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## **Binding of biguanides to $\beta$ -lactoglobulin: molecular-docking and molecular dynamics simulation studies**

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### **Abstract**

Biguanides are a class of drugs derived from biguanide and they are the most widely used drugs for diabetes mellitus or pre-diabetes treatment. An investigation of their interaction and a transport protein such as  $\beta$ -lactoglobulin (BLG) at atomic level could be a valuable factor in controlling their transport to biological sites. Molecular-docking and molecular dynamics simulation methods were used to study the interaction of metformin, phenformin and buformin as biguanides and BLG as transport protein. The molecular-docking results revealed that these biguanides bind to BLG and that the BLG affinity for binding the biguanides decreases in the following order: phenformin — buformin — metformin. The docking results also show the hydrophobic interactions to have a significant role in the BLG-biguanides complex stability. Analysis of molecular dynamic simulation trajectories shows that the root mean square deviation of various systems attained equilibrium and fluctuated around the mean value at various times. The time evolution of the radius of gyration and the total solvent-accessible surface of the protein showed that BLG and BLG-biguanide complexes became stable at approximately 2500 ps and that there was not any conformational change in the BLG-biguanide complexes. In addition, the profiles of atomic fluctuations show the rigidity of the ligand-binding site during the simulation.

**Keywords:** biguanides,  $\beta$ -lactoglobulin, binding affinity, molecular-docking, molecular dynamics simulation

## Introduction

Two types of diabetes mellitus are recognised: type I or insulin-dependent diabetes and type II or insulin-independent diabetes. Biguanides are a class of drugs derived from biguanide and they are the most commonly used drugs for diabetes mellitus or pre-diabetes treatment. In addition, their ability to inhibit the growth and development of cancer and the antimalarial effect of some of these molecules have been confirmed (Saito et al., 2009; Anisimov, 2003; Alexandrov et al., 1980; Anisimov et al., 1980). Metformin (Fig. 1a) is one of the best-known biguanide drugs used in type II diabetes. Studies on metformin have also shown its benefits for patients with specific types of cancer (Jiralerspong et al., 2009). Buformin and phenformin (Figs. 1b and 1c, respectively) are other anti-diabetic biguanides which also have an inhibitory effect on cancer development (Caraci et al., 2003; Anisimov, 2003; Alexandrov et al., 1980). The delivery of these drugs to their targets is of great importance and little understood. The interaction of the biguanides referred to above and a transport protein such as  $\beta$ -lactoglobulin (BLG) could be a valuable factor in controlling their transport to biological sites.

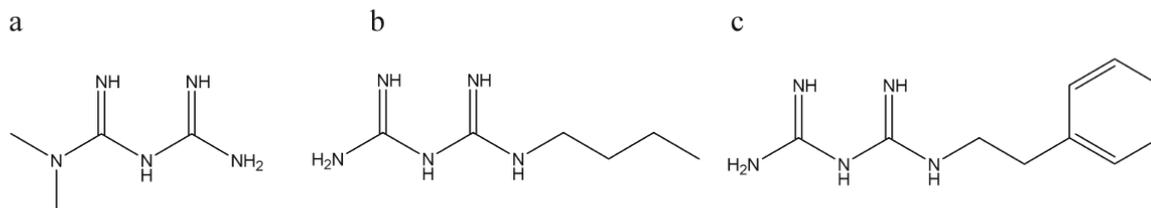


Fig. 1. Chemical structure of biguanides; metformin (a), buformin (b) and phenformin (c).

Lipocalins are small proteins that have the ability to bind and transfer small hydrophobic ligands (Flower et al., 2000; Schlehuber & Skerra, 2005). The core structure of these proteins consists of an eightstranded anti-parallel  $\beta$ -barrel that defines an internal cavity for binding the ligands. BLG is a major whey protein which has several genetic variants (Sawyer & Kontopidis, 2000; Dong et al., 1996). It consists of 162 amino acids with approximately 18.4 kDa of molecular mass (Brownlow et al., 1997). This lipocalin protein is capable of binding hydrophobic ligands such as steroids, fatty acids, retinoids, vitamin D and cholesterol (Kontopidis et al., 2002, 2004; Wu et al., 1999). Identifying novel bioactive ligand molecules of BLG is of biotechnological and pharmacological importance and contributes to further understanding of the molecular-recognition

properties of this lipocalin. However, BLG exhibits significant resistance against both gastric- and simulated duodenal digestions. Accordingly, it appears a prime candidate for the safe delivery and protection of pH-sensitive drugs in the stomach. The interaction and protein conformation of BLG with metformin, buformin and phenformin were investigated in the present study, using molecular-docking and molecular dynamics simulation studies. This may contribute not only to the better understanding of the molecular properties of this protein but might also influence future studies of the transport of drugs to biological sites.

## **Theoretical**

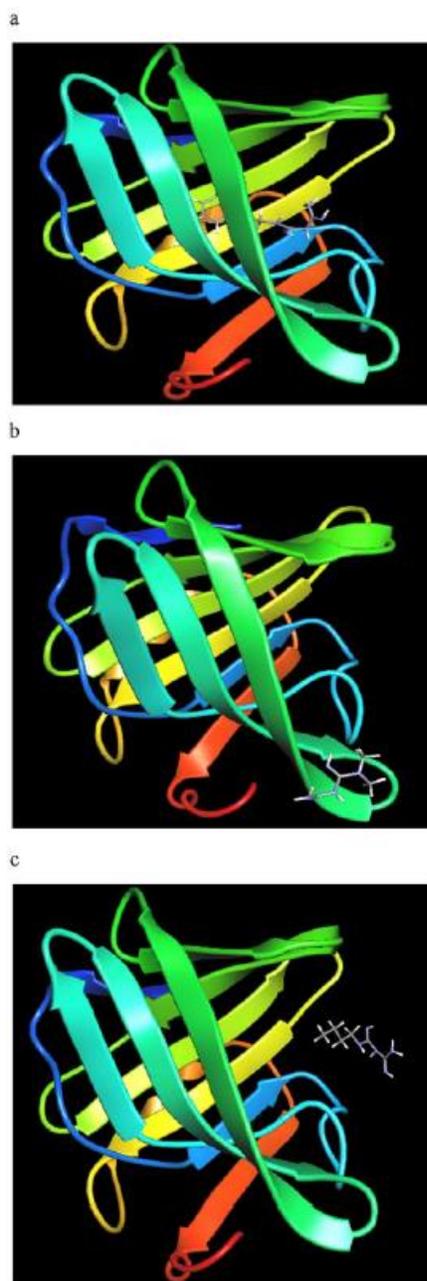
### **Molecular-docking**

All of the docked conformations of biguanide-BLG complexes were generated using the ArgusLab 4.0.1 molecular-docking program (Thompson, 2004). A comparison of the results of ArgusLab and GOLD (Genetic Optimisation for Ligand-Docking) shows a little difference in their docking accuracies (Thompson, 2004; Nikolic et al., 2008). The known crystal structure of BLG (PDB ID: 3NPO) was taken from the RSCB protein data bank. The ArgusLab suite was used to remove its water molecules. The Becke three-parameter Lee–Yang–Parr (B3LYP) hybrid density functional theory was employed to optimise the biguanides' structures, using the GAMESS (USA) quantum chemistry software (Schmidt et al., 1993). The uncharged form of biguanides was selected for the docking calculations. The blind-docking approach was used and the whole protein was taken as a potential binding site. The Ascore scoring method in ArgusLab, using high precision 0.4 °Å grid resolution and a maximum of 200 candidate poses was employed for the docking calculations. The Ascore scoring function ranked the docked conformations on the basis of their free-binding energy. The lowest free-binding energy conformation of each biguanide-BLG complex was selected for further studies.

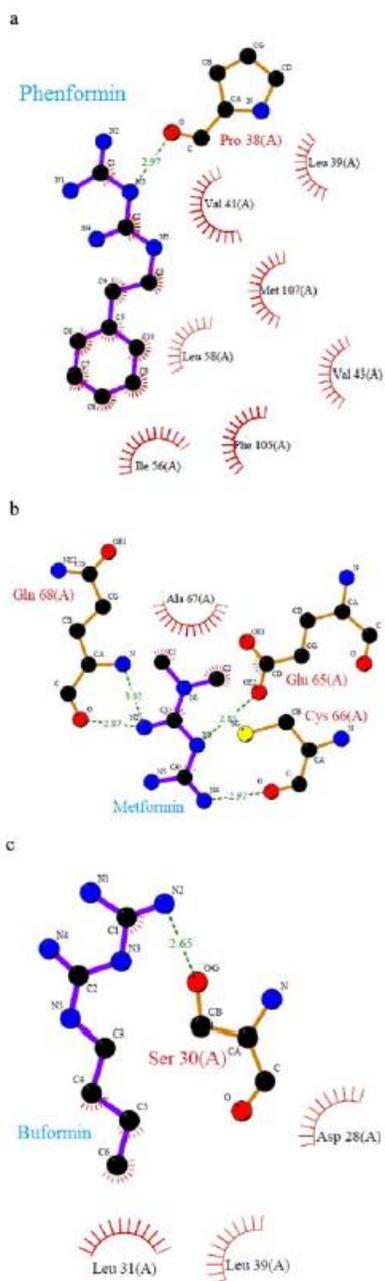
### **Molecular dynamics simulation**

The lowest free-binding energy conformation of each complex was selected as the initial conformation for the molecular dynamics (MD) studies. All MD studies were carried out using GROMACS4.5.6 (University of Groningen, Groningen, The Netherlands) package (Berendsen et al., 1995; Lindah et al., 2001) and GROMOS96 43a1 force field (van Gunsteren, 1996; Schleyer et al., 1998). The Dundee PRODRG2.5 server was used to generate the topology parameters of

biguanides (Schüttelkopf & van Aalten, 2004). The partial atomic charges of biguanides were calculated using GAMESS quantum chemistry software (Schmidt et al., 1993) at the level of HF/6-31G\*\*. The complex was located in a cubic box with the periodic boundary conditions. The box volume was 294.14 nm<sup>3</sup> and filled with extended simple-point charge (SPC) water molecules (Pullman et al., 1981) and the solvated systems were neutralised by adding 8 sodium ions. Energy minimisation was performed using the steepest descent method for the 4000 time-step. Next, the system was equilibrated for 40 ps at a temperature of 300 K. Finally, a 10 ns MD simulation was carried out at 0.1 MPa and 300 K. A Berendsen thermostat (Berendsen et al., 1984) at 300 K, the particle mesh Ewald (PME) method (Darden et al., 1993; Essmann et al., 1995) for long-range electrostatics, a 7 Å cutoff for the van der Waals interactions and Coulomb interactions were used. The equation of motion was integrated by the leap-frog algorithm with the 2 fs time-step. The atomic coordinates were recorded to the trajectory file every 0.5 ps for subsequent analysis.



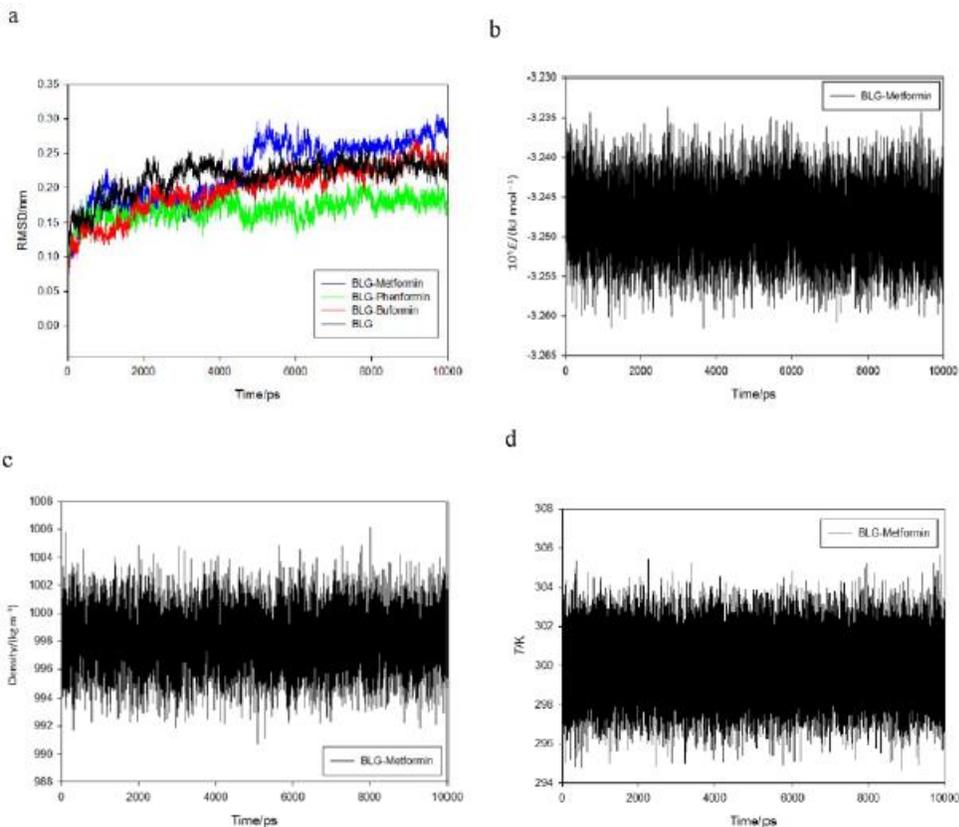
**Fig. 2.** Phenformin (a), metformin (b) and buformin (c) docked in BLG using ArgusLab 4.0.1. Ligands, depicted in a cylinder model (light grey), and BLG, represented in cartoon ribbon.



**Fig. 3.** Docking poses of BLG-phenformin (a), BLG-metformin (b) and BLG-buformin (c) complexes. H-bonds (highlighted by the green line) formed between ligands and BLG.

**Table 1.** Residues within 3 Å distance of each biguanide

| Biguanide  | Residues   |
|------------|--|
| metformin  | Ala(67), Asn(63), Cys(66), Gln(68), Glu(62), Glu(65), Lys(69)  |
| phenformin | Arg(40), Asn(90), Gln(120), Ile(56), Ile(71), Ile(84), Leu(39), Leu(46), Leu(54), Leu(58), Leu(122), Lys(60), Met(107), Phe(105), Pro(38), Val(41), Val(43), Val(92) |
| buformin   | Asn(109), Asp(28), Glu(114), Leu(31), Leu(39), Pro(38), Ser(30), Ser(116)  |



**Fig. 4.** Time-dependence of RMSD. RMSD values for BLG and BLG-biguanide complexes during 10 ns MD simulation using GROMACS 4.5.6 package and GROMOS96 43a1 force field at 300 K and 0.1 MPa (a); variation in total energy for BLG-metformin complex over 10 ns MD simulation (b); time-dependence of density over 10 ns MD simulation for BLG-metformin complex (c); time-dependence of temperature over 10 ns MD simulation for BLG-metformin complex (d). (Data for free BLG and other BLG-ligands are not given in Figs. 4b-4d).

## Results and discussion

### Molecular-docking studies

Three potential binding sites have been reported for ligand-binding to BLG: (i) the internal cavity of the  $\beta$ -barrel; (ii) the surface hydrophobic pocket in a groove between the  $\alpha$ -helix and the  $\beta$ -barrel and; (iii) the outer surface near Trp19-Arg124 (Roufik et al., 2006). In this study, the binding mode of biguanides: metformin, buformin and phenformin, at the active site of BLG was examined. The docking results show that phenformin binds in the internal cavity of BLG (Fig. 2a) but metformin and buformin bind to the surface of BLG (Figs. 2b and 2c). Fig. 3a shows that there is one hydrogen bond interaction between phenformin and Pro (38) with 2.97  $\text{\AA}$  distance. In addition, there are 4 hydrogen bond interactions between metformin and Cys(66), Glu(65) and Gln(68) with 2.97  $\text{\AA}$ , 2.85  $\text{\AA}$ , 3.02  $\text{\AA}$  and 2.87  $\text{\AA}$ , respectively. Fig. 3b shows that Gln(68) interacts with metformin by 2 hydrogen-bond interactions. One hydrogen bond interaction

between buformin and Ser(30) and with 2.65 °A distance is shown in Fig. 3c. The near residues for each biguanide are listed in Table 1. It may be concluded that, in addition to the hydrophobic interactions which stabilise the proteindrug complexes, the hydrogen-bond interactions also play an important role in the stability of BLGbiguanide complexes. The binding constant ( $K_a$ ) and free-binding energy ( $\Delta G^\circ$ ) of biguanides are listed in Table 2. The results showed that the interaction of BLG with phenformin was more favourable than those with metformin and buformin. This is due to the hydrophobic nature of the central cavity of BLG. Hence, it may be concluded that the hydrophobic interactions have a significant role in the binding of these drugs to BLG. The interaction of novel anti-diabetic pyrimidine-fused heterocycles and BLG was recently investigated by Mehraban et al. (2013). Their fluorescence spectroscopic results show that these drugs bind in the hydrophobic pocket (calyx) of the protein with a binding constant of from approximately  $11 \times 10^3 \text{ M}^{-1}$  to  $300 \times 10^3 \text{ M}^{-1}$ . Hence, the affinity of BLG for binding the biguanides are greater than that of the pyrimidine-fused heterocycles.

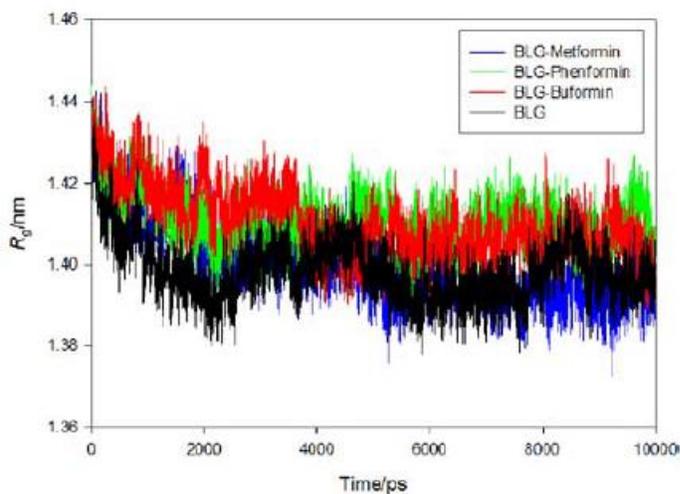
**Table 2.** Docking summary of BLG with biguanides

| Biguanide  | $10^5 \cdot K_a/\text{M}^{-1}$ | $\Delta G^\circ/(\text{kJ mol}^{-1})$ |
|------------|--------------------------------|---------------------------------------|
| metformin  | 1.4                            | -29.33                                |
| phenformin | 250.0                          | -42.18                                |
| buformin   | 3.0                            | -31.21                                |

## MD simulation studies

The root mean square deviation (RMSD) of the backbone of BLG, the total energy of the system, the system temperature and density of the system were obtained to examine the stability of trajectories for free BLG and BLG-biguanide complexes (Fig. 4). Analysis of Fig. 4a reveals that the RMSD of various systems achieved equilibrium and fluctuated at around the mean value at various times. The equilibration time is approximately 3500 ps for free BLG, BLGphenformin and BLG-buformin, whereas the BLGmetformin complex attained equilibrium after 5000 ps. The radius of gyration ( $R_g$ ) for BLG and BLGbiguanide complexes was also determined and plotted against the simulation time to examine the protein compactness (Fig. 5). The results show that the  $R_g$  of all systems stabilises after approximately 2.5 ns, indicating that the MD simulation equilibrated after 2.5 ns. The initial value of  $R_g$  for all systems is approximately 1.44 nm, which accorded with earlier experimental results (Renard, 1994). Fig. 5 also shows that the  $R_g$  value of

BLG does not depend upon complexation with biguanides. This indicates that the environment of BLG did not change during its interaction with biguanides. Fig. 6 shows the changes in the total solvent accessible surface (SAS) of the protein during the 10 ns simulation-time. The variation in SAS reveals that the trends for BLG and BLG-biguanide complexes are similar to the variation of their  $R_g$ . This similarity validates the accuracy of the MD simulation results. In addition, the amount of secondary structure components of BLG and BLG-biguanide complexes revealed that the conformation of BLG did not change during biguanides complexation (figure is not shown). The time-averaged root mean square fluctuations (RMSF) of protein residues was obtained to analyse the local mobility of the protein. The RMSF values versus the residue numbers are shown in Fig. 7. These values are based on the 6.5 ns, 6.5 ns, 6.5 ns and 5.0 ns of trajectory for BLG, BLG-phenformin, BLG-buformin and BLG-metformin complexes, respectively. The fluctuations of residues in the binding sites are lower than the others. This clearly indicates that the binding site remains approximately rigid during the MD simulation.



**Fig. 5.** Time-evolution of radius of gyration ( $R_g$ ) during 10 ns of MD simulation of BLG and BLG-biguanides complexes using GROMACS 4.5.6 package and GROMOS96 43a1 force field at 300 K and 0.1 MPa.

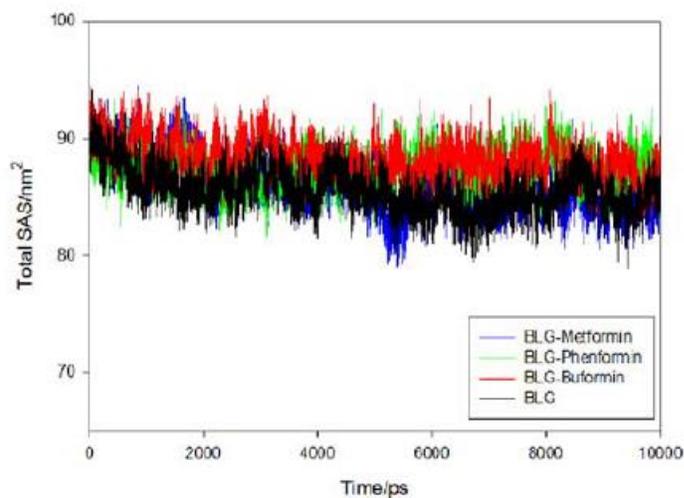


Fig. 6. Time-evolution of total solvent-accessible surface (SAS) during 10 ns of MD simulation of BLG and BLG-biguanides complexes using GROMACS 4.5.6 package and GROMOS96 43a1 force field at 300 K and 0.1 MPa.

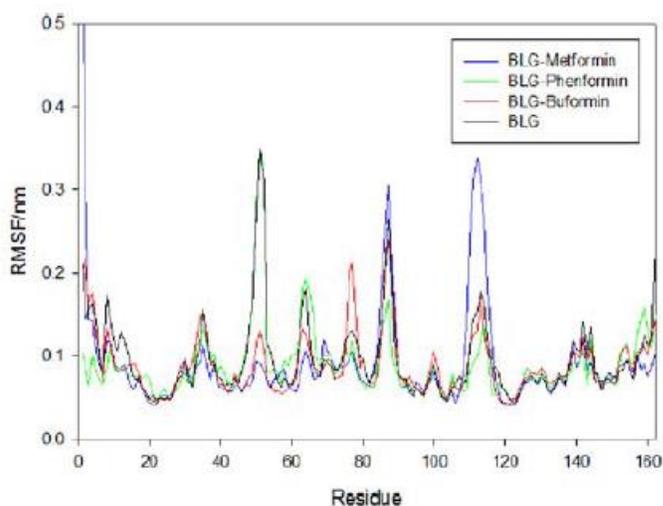


Fig. 7. RMSF values of BLG and BLG-biguanides complexes plotted against residue numbers.

## Conclusions

On the basis of the molecular-docking results, metformin and buformin bind to the surface of BLG and phenformin binds in the internal cavity of BLG. Of these biguanides, phenformin has the most negative free-binding energy. In addition, the docking results reveal that the hydrophobic interactions play a major role in the BLG-biguanide complex stability. MD simulation studies

showed the stabilisation of BLG and BLG-ligand complexes at around 2.5 ns. The BLG did not exhibit any conformational changes during the biguanides-binding. Also, the profiles of atomic fluctuations show the rigidity of the ligand-binding site during the simulation.

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