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Mucin-drugs interaction: the case of theophylline, prednisolone and cephalexin

Carlotta Pontremoli\textsuperscript{a}, Nadia Barbero\textsuperscript{b}, Guido Viscardi\textsuperscript{b} and Sonja Visentin\textsuperscript{a,\textsuperscript{b}}

\textsuperscript{a}Molecular Biotechnology and Health Sciences Department, University of Torino, via Quarello 15, 10135 Torino, Italy

\textsuperscript{b}Department of Chemistry and NIS Interdepartmental Centre, University of Torino, via Pietro Giuria 7, 10125 Torino, Italy

1. Introduction

Mucus is a viscoelastic gel secreted by epithelial cells to protect the epithelium from environmental factors [1]. Accumulation of mucus with abnormal viscosity in the airways is a central pathological feature of cystic fibrosis, asthma, and chronic obstructive pulmonary disease [2, 3]. Mucus is composed of water, ions, lipids, and approximately 2% of protein: the protein mainly expressed is mucin, an high molecular O-glycosylated protein. It plays an important role in the defence of epithelia, forming a protective extracellular mucin gel, a steric barrier against assault. Qualitative and quantitative anomalies of mucin in many diseases like cancers or chronic airway diseases (BPCO against assault. Qualitative and quantitative anomalies of mucin in many diseases like cancers or chronic airway diseases (BPCO)) in many diseases like cancers or chronic airway diseases (BPCO) in many diseases like cancers or chronic airway diseases (BPCO)) in many diseases like cancers or chronic airway diseases (BPCO) allowed to identify this protein as an important marker of adverse broncho-pneumopathie chronique obstructive or cystic fibrosis)

Against assault. Qualitative and quantitative anomalies of mucin in many diseases like cancers or chronic airway diseases (BPCO)

In this paper we analyzed the interaction of mucin with some drugs used to treat the symptomatology of cystic fibrosis, in particular theophylline for airway obstructions and asthma conditions, cephalexin for infection and prednisolone for inflammation. (Fig.1)

2. Materials and methods

2.1. Materials

Mucin from porcine stomach (type III, bound sialic acid 0.5-1.5%, partially purified powder) was purchased from Sigma Aldrich. The stock solution (1 mg/mL) was prepared by dissolving mucin in PBS (phosphate buffer solution, 2 mM, pH = 7.4).

Theophylline (≥99%), cephalexin (CAS# 23325-78-2) and prednisolone (≥98%) were all purchased from Sigma Aldrich; the stock solutions (3 mM) of theophylline and cephalexin were prepared by dissolving drugs in water, and the stock solution (3 mM) of prednisolone was prepared by dissolving drug in 1:1 of ethanol 96% and PBS.

2.2. Apparatus

All fluorescence spectra were recorded with a Horiba Jobin Yvon Fluorolog3 TCSPC spectrophotometer with 1.0 cm quartz cells. UV-Vis spectra were recorded on a UH5300 Hitachi spectrophotometer. The pH measurements were made with a Eutech Instruments pH2700.

2.3. Measurements of spectra

Mucin 0.05 mg/mL was titrated by successive additions of theophylline solutions at different concentrations: over a range from 50 μM to 600 μM for theophylline, from 3 μM to 400 μM for cephalexin and over a range from 20 μM to 500 μM for prednisolone. Titrations were done manually using microinjectors. Fluorescence quenching spectra were measured in the range of 285-500 nm at the excitation wavelength of 265 nm. The excitation and emission slits were respectively 6 nm and 10 nm. The fluorescence spectra were performed at three temperatures (296 K, 303 K, 310 K).

![Fig. 1 Chemical structures of: A) theophylline. B) prednisolone. C) cephalexin.](Image)
3. Results and discussion

3.1. UV-Vis absorption studies

UV-Vis absorption measurement is a simple and applicable method to investigate the formation of a complex [11, 12]. The absorption spectra of mucin in absence and in presence of different concentrations of drugs were recorded and presented in Fig.2: the absorption intensity of mucin at around 260 nm increased with the addition of increasing concentrations of drugs. Moreover, the absorption spectrum of mucin-drug complex is different from that of mucin and drugs alone. The maximum peak position of mucin-drugs complex was slightly shifted towards lower wavelength region, as shown in the reported spectra (Fig.2).

An explanation for this phenomenon may come from the formation of a new drug–protein complex with a new structure. This behavior may also indicate a change in polarity around the tryptophan residue and a change in peptide strand of mucin and thus the change in hydrophobicity as described in literature [13].

3.2. Fluorescence quenching mechanism of mucin-drugs system

The fluorescence spectra of mucin in absence and in presence of drugs at different concentrations are shown in Fig.3. Mucin shows a strong fluorescence emission at 360 nm and its fluorescence intensity decreases gradually with the increase of drug’s concentration; from the spectra it is possible to observe a hypsochromic effect for the complex mucin-theophylline (from 360 nm to 348 nm), a bathochromic effect for the complexes mucin-cephalexin (from 356 nm to 362 nm) and mucin-prednisolone (from 360 nm to 371 nm).

The quenching of mucin fluorescence by drugs was due to the formation of a protein-drug complex and the microenvironment of mucin was changed during the binding interaction. Binding studies were performed and the obtained steady-state maximum fluorescence intensity was recorded. Data were treated by two different methods, to obtain the evaluation of the equilibrium association (K_A) and dissociation (K_D) constants.

3.3. Stern-Volmer equation

The fluorescence quenching of mucin is analyzed by Stern-Volmer equation (1) [14]:

\[
F_0 / F = 1 + K_{SV} [Q]
\]  (1)

where F_0 is the fluorescence intensity of mucin alone and F is the fluorescence intensity of mucin in the presence of increased concentration of quencher. K_{SV} is the Stern–Volmer quenching constant, which describes a collisional quenching of fluorescence and [Q] is the quencher concentration. Quenching data are presented as plots of F_0/F vs. [Q], yielding an intercept of one on the y- axis and a slope equal to K_{SV}. Stern-Volmer plots at different temperatures are reported in Fig.4.

It is important to recognize that observation of a linear Stern-Volmer plot does not prove that collisional quenching of fluorescence has occurred. Static and dynamic quenching can be distinguished by their differing dependence on temperature: higher temperatures result in faster diffusion and hence larger
amounts of collisional quenching. Higher temperature will typically result in the dissociation of weakly bound complexes, and hence smaller amounts of static quenching. [14]

As shown in Table 1, the $K_{SV}$ of the complex mucin-cephalexin decreases with increasing temperature. This indicates a static quenching interaction between protein and drug. [12] The $K_{SV}$ of theophylline-mucin and prednisolone-mucin complexes are similar with negligible variations by changing the temperature.

### Table 1. The quenching constants of mucin and drugs: A) theophylline, B) prednisolone and C) cephalaxin at different temperatures.

<table>
<thead>
<tr>
<th></th>
<th>296 K</th>
<th>303 K</th>
<th>310 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20415</td>
<td>19628</td>
<td>20195</td>
</tr>
<tr>
<td>B</td>
<td>6136</td>
<td>4120</td>
<td>5332</td>
</tr>
<tr>
<td>C</td>
<td>9262</td>
<td>3385</td>
<td>1753</td>
</tr>
</tbody>
</table>

3.4. **Non linear least squares**

Fluorescence data at different temperatures were also analysed with a non-linear least-squares fit procedure, [15] based on equation 2:

$$ y = \frac{B_{max}[Q]}{K_D} $$

where, acting as a quencher, [Q] is the drug concentration, y is the specific binding derived by measuring fluorescence intensity, $B_{max}$ is the maximum amount of the complex protein/drug formed at saturation and $K_D$ is the equilibrium dissociation constant. The binding curves are reported in Fig.5: the percentage of bound mucin, i.e. y, derived from the fluorescence intensity emission maximum, is plotted against the drug concentration. The corresponding $K_D$ and $K_A$ at different temperatures are shown in Table 2.

3.5. **Lineweaver-Burk**

The other method used to analyse fluorescence data is the Lineweaver-Burk equation [14] based on Eq. (3):

$$ \frac{1}{F_0-F} = \frac{1}{F_0} + \frac{K_D}{[Q]} $$

where $F_0$ is the steady-state fluorescence intensity of mucin alone, F is the steady-state fluorescence intensity of the complex at the increasing quencher concentration, [Q] is the drug concentration in solution and $K_D$ is the equilibrium dissociation constant. As shown in Fig.6 reciprocals of $F_0-F$ are plotted against reciprocals of [Q]. The slope of the line is the $K_D/F_0$ ratio while the intercept is the reverse of $F_0$. The equilibrium dissociation ($K_D$) and association ($K_A$) constants at different temperatures are thus easily calculated and reported in Table 2. The results can be compared with the data obtained with the non linear fit equation.

As shown in figures 4, 5 and 6, for all the equations used, the difference of binding at different temperatures is greater for cephalaxin, while the binding constants remain nearly unchanged for theophylline and prednisolone. This can mean that the binding of cephalaxin is more...

3.6. **Binding parameters**

To obtain the number of binding sites (n), it is possible to use the double logarithm regression curve (shown in equation 4) [14], which describes the relationship between the fluorescence intensity and the concentration of the quencher.
Fig. 6 The Lineweaver-Burk plots of mucin-drugs complex at different temperatures. A) theophylline; B) prednisolone; C) cephalixin.

\[
\log \left( \frac{F_0}{F} \right) = \log K_A + n \log [Q] \tag{4}
\]

where \( F_0 \) is the fluorescence intensity of mucin alone, \( F \) is the fluorescence intensity after the addition of the quencher and \([Q]\) is the quencher concentration. The slope of the line is the \( n \) value. If the value of \( n \) is equal to 1, it means that a strong binding exists between the protein and the drugs. [13]

The number of binding sites (\( n \)) for mucin-cephalexin complex is 1.65 (296 K), 0.70 (303 K), 0.99 (310 K), for mucin-theophylline is 1.67 (296 K), 1.78 (303 K), 1.66 (310 K) and for mucin-prednisolone is 1.80 (296 K), 1.51 (303 K) 0.90 (310 K). Almost all values are approximately equals to 1, indicating that there is one independent binding site on mucin for every analyzed drug. [16]

Table 2: Values of the equilibrium dissociation and association constants of mucin-drugs complex at different temperatures obtained by non linear fit equation and by Lineweaver-Burk equation. A) theophylline. B) prednisolone. C) cephalixin

<table>
<thead>
<tr>
<th></th>
<th>296 K</th>
<th>303 K</th>
<th>310 K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_d</td>
<td>K_a</td>
<td>K_d</td>
</tr>
<tr>
<td></td>
<td>(M^{-1})</td>
<td>(M^{-1})</td>
<td>(M^{-1})</td>
</tr>
<tr>
<td>Non linear fit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6329</td>
<td>1.50</td>
<td>8130</td>
</tr>
<tr>
<td>B</td>
<td>4098</td>
<td>2.44</td>
<td>4760</td>
</tr>
<tr>
<td>C</td>
<td>10482</td>
<td>0.96</td>
<td>5780</td>
</tr>
<tr>
<td>Lineweaver-Burk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6808</td>
<td>1.47</td>
<td>8203</td>
</tr>
<tr>
<td>B</td>
<td>7049</td>
<td>1.42</td>
<td>6156</td>
</tr>
<tr>
<td>C</td>
<td>14902</td>
<td>0.23</td>
<td>12730</td>
</tr>
</tbody>
</table>

3.7. Thermodynamic parameters

The interaction forces between small molecules and macromolecules include four binding modes: H-bonding, Van der Waals, electrostatic, and hydrophobic interactions [17]. According to the data of enthalpy change (\( \Delta H \)) and entropy change (\( \Delta S \)), the model of interaction between drug and biomolecule can be concluded [18]: 1- \( \Delta H > 0 \) and \( \Delta S > 0 \), hydrophobic forces; 2- \( \Delta H < 0 \) and \( \Delta S < 0 \), van der Waals interactions and hydrogen bonds; 3- \( \Delta H < 0 \) and \( \Delta S > 0 \), electrostatic interactions. The thermodynamic parameters, enthalpy (\( \Delta H \)) and entropy (\( \Delta S \)) of the reaction, of mucin and drugs complex are important to confirm binding modes. The temperature-dependence of the binding constant was studied at 296, 303, and 310 K and was calculated from the following Van’t Hoff equation [14]:

\[
\Delta G = -RT \ln K_A
\]

\[
\Delta S = \frac{\Delta H - \Delta G}{T}
\]

From Table 3 it can be seen that for mucin-theophylline complex both \( \Delta H \) and \( \Delta S \) have a positive value. For this drug–protein interaction, positive entropy is frequently taken as evidence for hydrophobic interaction, but it has been pointed out that positive entropy may also be a manifestation of electrostatic interaction. Conversely, it can be seen that for mucin-prednisolone and mucin-cephalexin complexes both \( \Delta H \) and \( \Delta S \) have a negative value. This indicates that van der Waals interactions and hydrogen bonds may play a major role in the binding [17]. The negative sign for \( \Delta G \) means that the binding process is spontaneous.

3.8. Energy transfer

FRET (Fluorescence Resonance Energy Transfer) is a simple method to measure the distance between protein and drug [19]. According to Förster’s non-radiative energy transfer theory, energy efficiency \( E \), critical energy- transfer distance \( R_0 \) (E= 50%), the energy donor and the energy acceptor distance \( r \) and the overlap integral between the fluorescence emission spectrum of donor and the absorption spectrum of the acceptor \( J \) can be calculated by the following equations [14]:

\[
E = 1 - \left( \frac{r}{R_0} \right)^6 = \frac{n_0^2}{n_d^2 + r^2}
\]
The overlaps of emission spectra of mucin and absorption spectra of theophylline (A), prednisolone (B) and cephalexin (C) at 296 K.

\[
R_0^D = 8.79 \times 10^{-5} \left[ \kappa^2 n^4 \Phi \right] \tag{9}
\]

\[
J = \frac{\int \int \Phi(\lambda) \epsilon(\lambda) \Delta \lambda}{\sum \Phi(\lambda) \epsilon(\lambda) \Delta \lambda} \tag{10}
\]

where \(\kappa^2\) is the orientation factor, \(\Phi\) is the fluorescence quantum yield of the donor, \(N\) is the refractive index of the medium, \(\Phi(\lambda)\) is the fluorescence intensity of the donor at wavelength \(\lambda\) and \(\epsilon(\lambda)\) is the molar absorption coefficient of the acceptor at wavelength \(\lambda\). In this case, \(k^2=2/3, N=1.336\) and \(\Phi=0.118\) [11].

The overlaps of emission spectra of mucin and absorption spectra of theophylline, cephalaxin and prednisolone at 296 K were obtained (Fig.7).

Using the data equations, \(J, E, R_0\) and \(r\) were obtained for every interaction and they were reported in Table 4.

<table>
<thead>
<tr>
<th>J (cm³ L mol⁻¹)</th>
<th>E (%)</th>
<th>R₀ (nm)</th>
<th>r (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1.91 \times 10^{11}</td>
<td>0.96</td>
<td>0.85</td>
<td>0.5</td>
</tr>
<tr>
<td>B 2.51 \times 10^{13}</td>
<td>0.93</td>
<td>1.93</td>
<td>1.26</td>
</tr>
<tr>
<td>C 3.10 \times 10^{12}</td>
<td>0.92</td>
<td>1.36</td>
<td>0.90</td>
</tr>
</tbody>
</table>

The distance \(r<7\) nm indicates that the energy transfer between protein and drugs occurred with a high probability. [11, 20]

4. Conclusions

In this paper, the interaction of mucin with three different classes of drugs (theophylline, prednisolone and cephalaxin) was investigated at different temperatures by different spectroscopic methods.

UV–Vis spectroscopy showed that all the three investigated drugs can bind to mucin to form a protein-drug complex. Fluorescence data proved that mucin fluorescence can be quenched by the studied drugs and that the quenching is governed by a static quenching for mucin and cephalaxin interaction. According to thermodynamic parameters (positive \(\Delta H\) and \(\Delta S\) value) hydrophobic forces played a major role in the binding process between mucin and theophylline, while the hydrogen bonds and van der Waals forces may play a major role in stabilizing mucin-prednisolone and mucin-cephalexin complex.

The evaluation of the equilibrium association (\(K_a\)) and dissociation (\(K_d\)) constants was obtained by two different methods (comparable to each other) at different temperatures.

The data showed that temperature does not influence the formation of mucin-theophylline and mucin-prednisolone complex, while it can influence the interaction between mucin and cephalaxin. However, the difference in the \(K_a\) and \(K_d\) values observed at different temperatures is not due to degradation of mucin, that results thermally stable at the temperatures used in the experiment. [21]

The obtained results on the investigation of mucin-drugs interaction can facilitate the interpretation of absorption and distribution process of the drugs used in CF and could be helpful in the future to explain the structure-activity relationship (SAR) of new therapeutic molecules to identify mucin as a therapeutic target.

5. Acknowledgements

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6. References


