RESEARCH ARTICLE

Spectroscopic study on the mechanism of meloxicam and α-amylase

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Abstract: In order to explore the mechanism of action of meloxicam and α-amylase. The interaction between the rheumatoid arthritis drug meloxicam and α-amylase was studied by fluorescence spectroscopy, synchronous fluorescence spectroscopy and molecular docking under the experimental conditions of pH=6.8. The results showed that meloxicam was able to effectively quench the endogenous fluorescence of α-amylase in a static quenching form a 1:1 complex and change the conformation of α-amylase. Thermodynamic results indicated that the main type of meloxicam and α-amylase system was hydrophobic interaction. Molecular docking indicated that the binding system had hydrogen bonds in addition to hydrophobic interaction and meloxicam was surrounded by the active amino acid residues Trp13 and Trp263 of α-amylase, which changed the microenvironment of amino acid residues at the active center of α-amylase. By establishing the binding model, it can be seen that the protein binding rate W(B) of meloxicam to -amylase was 2.76%-41.79% under the experimental conditions. The results showed that the binding of meloxicam to -amylase had an effect on the number of free -amylase. The drug binding rate W(Q) of the system was 2.76%-1.67%, which indicated that the combination of α-amylase and meloxicam would not affect the efficacy of meloxicam.

Keywords: drug, protein, fluorescence analysis, conformation, molecular docking, binding rate

1 Introduction

Non-steroidal anti-inflammatory drugs can relieve pain and edema to play a role in the treatment of inflammation, however, patients taking non-steroidal anti-inflammatory drugs often cause gastritis, gastric ulcer, kidney and liver damage and other adverse symptoms[1]. Meloxicam (MEL) is one of the non-steroidal anti-inflammatory drugs[2] whose molecular structure is shown in Figure 1. Therefore, the adverse effect of MEL on gastrointestinal function was much smaller[3]. This drug is widely used in daily life and it is a very common drug for the treatment of rheumatoid arthritis. In addition to the treatment of inflammatory diseases, MEL also has a good effect on relieving physical pain in patients[4].

α-Amylase (AMS) belongs to a famous group of enzymes, which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units[5]. They can be found in the body of microorganisms, plants and all of the higher body organisms that use carbohydrates in their metabolism process. Thus, the amylases can be derived from several sources, including plants, animals and microorganisms; microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry[6]. Over the past few decades, considerable research has been undertaken with the extracellular AMS being produced by a wide variety of microorganisms[7].

In recent years, fluorescence spectroscopy has become an important means to study the mechanism of ligand-
protein system. So far, R. Omidiyan[8] has studied the interaction between AMS and cetylimethyammonium bromide by spectroscopy, and He Qiang[9] has studied the interaction between AMS and tannic acid, a hypoglycemic drug, by spectroscopy. There are many reports on the interaction between small molecular drugs and AMS by spectroscopy. Most of the studies only focus on the binding mechanism of ligands and AMS. However, there are relatively few studies on the binding of AMS to drug molecules by spectroscopy, and then infer the effect of MEL on the efficacy and properties of AMS. In this paper, a series of experimental data were obtained by fluorescence experiments and molecular docking, which revealed the binding mechanism of the system and predicted the drug efficacy and enzyme properties of MEL combined with AMS in human body. The qualitative effect provides a useful reference for the rapid prediction of the interaction between drugs and proteins in vivo.

2 Experimental

2.1 Apparatus

RF-5301PC fluorometer (Shimadzu, Japan); UV-3600 UV-vis spectrophotometer (Shimadzu, Japan); SYC-15 super constant temperature water bath (Nanjing Sanli Electronic equipment Factory); SZ-93 automatic double Pure Water Distiller (Shanghai Yarong biochemical instrument Factory).

2.2 Materials

AMS (purity grade inferior 99%, Sigma), reserve solution (1.0 × 10⁻⁵ mol/L); MEL (CAS# 71125-28-7), reserve solution (4.0 × 10⁻⁴ mol/L), phosphate buffer solution of pH=6.80 was prepared. The water used in the experiment was secondary quartz distilled water, and the above storage solution was kept away from light at 277 K. The fluorescence signal measured in the experiment was corrected by the “internal filter effect” Equation 1:[10]

\[
F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2}
\]

Where \(F_{cor}\) and \(F_{obs}\) are the corrected and observed fluorescence signals, respectively, and \(A_{ex}\) and \(A_{em}\) are the absorbance values of MEL-AMS system at excitation and emission wavelengths, respectively. The fluorescence signal used in this article was corrected.

2.3 Experiment procedure

2.3.1 Fluorescence experiment

At 298 K, 310 K and 318 K, 1.0 mL phosphate buffer solution, 2.0 mL AMS solution and different volume of MEL solution were added to the 10.0 mL colorimetric tube at constant volume and constant temperature of 30 min. The slit width was 5 nm, \(\lambda_{ex}\) and the scanning fluorescence spectra were 280, 295 nm, respectively. When \(\Delta \lambda=15\) nm or 60 nm, scanning synchronous fluorescence spectroscopy.

2.3.2 UV-Vis measurements

At 298 K, 1.0 mL phosphate buffer solution, 2.0 mL AMS solution and different volume MEL solution was added to the 10.0 mL colorimetric tube at constant volume and constant temperature of 30 min. The absorbance of the system was determined by using the corresponding concentration of MEL solution as the blank reference, and the UV absorption spectrum of the system was drawn.

2.3.3 Molecular docking

The crystal structure (PDB ID: 1BLI of AMS comes from the protein database (Protein Data Bank). The ChemDraw Pro 14.0 and ChemBio 3D Ultra 14.0 are used to draw the MEL structure, and the energy minimization of the three-dimensional structure is carried out. AutoDock 4.2.6 was used to study the molecular docking of MEL and AMS, and genetic algorithm was used to calculate the binding conformation of MEL and AMS[11].

3 Results and discussion

3.1 Fluorescence quenching mechanism studies of MEL-AMS system

The fluorescence effect of protein is produced by the chromophore of Trp, Tyr and Phe residues. The Trp and Tyr residues in protein are excited together at 280 nm wavelength, while at 295 nm wavelength, only Trp residue is excited[12]. Figure 2 showed the fluorescence spectra of the interaction between MEL and AMS (at \(\lambda_{ex}=295\) nm, the fluorescence spectra of MEL and AMS are similar, but the fluorescence intensity is low). Figure 2 showed that the fluorescence peak of AMS at 343 nm quenched and the emission peak shifts blue with the increase of MEL concentration, indicating that the MEL-AMS system interacted and formed a stable complex.[13]

The Stern-Volmer[14] equation was shown below, through which the quenching constant \(K_{sv}\) and the quenching rate constant \(k_q\) can be calculated by using the fluorescence signal data obtained from the experiment:

\[
F_0/F = 1 + k_q\tau_0[L] = 1 + K_{SV}[L]
\]

Where \(F_0\) and \(F\) represent the fluorescence signals in the absence and presence of quencher, respectively. \(\tau_0\) is the average lifetime of fluorescence without quencher,
The binding constant \[ K_a \] of MEL to AMS decreased with the increase of temperature, which further proved that the main type of force between the binding systems was electrostatic interaction. Based on this, the hydrophobic interaction between drug molecules and protein molecules, but now some reports believed that when \( \Delta S > 0 \), there was electrostatic attraction between drug molecules and biomolecules, but now some reports thought that when \( \Delta H < 0 \), \( \Delta S > 0 \) could directly judge that the main type of force between the binding systems is electrostatic interaction. In order to further verify whether the main force between MEL and AMS is elec-

\[ R \ln K = \Delta S - \Delta H / T \]

\[ \Delta G = -RT \ln K = \Delta H - T \Delta S \]

For static quenching, the Equation 3 is generally used to calculate the binding constant \( K_a \) and the number of binding sites \( n \):

\[ \log \left( \frac{F_0 - F}{F} \right) = n \log K_a + n \log \left\{ \frac{[L]}{[B_i]} + n \frac{F_0 - F}{F_0} [B_i] \right\} \]

\[ (3) \]

\([B_i]\) represents the concentration of AMS, and the results were shown in Table 1. From Table 1, \( n \approx 1 \) at the experimental temperature indicates that there is only one high affinity binding site for MEL to bind to AMS, that is, MEL forms a 1:1 complex with AMS. The binding constant \( K_a \) of MEL to AMS decreased with the increased of temperature, which further proved that the fluorescence quenching type of MEL-AMS system was static quenching. Figure 3 showed the participation of Tyr residues and Trp residues of AMS in MEL-AMS system. The results showed that when \( \lambda_{ex} = 280 \) nm and \( \lambda_{ex} = 295 \) nm, the quenching curve of MEL-AMS system was separated. This indicated that both Tyr residues and Trp residues in AMS participated in the reaction. However, the slope of the quenching curve of MEL-AMS system at \( \lambda_{ex} = 295 \) nm was obviously smaller than that of the binding system at \( \lambda_{ex} = 280 \) nm, which indicated that the fluorescence quenching degree of AMS was stronger at \( \lambda_{ex} = 280 \) nm.

**3.2 Type of interaction force of MEL-AMS system**

The thermodynamic parameters of MEL-AMS system are calculated according to van’t Hoff equation, and the calculated results were shown in Table 2.

\[ R \ln K = \Delta S - \Delta H / T \]

\[ \Delta G = -RT \ln K = \Delta H - T \Delta S \]

Where \( R \) is a gas constant (\( \Delta H \) and \( \Delta S \) with a value of about 8.314), MEL and AMS could be calculated by a linear relationship between the natural logarithm (\( \ln K_a \)) of the binding constant and the reciprocal (1/T) of the temperature. The results were shown in Table 2. It could be seen from Table 2 that \( \Delta G < 0 \) indicated that the binding reaction between MEL and AMS was spontaneous, and \( \Delta H < 0 \) indicated that the formation of MEL-AMS complex was exothermic. The arrangement of water molecules creates a more random configuration around drugs and proteins in a more orderly manner. Therefore, \( \Delta S > 0 \) is usually used as evidence of hydrophobic interaction between drug molecules and protein molecules. Based on this, the hydrophobic interaction between MEL and AMS could be judged.

Ross and Subramanian believed that when \( \Delta H \approx 0, \Delta S > 0 \), there was electrostatic attraction between drug molecules and biomolecules, but now some reports thought that when \( \Delta H < 0, \Delta S > 0 \) could directly judge that the main type of force between the binding systems is electrostatic interaction. In order to further verify whether the main force between MEL and AMS is elec-
Table 1. Quenching reactive parameters of MEL-AMS system at different temperatures

<table>
<thead>
<tr>
<th>λ&lt;sub&gt;ex&lt;/sub&gt; (nm)</th>
<th>T/(K)</th>
<th>K&lt;sub&gt;sv&lt;/sub&gt; (L/mol·s)</th>
<th>k&lt;sub&gt;q&lt;/sub&gt; (L/mol)</th>
<th>r&lt;sub&gt;1&lt;/sub&gt;</th>
<th>K&lt;sub&gt;a&lt;/sub&gt; (L/mol)</th>
<th>n</th>
<th>r&lt;sub&gt;2&lt;/sub&gt;</th>
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<td>298</td>
<td>1.70×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.70×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9932</td>
<td>1.81×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.03</td>
<td>0.9922</td>
<td></td>
</tr>
<tr>
<td>310</td>
<td>1.38×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.38×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9939</td>
<td>1.46×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.96</td>
<td>0.9969</td>
<td></td>
</tr>
<tr>
<td>318</td>
<td>1.13×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.13×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9957</td>
<td>1.17×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.98</td>
<td>0.9956</td>
<td></td>
</tr>
<tr>
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<td>1.36×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.36×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9938</td>
<td>1.43×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.06</td>
<td>0.9942</td>
<td></td>
</tr>
<tr>
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<td>1.12×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9965</td>
<td>1.08×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.99</td>
<td>0.9969</td>
<td></td>
</tr>
<tr>
<td>318</td>
<td>0.91×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.91×10&lt;sup&gt;12&lt;/sup&gt;</td>
<td>0.9974</td>
<td>0.88×10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.02</td>
<td>0.9915</td>
<td></td>
</tr>
</tbody>
</table>

Note: r<sub>1</sub> is the linear relative coefficient of F<sub>0</sub>/F<sub>0</sub>-[L]; r<sub>2</sub> is the linear relative coefficient of lg[(F<sub>0</sub>-F)/F<sub>0</sub>]-n[Bt](F<sub>0</sub>-F)/F<sub>0</sub>

3.3 Conformation studies of MEL-AMS system

3.3.1 Synchronous fluorescence studies of MEL-AMS system

By measuring the shift of the maximum emission wavelength of the synchronous fluorescence spectrum of the binding system, the environmental information of amino acid residues near the fluorescent luminescence group can be explored[23]. As shown in Figure 5, when Δλ<sub>λ</sub>=60 nm and Δλ<sub>λ</sub>=15 nm, the intensity of the fluorescence signal of the AMS tended to decrease, and the fluorescence peaks of both the Trp residue and the Tyr residue shifted very slightly, this means that the binding of MEL to AMS changes the microenvironment of amino acid residues in small molecular proteins such as AMS, reducing its hydrophobicity. The extension of peptide chain increased[24] and Tyr and Trp residues were involved in the binding reaction. This result was consistent with conclusion 3.1.

3.3.2 UV-vis absorption spectra studies of MEL-AMS system

UV-vis absorption spectra can be used to explore the structural changes of proteins and to study the formation of protein-ligand complexes[25]. Figure 6 was an ab-
Figure 5. Synchronous fluorescence spectra of MEL-AMS system (T=310 K). (A) $\Delta \lambda=60$ nm; (B) $\Delta \lambda=15$nm; $C_{AMS}=2.0 \times 10^{-6}$ mol/L, $C_{MEL}=(0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0) \times 10^{-5}$ mol/L

Figure 6. Fluorescence intensity of MEL-AMS system as a function of NaCl concentration (Absorption spectra of MEL-AMS system (T=298 K); $C_{AMS}=2.0 \times 10^{-6}$ mol/L, $C_{MEL}=(0, 0.2, 0.5, 1.0, 2.0) \times 10^{-5}$ mol/L

Absorption spectrum of the MEL-AMS system. From the figure, it could be seen that AMS has two absorption peaks, and its strong absorption peak near 208 nm reflected the frame conformation of the protein. The weak absorption peak at about 280 nm was due to aromatic amino acids (Trp, Tyr and Phe)\[26\]. With the increased of MEL concentration, Figure 6 showed that the intensity of the absorption peak at 208nm decreased with the blue shifted, and the absorption peak at 280nm also decreased slightly. This result indicated that the interaction between MEL and AMS led to the formation of new complexes, and the AMS molecule tends to fold, the hydrophobicity of AMS microenvironment was enhanced.

3.4 Molecular docking

Molecular docking plays an important role in exploring the interaction between ligands and receptors. In order to further determine the binding position of MEL-AMS system and the effect of MEL binding to AMS on ligands and receptors. In this paper, the binding model of MEL and AMS was established by molecular docking method. By this method, the type of force and the lowest binding energy of MEL and AMS binding system could be obtained. Figure 7(A) showed the optimal binding position after MEL binds to AMS, the Asp328 residue forms two hydrogen bonds with MEL with bond lengths of 2.138 Å and 2.144 Å respectively, and the His253 residue formed a hydrogen bond with MEL. The bond length is 1.791 Å, which showed that the hydrogen bond played an important role in the binding of MEL to AMS. Figure 7(B) showed a plurality of hydrophobic amino acid residues such as Ala232, Tyr262, Trp13, Trp263, Leu196, Leu335 and Val233 around MEL, further indicated that there was hydrophobic force in the binding process between MEL and AMS. The amino acid residues such as Tyr262, Trp13 and Trp263 were relatively close to the binding position of MEL and AMS, which led to the binding could effectively quench the endogenous fluorescence of AMS, which was consistent with the conclusion of fluorescence quenching experiment. Trp13 and Trp263 were the key residues of the catalytic active center of AMS\[27\]. The results of molecular docking also showed that the binding of MEL and AMS could change the microenvironment of the catalytic active center of AMS. In other words, the binding of the system might affect the catalytic activity of AMS.

The binding energy obtained from molecular docking for MEL and AMS interaction was -25.89 kJ/mol. Whereas, the free energy change calculated from fluorescence quenching results was -24.66 kJ/mol at 310 K.
rate and protein binding rate of MEL to AMS can be calculated. When \( n=1 \), the binding rate \((W)\) formula of MEL-AMS system was as follows\(^{[29]}\).

**Drug binding rate:**
\[
W(Q) = \frac{x}{Q} \times 100\%
\]
\[
= \frac{K_a(Q + B) + 1 - \sqrt{K_a^2(Q - B)^2 + 2K_a(Q + B) + 1}}{2K_aQ} \times 100\%
\] \( (6) \)

**Protein binding rate:**
\[
W(B) = \frac{x}{B} \times 100\%
\]
\[
= \frac{K_a(Q + B) + 1 - \sqrt{K_a^2(Q - B)^2 + 2K_a(Q + B) + 1}}{2K_aB} \times 100\%
\] \( (7) \)

Wherein \( Q \) represents the total concentration of MEL and \( B \) represents the total concentration of AMS. At the three temperatures of 298K, 310K and 318K, the drug binding rate \( W(Q) \) of MEL and AMS calculated according to formulas \((6)\) and \((7)\) was 3.38%-1.88% (298K), 2.76%-1.67% (310K), 2.24%-1.46% (318K), protein binding rate \( W(B) \) was 3.38%-47.03% (298K), 2.76%-41.79% (310K), 2.24%-36.57% (318K). At three temperatures, the drug binding rate and protein binding rate of MEL to AMS decreased with the increase of temperature, and the content of free drugs and proteins increased with the increase of temperature, which indicated that the stability of the binding system decreased with the increase of temperature. The results were consistent with the experimental results of fluorescence quenching.

The ratios of \( W(Q) \) and \( W(B) \) to drug concentration and protein concentration were plotted, respectively, as shown in Figure 8. It could be seen from Figure 8 that the \( W(Q) \) value decreased with the increased of temperature, while the \( W(B) \) value was large and increased. Taking 310K data close to human body temperature as an example: The \( W(Q) \) of MEL and AMS was 2.76%-1.67%, which indicated that the interaction between MEL and AMS had little effect on the efficacy of MEL as the

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**Table 3. Docking energy of MEL-AMS system (unit: kJ/mol)**

<table>
<thead>
<tr>
<th>Protein PDB ID</th>
<th>( \Delta G_0 )</th>
<th>( \Delta E_1 )</th>
<th>( \Delta E_2 )</th>
<th>( \Delta E_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1BLI</td>
<td>-25.89</td>
<td>-29.65</td>
<td>-28.81</td>
<td>-0.84</td>
</tr>
</tbody>
</table>

\( \Delta G_0 \) is the binding energy in the binding process.

\( \Delta E_1 \) denotes intermolecular interaction energy, which is a sum of van der Waals energy, hydrogen bonding energy, desolvation free energy and electrostatic energy.

\( \Delta E_2 \) is the sum of van der Waals energy, hydrogen bonding energy and desolvation free energy.

\( \Delta E_3 \) is the electrostatic energy.
amount of MEL added increases. The \( W(B) \) of MEL and AMS was 2.76%-41.79%, which indicated that the protein binding rate varies greatly with the increase of MEL addition. Combined with the results of 3.4, MEL might have an effect on the activity of AMS, which was not conducdive to the digestion and absorption of starchy substances. Therefore, the intake of drugs should be strictly controlled when taking MEL, so as to reduce the adverse effects of drugs on the digestive system.

![Graph](image)

**Figure 8.** The binding rate of MEL to protein in different temperature and the binding rate of AMS to drug (\( \lambda_{ex} = 280 \text{ nm} \))

### 4 Conclusion

In this paper, the interaction between MEL and AMS was studied by spectroscopic and theoretical modeling under simulated physiological conditions. The binding rate model of MEL and AMS was established. The interaction between MEL and AMS was used to treat drugs. The effects of AMS on the digestive function of starches were simply predicted. It provides a new idea to study the effect of the interaction between protein molecules and small drug molecules on the properties of proteins and the efficacy of drugs. The binding rate of ligand-protein system is studied by spectroscopy. Compared with other methods such as equilibrium dialysis, there will be some errors in the binding constant and the number of binding sites, but these commonly used binding rates are studied. The experimental equipment of the method is expensive, the experimental period is long and the concentration range of the experimental drug is narrow. However, the method of studying the binding rate by spectroscopy is simple, rapid and suitable for a wide range of applications, so this method is preferable.

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