor teneligliptin reduced xanthine dehydrogenase expression in the epididymal adipose tissue of the high fat diet-fed rats and 3T3-L1 adipocytes [16]. Since linagliptin contains xanthine scaffold structure (Fig. 1), antioxidative properties of this DPP-4 inhibitor might be ascribed, in part, to its inhibitory effects on XO activity, what was shown *in vitro* and in human serum with decreased uric acid levels [17]. Generally, it is known that framework with purine motif is responsible for some side effects (hypersensitivity reaction, gastrointestinal distress, worsening of renal function) and move from the purine-based structure might result in fewer effects, but anyhow some new types of toxicities are not excluded [18].

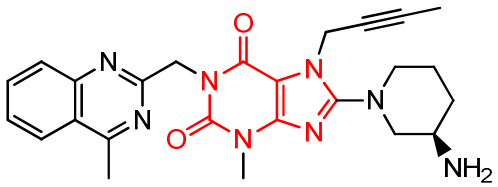


Fig. 1. Structure of linagliptin with marked xanthine core

Nongonierma and coworkers studied XO and DPP-4 inhibition by amino acids and dipeptides. Trp and Trp-Val were identified as multifunctional inhibitors of XO and DPP-4. This can be the example of food-derived peptides that may have significant impact on the reduction of ROS generation and the protection of incretin hormones from rapid degradation [19]. Hypouricemic effect of dual DPP-4 and XO inhibitors is potentiated due to decrease of XO substrate levels by inhibiting DPP-4 activity and its binding to ADA, as well as due to the inhibition of XO activity [20]. Wallace and coworkers showed that benzimidazole derivatives might have significant inhibitory potential against DPP-4 with the contribution of the bicyclic base (benzimidazole core) in interactions with the amino acid residues of the protease [21], while Nile and coworkers [22] reported that benzimidazole derivatives might be used as good source of XO inhibitors. Herein, inhibitory potential of 1,3-disubstituted-benzimidazole-2-imines and 1,3-thiazolo[3,2-*a*]benzimidazolones (synthesized as described in our previous study [23]) against DPP-4 and XO enzymes was evaluated in order to obtain/select the representatives of dual non-purine inhibitors of these two enzymes, which, rare in the literature, might be the starting point in the design of future multitarget candidates. Caco-2 cell line, well characterized, proven as *in vitro* model, among others, to assess potential toxicity of drug candidates on this biological barrier [24,25], e.g. so far done for metformin nanoparticles [26] and milk protein-derived peptides with DPP-4 inhibitory activity [27], was used to assess the potential cytotoxic effects of

the assayed compounds. So far, multiple computational methods have been utilized for the development of novel DPP-4 and XO inhibitors [28-30]. According to these studies, in order to provide insight into the key structural features required for the activity, molecular docking and dynamics simulation of selected dual DPP-4 and XO inhibitors was performed, too.

2. Materials and methods

2.1. Compounds

The synthesis of the studied 1,3-disubstituted-benzimidazole-2-imine and 1,3-thiazolo[3,2-*a*]benzimidazolone derivatives was performed as described in our previous study [23].

2.2. DPP-4 and XO inhibition assays

1,3-Disubstituted-benzimidazole-2-imines (**1-5**) and 1,3-thiazolo[3,2-*a*]benzimidazolones (**6-9**) were assayed *in vitro* for the inhibitory activity against DPP-4 and XO. Recombinant human DPP-4, Gly-Pro-*p*-nitroanilide *p*-toluenesulfonate, sitagliptin, diprotin A, bovine milk xanthine oxidase, xanthine and allopurinol were purchased from Sigma-Aldrich.

2.2.1. Evalutaion of DPP-4 inhibition

Inhibitory potential of a small series of benzimidazole derivatives (**1-9**) against DPP-4 was evaluated *in vitro* on recombinant human enzyme. Protease (0.005 units) in TRIS-HCl buffer (90 mM, pH 7.60) was treated with dimethyl sulfoxide (DMSO) solutions of compounds. The final concentration of DMSO in the assay was 5% v/v. After the incubation at room temperature for 15 min, substrate Gly-Pro-*p*-nitroanilide *p*-toluenesulfonate (260 μM) was added and reaction was carried out at 37°C for 60 min. The initial concentration of compounds for the evaluation was 200 μM. The effectivenes of the inhibition was determined on the basis of difference in absorbance at 385 nm, with corresponding blank samples for both, test and solvent control. Sitagliptin and diprotin A were used as reference inhibitors.

2.2.2. Evalutaion of XO inhibition

Inhibition of XO was evaluated *in vitro* on the commercial bovine milk enzyme by spectrophotometric measurement of uric acid formation at 293 nm, with allopurinol as standard inhibitor, as described in our previous study [31].

2.3. Cytotoxicity assay

Cytotoxicity of 1,3-disubstituted-benzimidazole-2-imine and 1,3-thiazolo[3,2-*a*]benzimidazolone that shown inhibitory activity toward DPP-4 and XO was determined in Caco-2 cells using MTT assay. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Switzerland) supplemented with 10% heat-inactivated fetal bovine

serum (FBS), 1% L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere containing 5% of CO_2.

Cells were seeded in 24-well plate (2×10^5 cells). Twenty-four hours before incubation with compounds **5** and **8** the medium was changed to a medium with 5% heat-inactivated FBS and 1% L-glutamine. Samples were added in different concentrations and incubated for 24 h at 37°C in a humidified atmosphere containing 5% of CO_2. After the incubation, samples were transferred from the well following the addition of 200 µl of MTT solution (Serva, Electrophoresis GmbH, Heidelberg, Germany) (10 × diluted MTT stock (5 mg/ml) in complete DMEM) and 4 h incubation at 37°C in a humidified atmosphere containing 5% of CO_2. Purple formazan was dissolved in 200 µl of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl and incubated overnight. The absorbance was measured at 570 nm on a microplate reader (Tecan Austria GmbH, Grödig, Austria).

2.4. Molecular docking and dynamics simulations

Molecular docking and dynamics simulations were performed using Schrödinger tools, a comprehensive platform that integrates predictive modeling and data analytics to enable research on chemical space, with validated strength of its approaches [32] (Supplementary data).

Selected benzimidazole derivatives were optimized, the X-ray crystallographic structures of DPP-4 (PDB code: 1X70) and XO (PDB code: 1FIQ) with identified possible ligand-binding pockets were prepared and subsequently selected benzimidazole derivatives were docked into the top-ranked binding pockets of these two enzymes. Additionally, molecular dynamics simulation of selected benzimidazole derivatives was carried out [32] (Supplementary data).

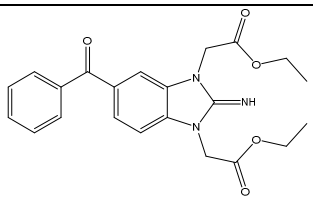
3. Results and Discussion

3.1. Evaluation of DPP-4 and XO inhibition

The previously synthesized 1,3-disubstituted-benzimidazole-2-imines (**1-5**) and 1,3-thiazolo[3,2-*a*]benzimidazolones (**6-9**) [23] were evaluated for inhibitory activity against DPP-4 and XO *in vitro* in the comparison with sitagliptin (0.05 ± 0.02 μM) and diprotin A (17.00 ± 5.15 μM) as reference inhibitors for DPP-4 and allopurinol (1.28 ± 0.17 μM) as standard inhibitor for XO. Two of synthesized compounds (**5** and **8**) inhibited DPP-4 with IC_{50} values below 200 μM and four compounds (**3**, **5, 8** and **9**) inhibited XO with IC_{50} values below 150 μM. Compounds **5** and **8** were shown as inhibitors of both enzymes (Tables 1 and 2).

Table 1

In vitro DPP-4 and XO inhibitory activity of 1,3-disubstituted-benzimidazole-2-imines

Compound	**Structure**	**IC_{50} values (DPP-4) (μM)**	**IC_{50} values (XO) (μM)**
1		> 200	> 150

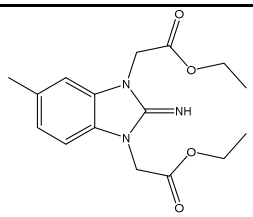
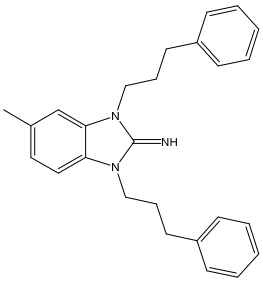
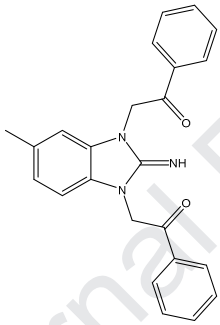
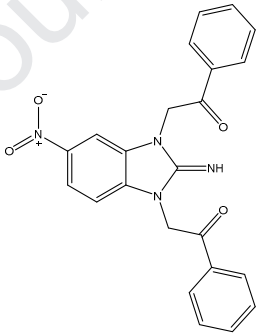
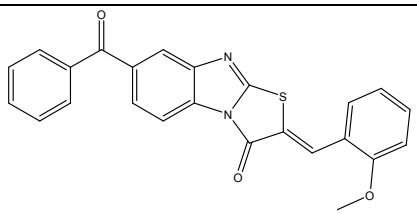
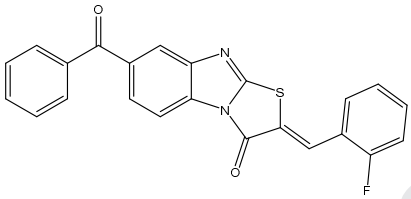
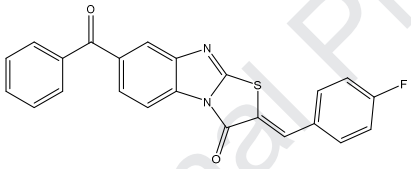
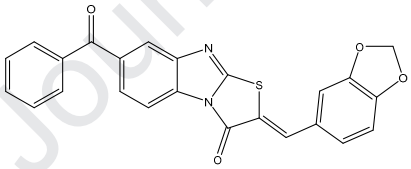
2		> 200	> 150
3		> 200	71.38 ± 6.59
4		> 200	> 150
5		151.04 ± 3.84	95.94 ± 3.16

Table 2

In vitro DPP-4 and XO inhibitory activity of 1,3-thiazolo[3,2-*a*]benzimidazolones

Compound	Structure	IC_{50} values (DPP-4) (μM)	IC_{50} values (XO) (μM)
6		> 200	> 150
7		> 200	> 150
8		177.96 ± 4.56	79.44 ± 7.97
9		> 200	108.69 ± 4.57

3.2. MTT toxicity assay

The cytotoxicity potential of the assayed compounds **5** and **8** as dual DPP-4 and XO inhibitors was assessed on Caco-2 cells. Compounds did not show cytotoxic effect expressed to a greater extent at concentrations below 250 μM after 24 h incubation (Fig. 2).

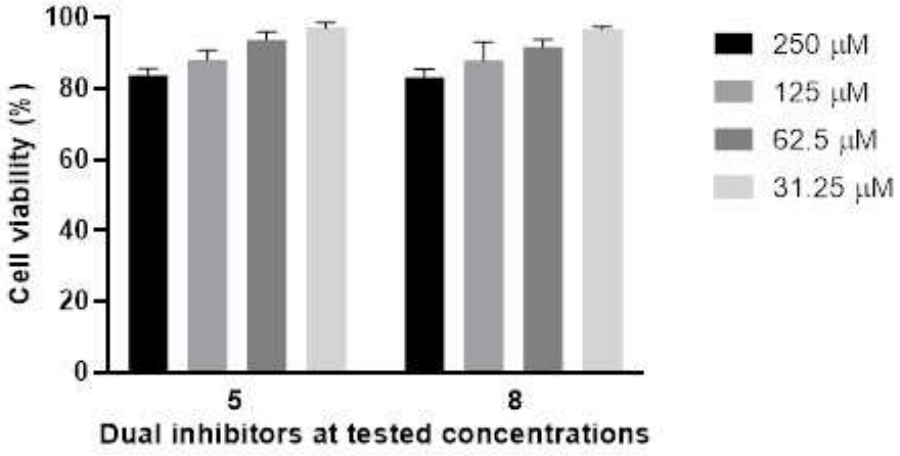


Fig. 2. Cytotoxicity of the assayed compounds **5** and **8** as dual DPP-4 and XO inhibitors at various concentrations (31.25, 62.50, 125 and 250 μM) on Caco-2 cell line. Error bars represent standard deviations.

3.3. Insights into the molecular mechanisms of DPP-4-ligand interactions

3.3.1. Molecular docking

The results from the SiteMap [33] analysis highlighted that amino acid residues Lys512, Tyr547, Lys554, Phe559, Arg560, Thr565, Trp629 and Ser630 constituted the top-ranked binding pocket of DPP-4 enzyme (Table S1, Fig. S1A, Supplementary data). Hydroxyl group of Tyr547 plays an oxyanion-stabilizing role, while residue Trp629 forms S2' pocket in DPP-4 enzyme. Ser630 is a part of catalytic triad, that nucleophilically attacks and cleaves peptide substrate [29]. A recent study has suggested that some naturally occurring compounds can exert DPP-4 inhibition through interactions with Phe559, Arg560 and Thr565 [34]. New mechanism to

enhance the bioactivities of DPP-4 inhibitors has been discovered, consisting of establishing interactions with Lys554 [35].

The intermolecular contacts between DPP-4 and compounds **5** and **8** were analyzed using the ligand interaction diagram in Maestro [32]. The interaction profile of selected DPP-4 inhibitors, illustrated the prevalent hydrogen bond interactions with Lys512, Arg560 and/or Thr565, accompanied by hydrophobic interactions with Phe559 (Fig. 3).

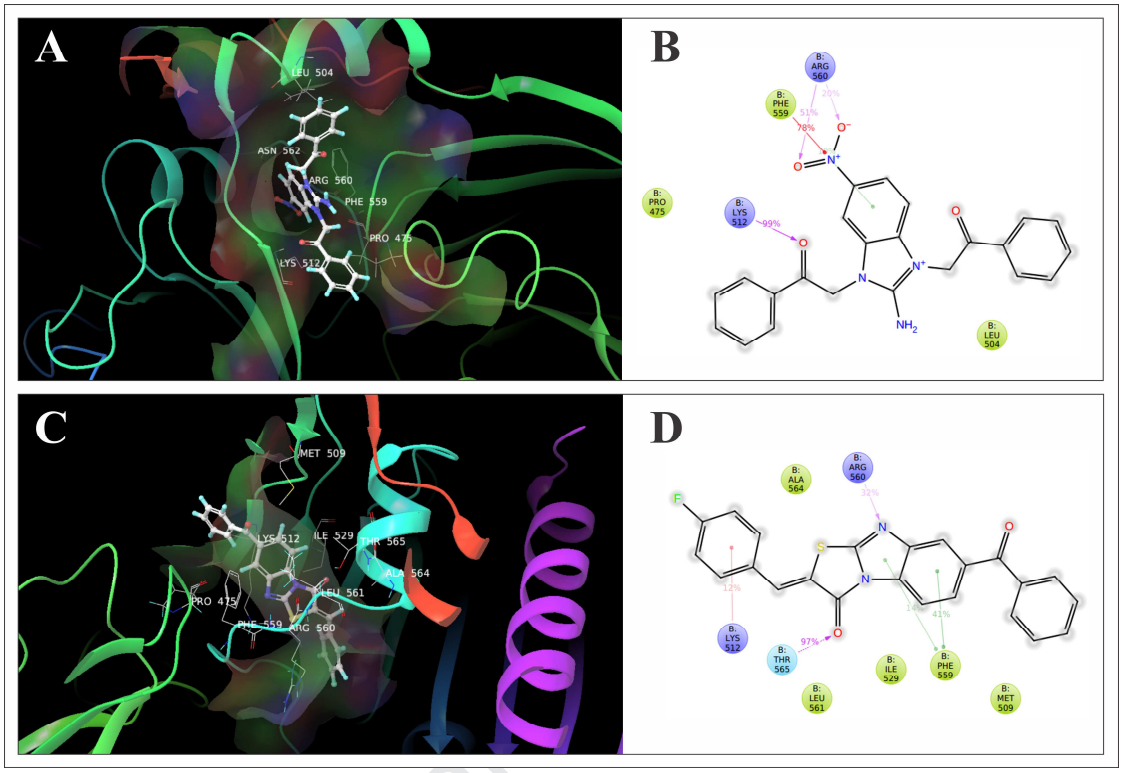


Fig. 3. 3D/2D view of compounds **5** (A and B) and **8** (C and D) bound to DPP-4. Interactions that occur more than 10% of the molecular dynamics simulation time are shown.

3.3.2. Molecular dynamics simulation

The study was further extended to assess the stability of docking DPP-4-inhibitor complexes through molecular dynamics simulations (Animation contents S1 and S2, Fig. S2 and S3, Supplementary data). The obtained results indicated small structural rearrangements, less conformational changes and confirmed stability of DPP-4-inhibitor complexes [32]. Once the conformational stability of the systems was established, the interaction stability of the systems was monitored. The interactions observed during 10 ns molecular simulation confirmed the

importance of Lys512, Phe559, Arg560 and/or Thr565 in the formation of DPP-4-inhibitor complexes (Fig. 3 and 4).

Examined DPP-4-inhibitor complexes, throughout the molecular dynamics simulation exhibited four types of interaction: hydrophobic, ionic, water-bridged and hydrogen bonds. π–π stacking interactions, π–cation and other non-specific interactions are classified into the category of hydrophobic interactions [32]. The molecular dynamics simulations of DPP-4-compound **5** complex (Fig. 3B and 4A and B), revealed prevalent hydrogen bonding interactions with Lys512 (99% of the simulation time) and Arg560 (71% of the simulation time), as well as hydrophobic interactions with Phe559 (99% of the simulation time). The molecular dynamics simulations of DPP-4-compound **8** complex revealed slightly different results (Fig. 3D and 4C and D). The molecular dynamics showed strong hydrogen bonding interactions with Thr565 (97% of the simulation time) and Arg560 (32% of the simulation time), as well as hydrophobic interactions with Phe559 (69% of the simulation time) and Lys512 (12% of the simulation time).

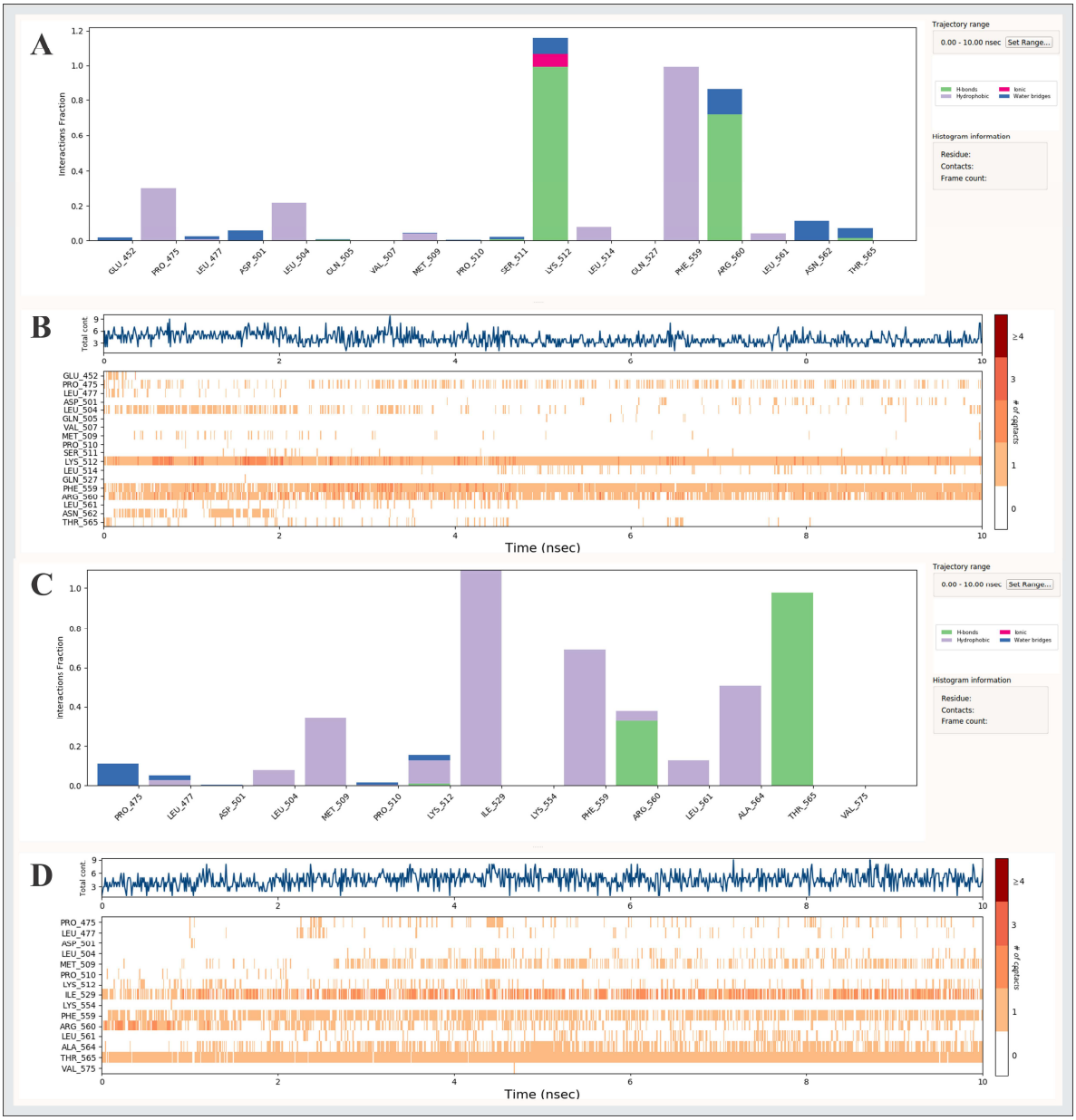


Fig. 4. Normalized stacked bar chart representation and timeline representation of interactions and contacts between DPP-4 and compounds **5** (A and B) and **8** (C and D), during the course of 10 ns molecular dynamics simulation.

3.4. Insights into the molecular mechanisms of XO-ligand interactions

3.4.1. Molecular docking

The results from the SiteMap analysis highlighted that amino acid residues Leu648, Phe649, Asn768, Leu873, Glu879, Arg880, Phe914, Phe1009, Thr1010, Phe1013, Leu1014, Gln1040, Gly1041, Ala1079, Ser1080, Gln1194 and Glu1261 constituted the top-ranked binding pocket of XO enzyme (Table S1, Fig. S1B, Supplementary data). Of note, the importance of Asn768, Arg880, Phe914, Phe1009 and Glu1261 residues in the catalytic site of XO enzyme has already been highlighted. It is worth mentioning that xanthine is hydroxylated catalytically in the Mo center surrounded by residues Leu648, Phe649, Asn768, Leu873, Arg880, Phe914, Phe1009, Thr1010, Phe1013, Leu1014, Ala1079 and Glu1261 [36-38]. Furthermore, it was shown that any conformational change on catalytic residues mentioned above, driven by inserted hydrogen bonds or Van der Waals forces, lead to inhibition of XO enzyme [39].

The predicted interaction profiles between XO and compounds **5** and **8** are displayed in Fig. 5. The interaction profile of compound **8**, illustrated the prevalent hydrophobic interactions with catalytic Phe914, Phe1009 and Phe1013, accompanied by water bridged interactions with Asn768 and Thr1010 (Fig. 5D). In addition, compound **5** showed prevalent ionic interactions with catalytic Glu1261, accompanied by hydrogen bond interactions with Ala1079 (Fig. 5B). Furthermore, it was shown that compounds **5** and **8**, also display certain number of interactions with non-catalytic residues of XO.

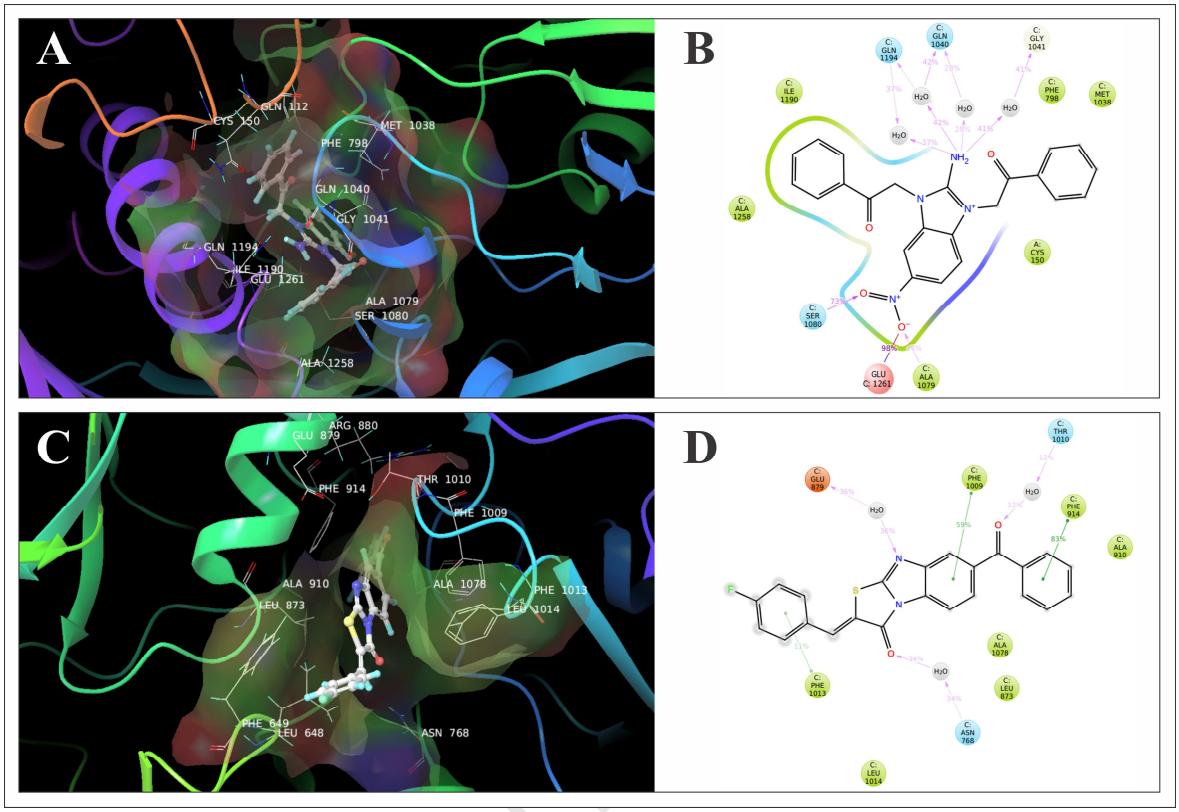


Fig. 5. 3D/2D view of compounds **5** (A and B) and **8** (C and D) bound to XO. Interactions that occur more than 10% of the molecular dynamics simulation time are shown.

3.4.2. Molecular dynamics simulation

The study was further extended to assess the stability of docking complexes between XO and compounds **5** and **8** through the molecular dynamics simulations (Animation contents S3 and S4, Fig. S4 and S5, Supplementary data). The interactions observed during 10 ns molecular simulation confirmed the importance of Leu648, Phe649, Asn768, Leu873, Arg880, Phe914, Phe1009, Thr1010, Phe1013, Leu1014, Ala1079 and Glu1261 in the formation of XO-inhibitor complexes (Fig. 5 and 6). The continuity of interactions between XO and the inhibitors are

clearly expressed by percentage of overall simulation time (Fig. 6). The molecular dynamics simulations of XO-compound **5** complex (Fig. 6A and B), confirmed prevalent ionic interactions with catalytic Glu1261 (98% of the simulation time). In addition, the simulations of XO-compound **5** complex also proved the hydrogen bonding interactions with catalytic Ala1079 (24% of the simulation time). Furthermore, it was shown a certain number of interactions with non-catalytic Gln 1040 (90 % of the simulation time), Gly1041 (48% of the simulation time), Ser1080 (84% of the simulation time) and Gln1194 (58% of the simulation time) were detected. The molecular dynamics simulations of XO-compound **8** complex revealed different results (Fig. 6C and D). The molecular dynamics showed strong hydrophobic interactions with catalytic Leu648 (12% of the simulation time), Phe649 (12% of the simulation time), Leu873 (50% of the simulation time), Phe914 (100% of the simulation time), Phe1009 (65% of the simulation time), Phe1013 (20% of the simulation time) and Leu1014 (68% of the simulation time). Furthermore, the simulations of XO-compound **8** complex confirmed water-bridged interactions with catalytic Asn768 (34% of the simulation time) and Thr1010 (36% of the simulation time).

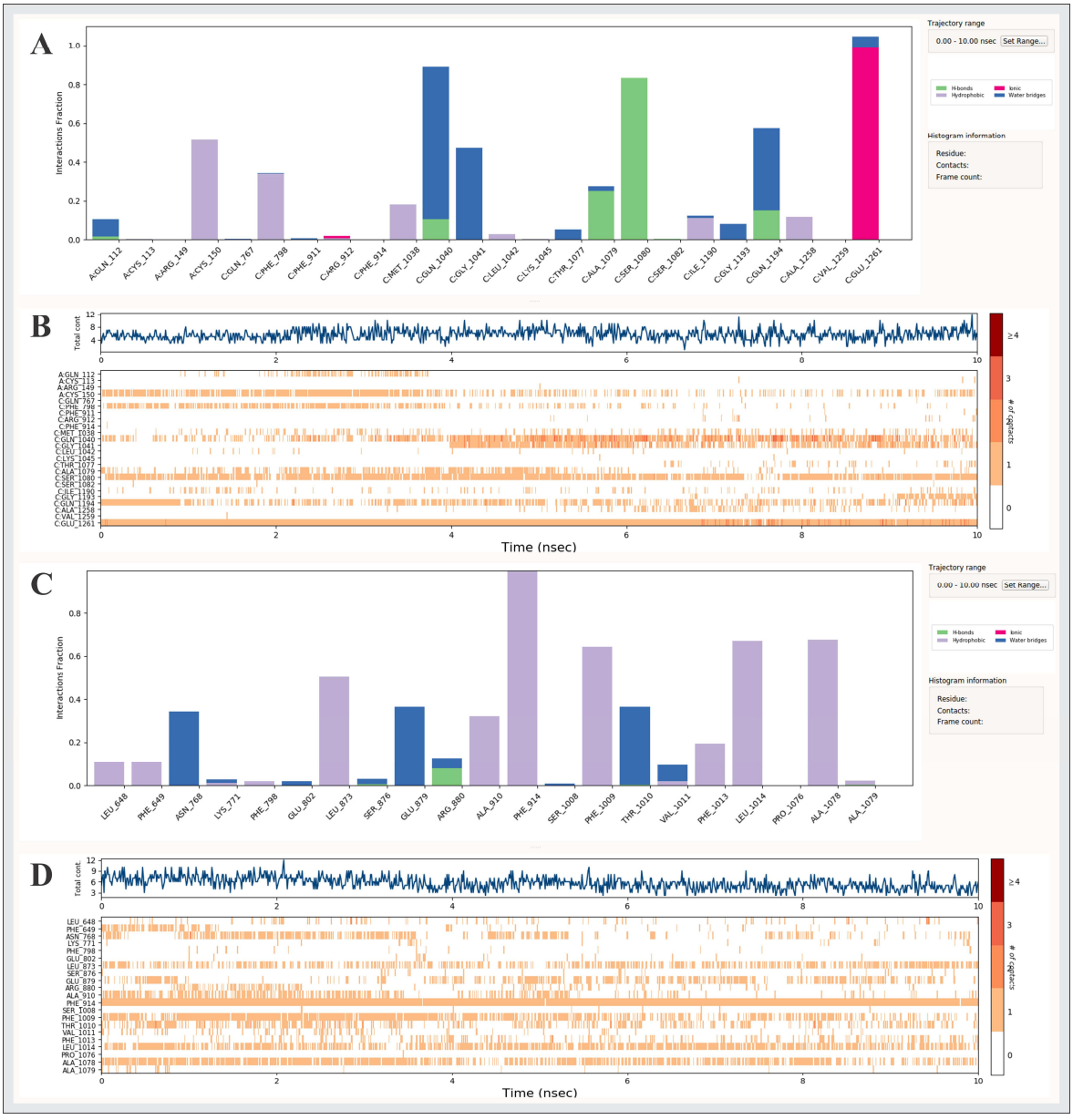


Fig. 6. Normalized stacked bar chart representation and timeline representation of interactions and contacts between XO and compounds **5** (A and B) and **8** (C and D), during the course of 10 ns molecular dynamics simulation.

3.5. Inhibition of xanthine oxidase as a mechanism contributing to vascular protection

Association of elevated serum uric acid levels with cardiovascular diseases has been epidemiologically evidenced, without established clear pathophysiological role of uric acid. Furthermore, XO-mediated oxidative stress is likely to have a significant role in the development of cardiovascular disorders. There are data that XO inhibition strategies and lower uric acid levels can benefit patients [40-42]. Serum uric acid bears significant positive correlation with hypertension, insulin resistance, hypertriglyceridemia. Besides production of ROS and uric acid, XO participates in pathogenic mechanisms of metabolic syndrome, including inflammation signalling, modulations of nitric oxide (NO) bioavailability by interfering with nicotinamide adenine dinucleotide phosphate oxidase and NO synthase activity, contributing to the regulation of endothelial function [43-45]. It was shown in hyperuricemic rats that decreased serum NO level might be reversed by inhibiting XO activity and lowering uric acid levels [46]. Oxidative stress plays a significant role in the pathogenesis of vascular complications in diabetes. Targeting ROS-producing enzymes, including XO, and treating endothelial dysfunction might suppress the progression of diabetes-associated vascular complications [47-50]. It was reported that elevated serum XO activity is associated with an increased risk of developing diabetes mellitus type 2 [51]. XO might represent a novel target for the treatment of vascular complications associated with insulin resistance and diabetes. Suggested mechanisms by which XO inhibition alleviates myocardial dysfunction and ischemia in insulin resistance could be by a reduction of the associated hyperuricemia, oxidative stress, low grade inflammation and angiotensin system [52-54]. Serum uric acid levels might be considered to be the cardiovascular risk marker, but published data overall do not support specifically targeting of this marker. Mechanistic studies suggest vascular beneficial effects from the inhibition of XO pathway rather than lowering serum

uric acid levels per se [55,56]. Therefore, antihyperglycemic agents such as DPP-4 inhibitors that additionally inhibit XO, besides potentiated antioxidative and antihyperuricemic properties, have advantageous profiles from the aspect of the therapeutic efficacy due to more pronounced cardiovascular protective effects.

4. Conclusions

Increasing disease complexity and increased/improved knowledge/understanding of its multiple pathophysiological mechanisms promote multitarget agents development [1]. XO as ROS-producing and hyperuricemia generating enzyme might provide a target for the treatment of vascular complications associated with insulin resistance and diabetes. New representatives of dual DPP-4 and XO inhibitors are 1,3-disubstituted-benzimidazole-2-imine and 1,3-thiazolo[3,2-*a*]benzimidazolone derivative (compounds **5** and **8**), with IC_{50} values below 200 μM *in vitro*, and predicted binding modes for both enzymes. Selected/assayed dual inhibitors (compounds **5** and **8**) were not cytotoxic to a greater extent for Caco-2 cells at concentrations up to 250 μM. Since there are not many data on dual DPP-4 and XO inhibitors in the literature, these results could be utilized as one of the guidelines for the rational design of novel dual inhibitors; some optimized structurally similar non-purine or different, more advantageous from the future therapeutic use point of view in comparison to DPP-4 inhibitors without inhibitory effect on XO due to additional pleiotropic effects as consequence of XO inhibition.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix: Supplementary data

Supplementary data

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Highlights

- Two tested benzimidazoles inhibited DPP-4 and XO, with IC_{50} value lower than 200 μM
- Binding modes of both dual inhibitors with two target enzymes were predicted
- Assayed dual inhibitors were not cytotoxic to a greater extent for Caco-2 cells

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: