EFFECT OF THE HYPOGLYCEMIC AGENT GLICLAZIDE ON THE GASTRIC DIGESTIVE FUNCTION: BINDING MECHANISM BETWEEN GLICLAZIDE AND PEPSIN**

L.-H. Ma, B.-Sh. Liu*, G. Bian, Ch.-D. Wang, H.-C. Zhang, X. Cheng

Hebei University, National Chemistry Experimental Teaching Demonstration Center Hebei University, College of Chemistry & Environmental Science, Key Laboratory of Analytical Science and Technology of Hebei Province, Baoding 071002, Hebei Province, P.R. China; e-mail: lbs@hbu.edu.cn

Herein, the interaction between gliclazide (GCZ) and pepsin (PEP) was explored under simulated physiological conditions by multiple spectroscopy methods and molecular docking. The results showed that a new complex of 1:1 was formed between GCZ and PEP, thereby quenching the endogenous fluorescence of PEP. The addition of GCZ changed the conformation of PEP and increased the α-helix content in PEP from 20.16% to 21.13%. Using the binding constant K_a of the reaction between GCZ and PEP and the number of binding sites n, the binding rate formulas of GCZ and PEP were deduced. It was estimated that when the patient takes 40 mg of GCZ, the PEP in the gastric juice will be reduced by 96.69%. That meant taking GCZ will seriously affect the patient's digestive function. The results of molecular docking indicated that the GCZ binding site was located in the active center of PEP. The interaction of the two was driven by electrostatic attraction and hydrogen bonding forces.

Keywords: *gliclazide, pepsin, spectroscopy, digestive function, binding mechanism.*

ВЛИЯНИЕ ГИПОГЛИКЕМИЧЕСКОГО АГЕНТА ГЛИКЛАЗИДА НА ПИЩЕВАРИТЕЛЬНУЮ ФУНКЦИЮ ЖЕЛУДКА: МЕХАНИЗМ СВЯЗЫВАНИЯ ГЛИКЛАЗИДА С ПЕПСИНОМ

L.-H. Ma, B.-Sh. Liu*, G. Bian, Ch.-D. Wang, H.-C. Zhang, X. Cheng

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Национальный химико-экспериментальный учебно-демонстрационный центр университета Хэбэя, Баодин 071002, Хэбэй, Китай; e-mail: lbs@hbu.edu.cn

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В смоделированных физиологических условиях различными методами спектроскопии и молекулярного докинга исследовано взаимодействие между гликлазидом (GCZ) и пепсином (PEP). Показано, что новый комплекс 1:1 образовался между GCZ и PEP, подавляя эндогенную флуоресценцию PEP. Добавление GCZ изменило конформацию PEP и увеличило содержание α-спирали в PEP с 20.16 до 21.13%. С использованием константы связывания (K_a) реакции между GCZ и PEP и количества мест связывания (n) получены формулы для скорости связывания GCZ и PEP. Подсчитано, что при приеме 40 мг GCZ PEP в желудочном соке снижается на 96.69%. Это означает, что прием GCZ серьезно влияет на пищеварительную функцию. Результаты молекулярного докинга показывают, что место связывания GCZ расположено в активном центре PEP. Их взаимодействие вызвано электростатическим притяжением и силами водородной связи.

Ключевые слова*: гликлазид, пепсин, спектроскопия, пищеварительная функция, механизм связывания.*

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Introduction. Proteins have a variety of biological functions in the human body. Research on the interactions between drugs and proteins not only helps in understanding the mechanism of action of drugs at the molecular level, but also has an important role in the *in vitro* screening of anticancer and antiviral drugs [1]. In general, protein molecules exhibit certain spectroscopic properties because they contain tyrosine, tryptophan, and phenylalanine in their composition and contain α-helices, β-sheets, etc. in their steric structures. The establishment of an *in vitro* drug-protein model by spectroscopic methods has become an effective way for studying the interaction of drugs and proteins [2].

Gliclazide (GCZ) is a second-generation oral sulfonylurea hypoglycemic agent, mainly used for the treatment of mild and moderate non-insulin-dependent diabetes. GCZ improves diabetic retinopathy, metabolism, and blood vessel function disorder and can also be combined with insulin to treat insulin-dependent diabetes and reduce the amount of insulin [3, 4]. However, many hypoglycemic and anti-hypertensive drugs often cause adverse reactions. GCZ can cause mild nausea, vomiting, stomach pain, diarrhea and other symptoms, resulting in the dysfunction of the gastrointestinal tract. The mechanism of these side effects has not been reported at present. The investigation quantitatively studied the mechanism of GCZ affecting the digestive ability.

Pepsin (PEP) is an aspartate endopeptidase; two aspartate residues form its active center [5]. The increases and decreases of its content have an important influence on the normal function of the stomach and human health. Drugs that enter humans orally are unavoidably in contact with PEP. The interaction between pepsin and various drugs has been reported in the relevant literatures [6, 7], but the attempts have not yet been made to investigate the binding mechanism of GCZ with PEP. In this study, the binding characteristics of GCZ and PEP were investigated using a variety of spectroscopic methods and the molecular docking technique, which would provide a more intuitive and detailed reference for revealing the mechanism of the drug action and GCZ clinical drug control.

Experiment. Pepsin (PEP, purity >99%), was purchased from Sigma Company (Shanghai, China). A PEP stock solution (5.0×10^{-5} M) was prepared. The gliclazide (GCZ, purity ≥98.5%) stock solution (2.5×10^{-4} M) was also prepared. All the stock solutions were further diluted as working solutions prior to use. We employed a citric acid/sodium citrate buffer solution (pH 2.00 ± 0.02, containing 0.10 mol/dm^3 NaCl). Doubly distilled water was used throughout the study; all aqueous solutions were stored at 277 K in a refrigerator.

A Shimadzu RF-5301PC spectrofluorophotometer (Kyoto, Japan) equipped with a thermostatic cell holder was used to record fluorescence spectra. The UV-Vis absorption spectra were obtained on a UV-Vis recording spectrophotometer (UV-3600, Shimadzu, Japan). Circular dichroism spectra were recorded on a MOS-450/SFM300 circular dichroism spectrometer (Bio-Logic, Claix, France).

Fluorescence spectra and synchronous fluorescence spectra. In each test, the buffer solution (1.0 mL), 2.0 mL 1.0×10^{-5} M of PEP, and different volumes of the GCZ solution were mixed in a 10 ml colorimetric tube and then incubated for 30 min at different temperatures. A 1.0 cm quartz cell was always used in the experiment. The excitation wavelength of fluorescence spectra was at 280 and 295 nm, respectively. The slit widths of excitation and emission were set at 5 nm. The synchronous fluorescence spectra of PEP were measured with $\Delta\lambda$ = 15 and 60 nm.

Molecular docking. The crystal structure of PEP (PDB code 5PEP) was taken from the RCSB Protein Data Bank (http://www.rcsb.org/pdb), and pretreatment to PEP was carried out using Discovery Studio 2.5 software. The structure of GCZ was drawn in ChemBioDraw Ultra 12.0 and then optimized for its three-dimensional structure in ChemBio 3D Ultra 12.0. GCZ was docked with PEP using AutoDock 4.2.6 [8]. Using the Lamarkian Genetic Algorithm, the optimal binding position of PEP and GCZ molecules was selected.

Results and discussion. The quenching mechanism of the GCZ-PEP system: As shown in Fig. 1, the addition of GCZ caused the fluorescence peaks of PEP at 348 nm to annihilate sequentially (λ_{ex} = 280 nm, similar to 295 nm), indicating that GCZ can interact with PEP [9, 10]. In a typical theory, there are two ways in which biologically active small molecules, such as drugs, quench the endogenous fluorescence of a protein. One is the static quenching due to the formation of a new compound that generates non-luminescence, the lower temperature favors the reaction; the other is a result of the quenching occurring due to the diffusion and collision between the drug and the protein molecules, and the extent of quenching increases with increase of temperature [11]. Besides these two mechanisms, there is the existence of a combined quenching process. The mechanism of action can be distinguished by the correlation of its Stern-Volmer quenching constant K_{SV} and quenching rate constant K_q with temperature [12]:

$$F_0/F = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q], \quad (1)$$

where F_0 and F are the fluorescence intensity in the absence and presence of the drug, respectively; [Q] is the concentration of the quencher, and τ_0 is the fluorescence lifetime in the absence of quencher (about 10^{-8} s). The calculated values are summarized in Table 1. The results displayed that K_{SV} and K_q decreased significantly as the temperature lose, and K_q at different temperatures were all larger than the maximum scatter collision quenching constant (2.0×10^{10} $M^{-1}\cdot s^{-1}$) [13]. Thus, the quenching mechanism between PEP and GCZ was static quenching [14].

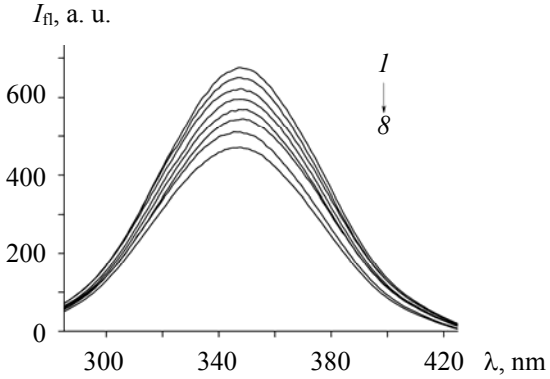

Fig. 1. Fluorescence spectrum of the GCZ-PEP system (T = 298 K, λ_{ex}= 280 nm) $C_{PEP} = 2.0\times10^{-6}$ M; 1–8: C_{GCZ} = (0, 0.2, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5)×10^{-5} M.

TABLE 1. Quenching Reactive Parameters of the GCZ-PEP System

λ_{ex}, nm	T, K	K_q, $M^{-1}\cdot s^{-1}$	K_{SV}, M^{-1}	r_1	K_a, M^{-1}	n	r_2
280	298	6.07×10^{11}	6.07×10^{3}	0.9903	1.33×10^{4}	1.01	0.9953
	310	4.69×10^{11}	4.69×10^{3}	0.9973	1.18×10^{4}	0.97	0.9964
	318	4.08×10^{11}	4.08×10^{3}	0.9959	9.31×10^{3}	0.81	0.9973
295	298	5.62×10^{11}	5.62×10^{3}	0.9946	1.21×10^{4}	0.92	0.9964
	310	4.31×10^{11}	4.31×10^{3}	0.9927	1.06×10^{4}	1.12	0.9975
	318	3.79×10^{11}	3.79×10^{3}	0.9984	7.82×10^{3}	0.92	0.9923

K_q is the quenching rate constant; K_{SV} is the Stern-Volmer quenching constant; K_a is the binding constant; n is the number of binding sites. r_1 is the linear relative coefficient of F_0/F~[L]; r_2 is the linear relative coefficient of $\lg[(F_0–F)/F]$~$\lg\{[D_t]$-$n[B_t](F_0–F)/F_0\}$.

Additionally, it is well known that the UV-Vis absorbance of proteins at 280 nm is due to the presence of aromatic amino acids phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp); thus the UV-Vis absorbance is usually used to confirm the quenching mechanism between the quencher and the fluorophore [15]. The solution was prepared according to the procedure of fluorescence experiments, and the reference was the corresponding concentration of GCZ solutions and buffer solutions, $C_{PEP} = 2.0\times10^{-6}$ M, C_{GCZ} = (0, 0.2, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5)×10^{-5} M. The UV-Vis absorption spectra of the GCZ-PEP system showed a strong absorption peak at around 210 nm and a weak absorption peak at around 280 nm. The addition of GCZ reduced the absorption peak of PEP at around 210 nm with a blue shift, and the characteristic peak at 280 nm of PEP reduced with a slight red shift. The results indicated that the addition of GCZ changed the conformation of the PEP molecule, and a new complex of GCZ and PEP was generated [16]. The results also proved that the quenching of the GCZ-PEP system was a static process.

The binding mechanism of the GCZ-PEP system: For a static quenching process, the number of binding sites n and the binding constant K_a can be determined by the following equation [17]:

$$\lg[(F_0 - F)/F] = n\lg K_a + n\lg\{[Q] - n[B_t](F_0 - F)/F_0\}, \quad (2)$$

where [B_t] and [Q] are the total concentrations of PEP and GCZ, respectively. As can be seen from the values of K_a and n (given in Table 1) the values of n obtained at three investigated temperatures were nearly equal to 1, which showed the presence of only one binding site with a high affinity for GCZ on PEP [18].

The value of K_a at λ_{ex} = 280 nm was obviously larger than that at λ_{ex} = 295 nm at the same temperature, showing that both tyrosine and tryptophan residues participated in the molecular interaction between PEP and GCZ [19]. The values of K_a increased with rising temperature, suggesting that the increase in temperature was not conducive to the binding of PEP and GCZ. This also demonstrated that the fluorescence quenching of PEP did not originate from dynamic collision but was initiated by the formation of a complex between GCZ and PEP [20].

In the case of a drug-protein binding homeostasis, one part of the protein is a free-form protein and the other part is bound-form. The reactions between drug Q and protein B can be expressed as

$$[B] + n[Q] \rightarrow [BQ_n],$$

where $[B]$ and $[Q]$ are the free concentration of protein and drug, respectively; n is the equivalent and independent number of drug binding sites. If the interaction of B with Q obeyed the Langmuir single molecule adsorption model [21], the equilibrium constant K_a can be given by the following equation:

$$K_a = [BQ_n]/([Q]^n[B]).$$

According to the above results, the drugs and the proteins were bound in a 1:1 ratio:

$$K_a = [BQ]/([Q][B]).$$

Assuming that the total drug concentration is Q, the total protein concentration is B, and the concentration of the drug-protein complex is x, the binding constant K_a is described by

$$K_a = x/\{(Q - x)/(B - x)\}.$$

This is a quadratic equation with one unknown:

$$K_a x^2 - (K_a Q + K_a B + 1)x + K_a QB = 0.$$

According to the root formula

$$x = \frac{K_a(Q+B)+1 \pm \sqrt{\left[K_a(Q+B)+1\right]^2 - 4K_a{}^2QB}}{2K_a}$$

we solve this equation and take a reasonable result:

$$x = \frac{K_a(Q+B)+1-\sqrt{K_a{}^2(Q-B)^2 + 2K_a(Q+B)+1}}{2K_a}.$$

So, the binding rate of the drug can be given by

$$W(Q) = \frac{x}{Q}\times 100\% = \frac{K_a(Q+B)+1-\sqrt{K_a{}^2(Q-B)^2 + 2K_a(Q+B)+1}}{2K_aQ}\times 100\%, \quad (3)$$

and the percentage of the protein that binds to the drug, that is, the binding rate of the protein is

$$W(B) = \frac{x}{B}\times 100\% = \frac{K_a(Q+B)+1-\sqrt{K_a{}^2(Q-B)^2 + 2K_a(Q+B)+1}}{2K_aB}\times 100\%. \quad (4)$$

The data at 298, 310, and 318 K were calculated. Under the experimental conditions, the concentration of GCZ was in the range from 2×10^{-6} to 1.5×10^{-5} M; the concentration of the protein was fixed at 2×10^{-6} M. The values of $W(Q)$ at different temperatures were 2.53–2.18, 2.25–1.97, and 1.80–1.61%, but the changes were not significant. However, $W(B)$ varied in the ranges 2.53–16.3, 2.25–14.8, and 1.80–12.1%, reduced with an increased temperature, and showed a significant change. These results showed that at the same temperature if the concentration of the drug increased, the binding rate of the drug decreased. Thus, the concentration of free GCZ weakly increased. Meanwhile, if the binding rate of the protein increased more obviously, the concentration of free PEP decreased significantly, which was unfavorable to digestion.

Let the ratio of the total concentration of the drug to the total concentration of the protein be R, $R = Q/B$. A non-linear fit of the curve at 310 K is shown in Fig. 2. The binding models were $W(Q) = 8.828\times10^{-6}R^2 - 5.106\times10^{-4}R + 0.02305$, and $W(B) = -3.990\times10^{-4}R^2 + 0.02265R + 3.397\times10^{-4}$, respectively. Both the correlation coefficients r was equal to 1.000. Referring to the amount of GCZ per patient as 40 mg each time [22] and the basal gastric acid secretion as 10–100 mL (its average value thus being 50 mL), the concentration of GCZ was approximately 2.476×10^{-3} M, and the concentration of PEP varied in the range

0.26×10^{-7}–0.43×10^{-7} M [23]. The calculated results were $W(Q)$ = 0.0010–0.0017% and $W(B)$ ≈ 96.69% (96.6906–96.6905%), where $W(Q)$ < 0.1%, small enough to be negligible, which showed that the binding of GCZ to PEP would not substantially affect the free concentration of GCZ, and the effect of the binding on the efficacy was negligible. However, the values of $W(B)$ were very high, which indicated that 96.69% of PEP would be consumed owing to the binding of GCZ to PEP, while the digestive PEP only takes part at 3.31%, which meant that taking GCZ will cause a serious weakness of the digestive capacity of the stomach. For patients with stomach diseases, taking GCZ can worsen the condition.

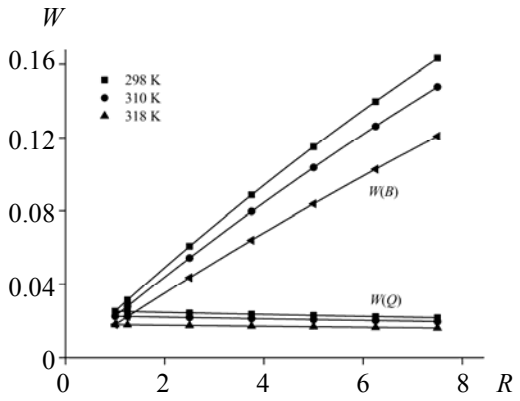

Fig. 2. The binding rate of the GCZ and the PEP.

Additionally, to clarify the major interaction mode of the GCZ-PEP system, the relevant thermodynamic parameters, free-energy change ΔG, entropy change ΔS, and enthalpy change ΔH, were obtained by the following equations [24]:

$$\ln K_a = -\Delta H/RT + \Delta S/R, \quad (5)$$

$$\Delta G = \Delta H - T\Delta S, \quad (6)$$

where T is the temperature and R is the gas constant. The calculated results are summarized in Table 2. Generally, four types of non-covalent interactions play an important role in the binding of different ligands of the drug to proteins. They are hydrogen bonds and van der Waals, electrostatic, and hydrophobic forces. As shown in Table 2, from a thermodynamic standpoint, the negative values obtained for ΔG indicated that the interaction between GCZ and PEP was spontaneous, while $\Delta H < 0$ demonstrated that the binding of GCZ to PEP was an exothermic reaction. $\Delta H < 0$ and $\Delta S < 0$, indicating that the electrostatic force might play the main role in the binding reaction [25]. In order to further determine the force of the GCZ-PEP system and find its binding position, molecular docking was used to simulate the binding of the GCZ molecule and the PEP molecule.

TABLE 2. Thermodynamic Parameters of the GCZ-PEP System at Different Temperatures (λ_{ex} = 280 nm)

λ_{ex}, nm	T, K	K_a, M^{-1}	ΔH, kJ/mol	ΔS, $J\cdot mol^{-1}\cdot K^{-1}$	ΔG, kJ/mol
280	298	1.33×10^4	–13.46	33.75	–23.51
	310	1.18×10^4		34.60	–24.18
	318	9.31×10^3		33.69	–24.17

N o t e. ΔH is the enthalpy change; ΔS is the entropy change; ΔG is the free-energy change.

According to the data of the docking results, the binding energy ΔG of GCZ and PEP was –23.95 kJ/mol, which was close to the thermodynamic parameters (ΔG = –24.18 kJ/mol) obtained from the experiment. As shown in Fig. 3, the binding site of GCZ was located within the active centre of PEP, which was formed by amino residues Asp32 and Asp215. This site is also the main binding site for small molecules of drugs on PEP molecules. GCZ was surrounded by residues Asp215, Gly34, Gly217, Asp32, Tyr75, Ile120, Ile30, Phe117, Trp39, Leu112, Gly78, Thr77, Phe111, Gly76, Met290, Val292, Thr222, Ile301, and

Thr218. GCZ formed hydrogen bonds with Gly76 and Asp215 residues, and the bond lengths were 2.09 and 1.87 Å, respectively. Combined with the experimental results of fluorescence spectroscopy, it is seen that the binding of the GCZ-PEP system is driven by the electrostatic attraction force and the hydrogen bonding force and spontaneously forms a new complex reaction process at a ratio of 1:1.

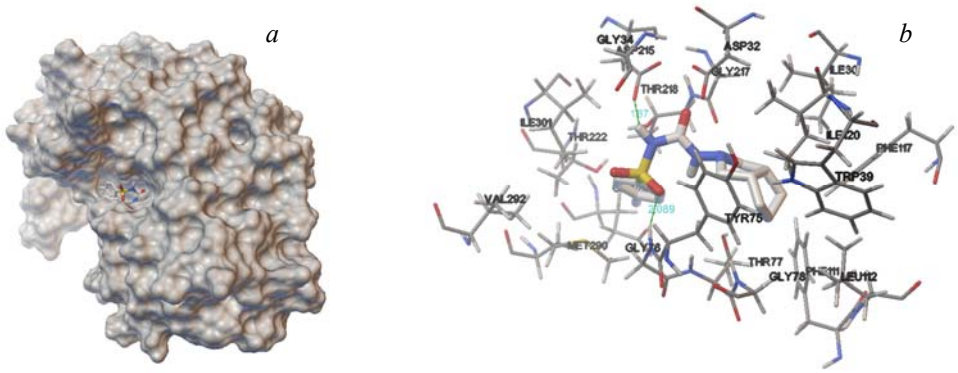

Fig. 3. The binding site of the GCZ in the PEP cavity (a) and detailed illustration of the amino acid residues lining the binding site in the PEP cavity (b).

Investigation of PEP conformation changes. In order to investigate the effects of GCZ on the conformation changes of PEP, a synchronous fluorescence measurement was performed. The synchronous fluorescence spectra of PEP upon the addition of different concentrations of GCZ are presented in Fig. 4. It was observed that the fluorescence intensity at $\Delta\lambda$ = 15 and 60 nm decreased, and the quenching degree of the latter was obviously larger than the former, which indicated that Trp residues in PEP molecules were more involved in the reaction than Tyr residues. Additionally, a slight blue shift in the maximum emission wavelength of Trp residues was detected upon the addition of GCZ, indicating that the micro-environment around the Trp residues of PEP changed slightly. This change meant that the addition of GCZ made the Trp residues of the PEP molecule more exposed to the hydrophobic environment, the polarity around the Trp residue was reduced, and the conformation of PEP was changed [26].

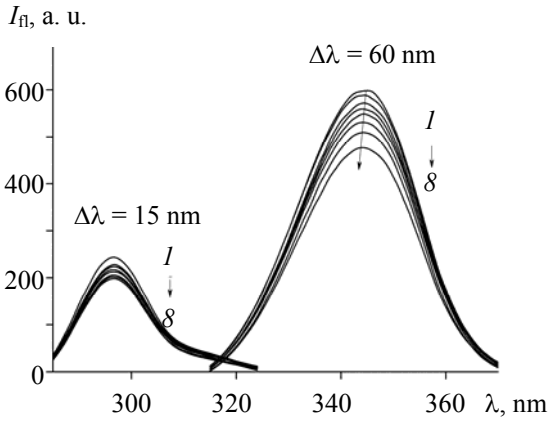

Fig. 4. Synchronous fluorescence spectrum of the GCZ-PEP system (T = 298 K), $C_{PEP} = 2.0\times10^{-6}$ M; 1–8: C_{GCZ} = (0, 0.2, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5)×10^{-5} M.

Circular dichroism (CD) is a fast, simple, and accurate method to investigate the secondary structure of proteins in an aqueous solution [27]. The characteristic peak around 204 nm in the far-ultraviolet region represented the α-helix in the PEP molecule. The content of the α-helix can be calculated according to the following formula [28]:

$$\alpha\text{-Helix} = \frac{-\text{MRE}_{204} - 4000}{33000 - 4000} \times 100\%, \quad (7)$$

$$\text{MRE}_{204} = I_{\text{CD }204}/10C_p nl, \quad (8)$$

where MRE_{204} is the observed mean residue ellipticity value at 204 nm, 4000 is the MRE of the form and random coil conformation cross at 204 nm and 33000 is the MRE value of a pure helix at 204 nm, n is the number of amino acid residues and l is the path length (cm), C_p is the molar concentration of the protein, and $I_{\text{CD }204}$ is the intensity of CD (degree) at 204 nm. The solution was prepared according to the procedure of fluorescence experiments. The buffer solution was selected as the blank under the same experimental conditions and was subtracted from the sample spectra. The speed of scanning was 1 nm/s, $C_{\text{PEP}} = 2.0\times10^{-6}$ M and $C_{\text{GCZ}} = (0, 6.0, 12.0)\times10^{-6}$ M. It was calculated that when the molar ratios of PEP to GCZ were 1:3 and 1:6, the band intensities of PEP at 204 nm increased with increasing in GCZ without causing any significant shift of the peaks, the content of α-helix structure of PEP molecules increased from 20.16 to 21.13%, indicating that the secondary structure of the protein to change. However, the peak shape and the peak position did not change, indicating that the α-helix structure in the PEP structure still dominated.

Hill's coefficient of the GCZ-PEP system. In biochemistry, the formation of a ligand-protein complex generally enhances the binding of a new ligand molecule to biomacromolecules. This is known as cooperative binding. Hill's coefficient (n_{H}) provides a way to quantify this effect. According to Archibald Vivian Hill's theory, when n_{H} is equal to 1, it indicates that the drug-protein binding is completely independent and there is no synergistic effect for the binding of later ligand and protein; when $n_H > 1$, there is a positive cooperativity for the later ligand and protein molecule; $n_{\text{H}} < 1$ reveals negative cooperativity, that is, it has a weakening effect on the binding of later ligand molecules and protein. Hill's equation is expressed as follows [29]:

$$\lg[L/(L_m - L)] = \lg K_a + n_{\text{H}}\lg[Q], \quad (9)$$

where $L = 1 - F/F_0$, L_m is obtained by plotting $1/L$ vs. $1/[Q]$. The values of n_{H} are given in Table 3. From the table, the values of n_{H} at different temperatures were all less than 1, indicating that the interaction between GCZ and PEP had a weak negative cooperative effect on the subsequent drug ligands, and the formation of the GCZ-PEP complex was not conducive to the binding of subsequent drugs and PEP and $n_{\text{H}(\lambda\text{ex} = 280\text{ nm})} < n_{\text{H}(\lambda\text{ex} = 295\text{ nm})}$, which showed that both Tyr and Trp residues contributed to this negative cooperative effect.

TABLE 3. Hill Coefficient of the GCZ-PEP System at Different Temperatures

T, K	$\lambda_{\text{ex}} = 280$ nm		$\lambda_{\text{ex}} = 295$ nm	
	n_{H}	r_3	n_{H}	r_3
298	0.75	0.9968	0.84	0.9977
310	0.82	0.9983	0.86	0.9945
318	0.78	0.9945	0.79	0.9966

N o t e. n_{H} is the Hill's coefficient; r_3 is the linear relative coefficient of $\lg[L/(L_m - L)]$~$\lg[Q]$.

Conclusion. The mechanism of the interaction between GCZ and PEP was explored in detail through a variety of spectroscopic methods and molecular modeling techniques. An *in vitro* binding model of the GCZ-PEP complex was established. The results showed that GCZ can spontaneously bind with PEP by electrostatic attraction and hydrogen bonding. The active center of PEP was the only binding site between GCZ and PEP. The formation of GCZ-PEP complexes changed the conformation of PEP and had a negative cooperative effect on subsequent drugs. The binding rate of GCZ and the binding rate of PEP were calculated from the values of binding constants and the number of binding sites. The result indicated that GCZ would cause a severe reduction in the digestive function of the stomach. For patients with stomach diseases, oral GCZ will further aggravate the condition. Investigation of the binding rate of drugs and proteins has significance for predicting the side effects of drugs and finding more reasonable modes of administration.

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