Research Article

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Comparison of pendimethalin binding properties of serum albumins from various mammalian species

Çeşitli memeli türlerinden serum albüminlerinin pendimetaline bağlanma özelliklerinin karşılaştırılması

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Abstract

Background: To investigate the interaction of pendimethalin (PM), a commonly used herbicide, with various mammalian serum albumins.

Methods: The interactions of PM with serum albumins of bovine (BSA), sheep (SSA), porcine (PSA) and rabbit (RbSA) were studied using fluorescence quenching titration and site marker displacement experiments.

Results: A comparison of the PM-induced quenching of the fluorescence of these albumins with that published for human serum albumin (HSA) showed similarity between BSA and HSA. The PM binding affinity of these albumins was found to follow the order: SSA > BSA > RbSA > PSA. Warfarin (WFN) displacement results also suggested similar displacing action of PM on WFN-BSA complex, when compared to the published results on WFN-HSA complex.

Conclusion: The results suggested close similarity between BSA and HSA in terms of PM binding characteristics and hence bovine can be selected as a suitable animal model for further toxicological studies of PM.

Keywords: Pendimethalin; Mammalian serum albumins; Fluorescence quenching; Warfarin displacement; Ligand-protein interaction.

Öz

Amaç: Yaygın olarak kullanılan bir herbisit olan pendimethalin (PM) ile çeşitli memeli serum albüminlerinin etkileşimini araştırmak.

Gereç ve Yöntem: PM’nin sığır (BSA), koyun (SSA), domuz (PSA) ve tavşan (RbSA) serum albüminleri ile etkileşimleri floresan söndürme titrasyonu ve yer işareti deplasman deneyleri kullanılarak incelenmiştir.

Bulgular: Bu albüminlerin PM kaynaklı floresan söndürmesi ile insan serum albümin (HSA) için yayılanmiş değerlerin karşılaştırılması, BSA ve HSA arasında benzerlik olduğunu gösterdi. Bu albüminlere PM bağlanma aфинitesinin sırı takip ettiği bulunmuştur: SSA > BSA > RbSA > PSA. Varfarin (WFN) deplasman sonuçları, WFN-HSA kompleksi üzerine yayınlanmış sonuçlarla karşılaştırıldığında, WFN-BSA kompleksi üzerinde PM’nin benzer yer değiştirme aktivitesini ortaya koymuştur.

Sonuç: Sonuçlar, PM bağlanma özellikleri açısından BSA ve HSA arasında yakın benzerlik olduğunu ve dolayısıyla sığırın, PM’nin ileri toksikolojik çalışmalar için uygun bir hayvan modeli olarak seçilebileceğini göstermiştir.

Anahtar Kelimeler: Pendimethalin; Memeli serum albümleri; Floresan söndürme; Varfarin yer değiştirmesi; Ligand-protein etkileşimi.
Introduction

Pendimethalin (3,4-dimethyl-2,6-dinitro-N-pentan-3-ylamine, PM) belongs to a class of dinitroanilinic herbicides and is being used to protect food crops (Figure 1). Its use in agriculture is widespread and is approved in most countries. However, PM is also known for its adverse effects towards various living organisms, which include soil fauna and fishes [1–4]. The degradation of PM in soil has been described as a slow process with a half-life period of 78–111 days [5]. The common use of PM in growing crops and its persistency in the soil contribute to soil and water contamination, through which it gets access to human and animal systems [6, 7]. Environmental Protection Agency of the United States of America (US-EPA) has classified PM as a Group C possible human carcinogen [8]. Liver enlargement and hepatic lesions have been noticed in dogs upon oral administration [9]. Toxicity studies of PM have shown increased level of reactive oxygen species in Chinese hamster lung fibroblast, V79 cells [10]. PM has also been found genotoxic to V79 cells as well as human peripheral lymphocytes [10]. Increased levels of antioxidant enzymes in rats exposed to PM have also indicated cellular toxicity of PM [11].

The toxicological effects of PM on the human system can be predicted from the animal model study, which may provide useful information on the compound’s toxicity [12, 13]. This makes the selection of an animal model an important subject. In principle, this selection is based on the similarity of the toxicokinetics/toxicodynamics of PM, including its transport in the blood circulation, between animal and human systems [14]. Most of the exogenous compounds including toxins are being transported in the circulation through serum albumin, which carries them to different target organs for further metabolism [15]. Therefore, it is imperative to check the similarity in the transport of any ligand in the blood circulation of human and animal systems [16]. Recently, we have published data on the PM binding characteristics of human serum albumin (HSA) [17]. However, results on PM binding to the mammalian albumins are lacking. In view of this, present study was undertaken and binding analysis of PM with serum albumins of different mammalian species was made. Here, we present our results on the binding characteristics of PM towards serum albumins of bovine (BSA), porcine (PSA), sheep (SSA) and rabbit (RbSA), using fluorescence spectroscopy as well as warfarin displacement experiments.

Materials and methods

Materials

Different serum albumins, viz. BSA (Lot # 011M7406V), SSA (Lot # 117K7540), PSA (Lot # 084K7636) and RbSA (Lot # 105K7565) were obtained from Sigma-Aldrich Co., (St. Louis, MO, USA). PM and warfarin (WFN) were also supplied by Sigma-Aldrich Co., (St. Louis, MO, USA). Other chemicals used were of analytical grade quality.

Sample preparation

Serum albumin stock solutions were prepared by dissolving ~22 mg of lyophilized powder in 10 mL of 60 mM sodium phosphate buffer, pH 7.4. The protein concentrations of different stock solutions were determined spectrophotometrically on a Shimadzu UV-2450 double beam spectrophotometer (Shimadzu Corp., Kyoto, Japan), using molar absorption coefficients at 280 nm, i.e. 43,827 M⁻¹·cm⁻¹ for BSA, 43,385 M⁻¹·cm⁻¹ for both PSA and RbSA and 42,925 M⁻¹·cm⁻¹ for SSA [18].

PM and WFN stock solutions were prepared by dissolving a known amount of these compounds in a fixed volume of absolute ethanol. The stock solutions were diluted before use to the desired concentrations, using 60 mM sodium phosphate buffer, pH 74.

PM-albumin titration

Binding of PM to different serum albumins was studied in the same way as described earlier [17], by titrating a fixed amount of serum albumin (3 μM) with increasing concentrations (0.9–4.5 μM with 0.9 μM intervals) of PM. The total volume of the mixture in each tube was made to 3.0 mL with 60 mM sodium phosphate buffer, pH 74. The fluorescence spectra were recorded after 1 h incubation at room temperature, in the wavelength range, 310–400 nm,
after exciting the samples at 295 nm. Jasco FP-6500 spectrofluorometer (Jasco Corp., Tokyo, Japan) was used for fluorescence measurements using quartz cuvette of 1 cm path length.

**Analysis of the titration data**

The fluorescence data, obtained above were subjected to the inner filter effect correction, as suggested by Lakowicz [19]:

$$F_{\text{cor}} = F_{\text{obs}} \times e^{-\left(A_{\text{ex}}+A_{\text{em}}\right)/2}$$  \hspace{1cm} (1)

where $F_{\text{cor}}$ and $F_{\text{obs}}$ refer to the values for the corrected and the observed fluorescence intensity, respectively, $A_{\text{ex}}$ and $A_{\text{em}}$ are the absorbance values at the excitation (295 nm) and the emission wavelengths (310–400 nm), respectively.

Values of the Stern-Volmer constant, $K_{sv}$, were obtained by treating the fluorescence quenching data according to the following equation [19].

$$F_0 / F = K_{sv} [Q] + 1 = k_q \tau_p [Q] + 1$$  \hspace{1cm} (2)

where $F_0$ and $F$ are the fluorescence intensity values of the serum albumin, obtained in the absence and the presence of the quencher (PM), respectively, $[Q]$ refers to the quencher concentration, $k_q$ the bimolecular quenching rate constant and $\tau_p$ is the fluorescence lifetime of serum albumins, taken as $10^{-8}$ s [19].

The double logarithmic plot, based on the following equation [20] was used to determine the values of the association constant, $K_a$.

$$\log \left( F_0 / F = n \log K_a - n \log \left[ 1 / ([L]_0 - (F_0 - F)[P]_0) / F_0 \right] \right)$$  \hspace{1cm} (3)

where $[L]_0$ and $[P]_0$ are the total ligand and protein concentrations, respectively.

The Gibbs free energy change ($\Delta G$) of the binding reaction was obtained from the following equation, using the values of the gas constant, $R$ and absolute temperature, $T$ as 8.3145 J⋅mol$^{-1}$⋅K$^{-1}$ and 298 K, respectively.

$$\Delta G = -RT \ln K_a$$  \hspace{1cm} (4)

**Warfarin displacement studies**

Equimolar concentrations (3 μM each) of WFN, Sudlow’s site I marker and albumin were incubated for 1 h at room temperature (25°C). Increasing concentrations (0–4.5 μM with 0.9 μM intervals) of PM were then added to WFN-albumin mixture and the fluorescence spectra were recorded in the wavelength range, 360–480 nm after 1 h incubation at room temperature, using an excitation wavelength of 335 nm.

**Results and discussion**

**Fluorescence quenching titration**

The fluorescence quenching titration results of different serum albumins, i.e. BSA, SSA, PSA and RbSA, obtained in the presence of increasing PM concentrations are shown in Figure 2A–D. Quantitative differences in the values of the fluorescence intensity, obtained with these albumins can be ascribed to the number of tryptophan (Trp) residues in these proteins, that contribute towards the fluorescence intensity upon excitation at 295 nm [19]. BSA, SSA and PSA with two Trp residues produced higher fluorescence intensity (Figure 2A–C, Table 1) compared to RbSA (Figure 2D, Table 1), which possesses single Trp residue [21]. On the other hand, the emission maxima of these albumins were found to fall in the range, 341–345 nm, characteristic of Trp residue(s) (Table 1) [22].

As evident from Figure 2A–D, addition of PM to these albumins produced quenching in the fluorescence intensity in a concentration dependent manner. Such quenching was suggestive of the binding of PM to these albumins, as similar quenching was observed with the binding of many ligands including PM to serum albumins [17, 23–27]. Values of the fluorescence intensity at the emission maxima, obtained at different PM concentrations were transformed into the relative fluorescence intensity, as described earlier [28]. Figure 3 shows change in the relative fluorescence intensity at the emission maxima with increasing PM concentrations, obtained with different serum albumins. All these albumins showed gradual decrease in the fluorescence intensity with increasing PM concentrations. However, a comparison of the quenching pattern, obtained with these albumins suggests lesser extent of the fluorescence quenching with PSA and RbSA compared to that observed with SSA and BSA at the highest PM/albumin molar ratio (1.5:1). Quantitatively, about 51% and 36% quenching was observed with SSA and BSA, respectively, compared to RbSA and PSA, showing 26% and 19% quenching, respectively (Figure 3, Table 1). Interestingly, magnitude of the fluorescence quenching characteristics of BSA (36%) was found similar to that observed with HSA (39%) [17].
Binding characteristics of PM-albumin interaction

Dynamic or static quenching mechanisms seem to be responsible for the ligand-induced quenching of the protein fluorescence. Molecular collisions between the fluorophore and the quencher may lead to dynamic quenching, while static quenching is resulted from the complex formation between the fluorophore and the quencher [19]. In order to characterize PM-induced quenching, fluorescence quenching data were treated according to Eq. 2 and the \( K_{SV} \) values were obtained (Table 2) from the slope of the linear Stern-Volmer plots (Figure 4A). In line to the quenching pattern (Table 1), SSA and BSA showed higher \( K_{SV} \) values compared to those obtained with PSA and RbSA (Table 2). The values of the bimolecular quenching rate constant, \( k_q \), as obtained from the \( K_{SV} \) values had fallen in the order of \( 10^{13} \text{ M}^{-1} \cdot \text{s}^{-1} \) for SSA and BSA, and \( 10^{12} \text{ M}^{-1} \cdot \text{s}^{-1} \) for RbSA and PSA. Values of \( k_q \) higher than \( 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1} \) (diffusion-controlled limit) simply reflected complex formation between the ligand and the protein [19]. Thus, it appears that PM-induced

**Table 1:** Fluorescence characteristics of different mammalian serum albumins in the absence and presence of PM.

<table>
<thead>
<tr>
<th>Albumin</th>
<th>Emission maxima (nm)</th>
<th>Fluorescence intensity (a.u.)</th>
<th>% Quenching ([PM]/[albumin]=1.5:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA</td>
<td>341</td>
<td>378</td>
<td>50.9 ± 2.3</td>
</tr>
<tr>
<td>BSA</td>
<td>345</td>
<td>424</td>
<td>36.3 ± 2.6</td>
</tr>
<tr>
<td>RbSA</td>
<td>343</td>
<td>125</td>
<td>26.0 ± 1.6</td>
</tr>
<tr>
<td>PSA</td>
<td>343</td>
<td>384</td>
<td>19.4 ± 1.6</td>
</tr>
</tbody>
</table>

PM, pendimethalin; SSA, sheep serum albumin; BSA, bovine serum albumin; RbSA, rabbit serum albumin; PSA, porcine serum albumin.
fluorescence quenching might have resulted from static quenching mechanism, which supported the complex formation between PM and serum albumins.

Binding affinity of PM towards different serum albumins was determined by analyzing the fluorescence quenching data according to Eq. 3. The values of the association constant, $K_a$, for different PM-albumin systems were obtained from the resulting double logarithmic plots (Figure 4B) and are given in Table 2. Based on the PM binding affinity, these albumins can be arranged in the order: SSA $>$ BSA $>$ RbSA $>$ PSA. Although the sequence and protein characteristics of these albumins show high degree of similarity [29], binding affinity of ligands varies considerably among the species according to the degree of phylogenetic relationship [30]. Such species differences in ligand-albumin interaction have been reported earlier [26, 31–34]. However, the binding affinity of BSA (Table 2) was revealed to be similar to the binding affinity of HSA [17]. The Gibbs free energy change ($\Delta G$) of the binding reaction was calculated using Eq. 4 and the negative values of $\Delta G$ for all PM-albumin systems (Table 2) suggested that the binding reactions were energetically feasible [35].

### Table 2: Binding parameters for the interaction between PM and different serum albumins.

<table>
<thead>
<tr>
<th>Albumin</th>
<th>$K_{sv}$ (M$^{-1}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$\Delta G$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA</td>
<td>$(2.44 \pm 0.04) \times 10^5$</td>
<td>$(3.27 \pm 0.24) \times 10^5$</td>
<td>$-31.5$</td>
</tr>
<tr>
<td>BSA</td>
<td>$(1.29 \pm 0.14) \times 10^5$</td>
<td>$(1.71 \pm 0.15) \times 10^5$</td>
<td>$-29.9$</td>
</tr>
<tr>
<td>RbSA</td>
<td>$(8.10 \pm 0.14) \times 10^4$</td>
<td>$(9.74 \pm 0.31) \times 10^4$</td>
<td>$-28.5$</td>
</tr>
<tr>
<td>PSA</td>
<td>$(5.57 \pm 0.30) \times 10^4$</td>
<td>$(8.88 \pm 0.08) \times 10^4$</td>
<td>$-28.2$</td>
</tr>
</tbody>
</table>

$K_{sv}$, Stern-Volmer constant; $K_a$, association constant; $\Delta G$, Gibbs free energy change; SSA, sheep serum albumin; BSA, bovine serum albumin; RbSA, rabbit serum albumin; PSA, porcine serum albumin.

**WFN displacement results**

Warfarin is a well-defined marker that binds to HSA at site I, located in subdomain IIA [36]. Therefore, it is commonly used in characterizing the binding site of ligands on serum albumins [30]. As PM was found to bind to site I of HSA, based on site marker displacement and molecular modeling study [17], a comparison of the WFN displacing action of PM on the complexes of WFN with other serum albumins was made. Spectrum 1 in Figure 5 shows the fluorescence spectrum of WFN-SSA complex,

**Figure 5:** Fluorescence spectra of WFN-SSA complex with increasing PM concentrations. [WFN] = [SSA] = 3 μM, [PM] = 0–4.5 μM with 0.9 μM intervals (1–6), $\lambda_{ex} = 335$ nm, $T = 25^\circ$C. The spectra labeled as ‘a’, ‘b’, ‘c’ and ‘d’ refer to the fluorescence spectra of WFN, SSA, PM and PM-SSA mixture, respectively.
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when excited at 335 nm. The spectrum was characterized by the presence of an emission maxima at 383 nm. Addition of increasing concentrations of PM displaced WFN from its binding site, as reflected from the progressive decrease in the fluorescence intensity with increasing PM concentrations (spectra 2–6 in Figure 5). In this range of wavelength, the fluorescence signals produced by SSA, PM and PM-SSA mixture (spectra 'b’–’d’ in Figure 5) were insignificant. Even free WFN (spectrum 'a' in Figure 5) produced relatively weaker signal at 383 nm compared to WFN-SSA complex. WFN displacement results, obtained with other serum albumins were qualitatively similar to those obtained with SSA, but showed significant differences in the extent of fluorescence quenching. This can be clearly seen from the decrease in the relative fluorescence intensity of different WFN-albumin (1:1) complexes at 383 nm with increasing PM concentrations, as shown in Figure 6. Values of the percentage fluorescence quenching of different WFN-albumin complexes induced by 4.5 μM PM are listed in Table 3. Complexes of WFN with BSA and RbSA showed close similarity to HSA in terms of percentage quenching, as 27% and 31% quenching were observed with BSA and RbSA, respectively, against 34% quenching observed with HSA [17]. On the other hand, SSA showed relatively higher degree (48%) of quenching, whereas a lesser degree (23%) of quenching was displayed by PSA.

Conclusion

Among the serum albumins of different mammalian species used in this study, BSA was found to be closely similar to HSA, as evident from the PM-induced fluorescence quenching pattern, binding affinity as well as WFN displacement results. Thus, bovine can be considered as a suitable animal model for further exploration on toxicological effects of PM.

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