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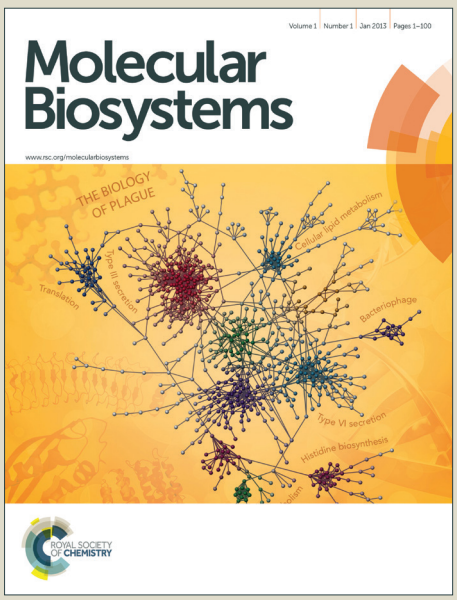

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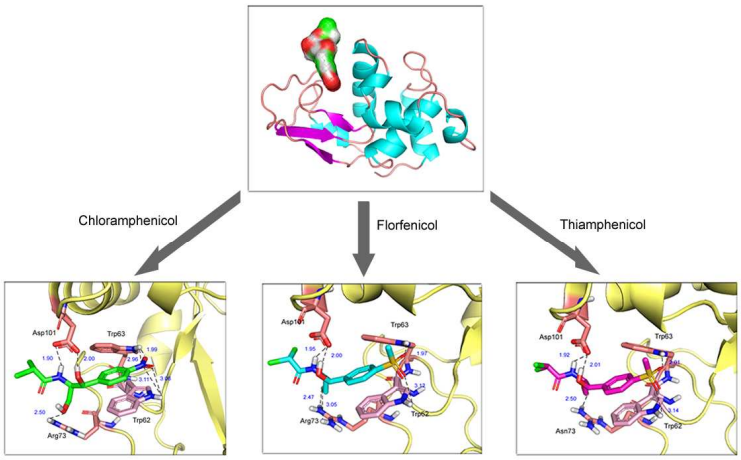
Graphic for Table of Contents

The molecular recognition of amphenicols by low-molecular-weight protein may have great impacts on the pharmacokinetics of amphenicols in the human body.



Renal Protein Reactivity and Stability of Antibiotic Amphenicols: Structure and Affinity

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ABSTRACT: In the present context, the molecular recognition of the oldest active amphenicols by the most popular renal carrier lysozyme was deciphered by using fluorescence, circular dichroism (CD) as well as molecular modeling at the molecular scale. The data of steady state fluorescence showed that the recognition of amphenicol with lysozyme yield fluorescence quenching by a static type, this corroborates time-resolved fluorescence that lysozyme-amphenicol adduct formation has a moderately affinity of 10^4 M^{-1}, and the driving forces were found to be chiefly hydrogen bonds, hydrophobic and π stacking. Far-UV CD spectra confirmed that the spatial structure of lysozyme was slightly changed with a distinct reduction of α-helix in the presence of amphenicol suggesting partial destabilization of the protein. Furthermore, via extrinsic 8-anilino-1-naphthalenesulfonic acid fluorescence spectral properties and molecular modeling, one could see that the amphenicol binding site was situated at the deep crevice on the protein surface, and ligand was also near to several crucial amino acid residues, such as Trp-62 and Trp-63 and Arg-73. Simultaneously, contrastive studies of protein-amphenicols revealed clearly that some substituting groups, e.g. nitryl in the molecular structure of ligands may possess vitally important for the recognition activity of amphenicols with lysozyme. Due to amphenicols connection with fatal detrimental effects and lysozyme has been applied as drug carriers for proximal tubular targeting, the discussion emerged herein is thereby necessary for rational antibiotic use, development of safe antibiotic and particularly a better appraisal of the risk associated with the human exposure of toxic agrochemicals.

KEYWORDS: *amphenicols, lysozyme, fluorescence, molecular modeling, structure and activity*

INTRODUCTION

The contamination of food/farm product by chemical contaminants is a globally public health suspense, and is a main reason of trade problems internationally. Contamination could arise by environmental pollution of the air, water and soil, such as the case with toxic metals, polychlorinated biphenyls and dioxins, or especially through the purposeful apply of varied compounds, e.g. pesticides and animal drugs.[1-4] In general, the use of antibiotics in livestock has been a mooted issue for many years, but antibiotics in livestock farming is necessary to assist avoid the broad and disastrous effects of diseases in herbs. Sometimes antibiotics have been appended to rear to facilitate growth. As Briggs has indicated,[5] human contact to antibiotics can take place in many different tracks, but food and drinking water are the most significant pathways of exposure for almost any antibiotics including amphenicols (structure shown in Fig. 1). This opinion held by Briggs was subsequently evidenced by certain studies, which endorsed that more than 90% of human exposure befalls mostly through foodstuffs, and those of animal source ordinarily account for nearly 80% of the total exposure.[6] However, pernicious effects are usually far from direct or apparent, owing to most of those antibiotics are seldom present in unduly large concentrations. Luckily, accumulating evidence strongly shows that low residues of the antibiotics may pile up in the fatty tissue, kidney and liver of animals and these are considered to be create several peril to human health.[7] Furthermore, the use of antibiotics such as amphenicols have plainly been doubted as one of the reasons of the

emersion of antibiotic resistant species of bacteria, although the most common root is drecky drug management in the handling of human health.[8,9] This in reverse sparks off human illness that can not be cured by traditional antibiotics.

Fig. 1 here about

It was apparent to all that the kidney plays a vital function in the human body's defense against potentially toxic xenobiotics and metabolic waste products through elimination routes. Particularly, secretory vectors in the proximal tubule are outstanding determinants of the treatment of xenobiotics, including many prescription drugs.[10,11] In accordance with a much recent notion proposed by Dolman et al.,[12] the effectiveness and toxicity of many clinically important drugs and poisonous environmental agents in the body is resolved by their interaction with the renal carriers system. As the proximal tubule is the main site of excretion, low-molecular-weight (LMW) proteins, especially lysozyme is the most famous renal transporter for the intracellular shipment of therapeutic compounds into proximal tubule cells.[13] In the kidney, the drug-LMW protein complex follows the same route as endogenous LMW protein and ends up in the lysosomes of the proximal tubular cells. Lysozyme is one of the proteins most delved in the drug-LMW protein investigations due to the wealth of data regarding it three-dimensional structure, folding and stability. It is an antibacterial enzyme present in a variety of organisms, which exercises its bacteriostatic role via hydrolyzing the β-1,4 linkage between *N*-acetylmuramic acid (NAM) and *N*-acetyl-glucosamine (NAG) of gigantic polymers $(NAM\text{-}NAG)_n$ in the peptidoglycan (murein), leading to lysis of Gram-positive

bacterial cell walls.[14] Body fluids, e.g. tears, saliva, urine and human milk comprise 2.6, 0.13, trace, and 0.2～0.4 mg mL^{-1} of lysozyme, respectively. The lysozyme from chicken egg white is structurally highly homologous to lysozyme human, whose diverse variants (Ile-56-Thr and Asp-67-His) from immense amyloid accumulates in the liver and kidneys of the individuals influenced by a hereditary systemic amyloidosis.[15] Therefore, lysozyme is frequently an essential target for toxicity because of its rich vehicle role and focused ability and has been advisedly used as drug transporters for proximal tubular targeting.

Currently, binding of a small drug molecule to a LMW protein is requested to alter the receptor's dynamics and conformations and affinities, aiming at governing the protein's functions. Concrete protein-ligand adducts can also be regulated by other small molecules that have similar physicochemical properties, begging for a quantitative estimation of binding specificity at the molecular level.[16] Furthermore, the intrinsic activity of the drug, together with the number of available coupling sites in the carrier macromolecule, determines and thereby also affects the essential amount of carrier protein. Thus both pharmacokinetic and biological parameters need to be taken into account when assessing a LMW protein-drug adduct.[17] For the above reasons, the scope of the present work was to scrutinize the complexation as well as the structure of the complexes formed between the dangerous antibiotic amphenicol and the LMW protein lysozyme, by means of steady state fluorescence, time-resolved fluorescence spectra and extrinsic 8-anilino-1-naphthalenesulfonic acid (ANS) probe. Moreover, the conformation of lysozyme after complexation was monitored by

circular dichroism (CD) and, in particular, two other amphenicols, that is thiamphenicol and florfenicol which are all powerful antibacterial compounds and developed in the United States for use exclusively in veterinary medicine, were chose for comparatively analyzing the binding mode between drug and lysozyme based on the computer-aided molecular modeling. Evidently, the study of protein-amphenicols could provide a better comprehension of the biointeraction happening between charged macromolecules and therefore giving perception into processes that occur in several biological systems.

EXPERIMENTAL

Materials. Lysozyme from chicken egg white (L4919, BioUltra, lyophilized powder, ⩾ 98%), chloramphenicol (C0378, 98%) and 8-anilino-1-naphthalenesulfonic acid (A1028, ⩾ 97%) were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification, and deionized water was generated by a Milli-Q Ultrapure Water Purification Systems from Millipore (Billerica, MA). Tris (0.2 M)-HCl (0.1 M) buffer of pH＝7.4, with an ionic strength 0.1 in the presence of NaCl, except where specified, was used, and the pH was measured with an Orion Star A211 pH Benchtop Meter (Thermo Scientific, Waltham, MA). Dilutions of the lysozyme stock solution (10 μM) in Tris-HCl buffer were prepared immediately before use, and the concentration of protein was determined by the method of Lowry et al.[18] All other reagents employed were of

analytical grade and received from Sigma-Aldrich.

Steady State Fluorescence. Steady state fluorescence was obtained on a F-7000 spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cell and a thermostatic cell holder with stirrer. The excitation and emission slits were set at 5.0 nm each, intrinsic fluorescence was recorded by exciting the continuously stirred protein solution at 295 nm to selectively excite the tryptophan (Trp) residues, and the fluorescence emission spectra were registered in the wavelength range of 300～450 nm at a scanning speed of 240 nm min^{-1}. The reference sample of the Tris-HCl buffer and chloramphenicol did not give any fluorescence signal.

Extrinsic ANS Displacement. In the first series of experiments, lysozyme concentration was kept fixed at 4.0 μM, and chloramphenicol/ANS concentration was varied from 5.0 to 35 μM, lysozyme fluorescence was gained (λ_{ex}＝295 nm, λ_{em}＝337 nm). In the second series of experiments, chloramphenicol was added to solutions of lysozyme and ANS held in equimolar concentration (4.0 μM), and the concentration of chloramphenicol was also varied from 5.0 μM to 35 μM, the fluorescence of ANS was recorded (λ_{ex}＝370 nm, λ_{em}＝465 nm).

Molecular Modeling. Molecular modeling of the lysozyme-amphenicols conjugation was conducted on SGI Fuel Workstation. The crystal structure of lysozyme (entry codes 4HP0),[19] determined at a resolution 1.19 Å, was retrieved from the Brookhaven Protein Data Bank. After being imported in the program Sybyl version 7.3, protein structure was carefully inspected for atom and bond type correctness assignment. Hydrogen atoms were computationally added using the Sybyl

Biopolymer and Build/Edit menus. To avoid negative acid/acid interactions and repulsive steric clashes, added hydrogen atoms were energy minimized with the Powell algorithm with a convergence gradient of 0.5 kcal (mol Å)$^{-1}$ for 1,500 cycles; this procedure does not change positions of heavy atoms, and the potential of the three-dimensional structure of lysozyme was assigned according to the AMBER force field with Kollman all-atom charges. The two-dimensional structures of amphenicols were downloaded from PubChem, and the initial structures of these molecules were generated by Sybyl 7.3. The geometry of amphenicols was subsequently optimized to minimal energy using the Tripos force field with Gasteiger-Hückel charges, the Surflex-Dock program that employs an automatic flexible docking algorithm was applied to calculate the possible conformation of the ligand that binds to the protein, and the program PyMOL (http://www.pymol.org/) was used for visualization of the molecular docking results.

Statistical Analysis. All assays were executed in triplicate, the mean values, standard deviations, and statistical differences were estimated using analysis of variance (ANOVA). The mean values were compared using student's *t*-test, and all statistic data were treated using the OriginPro Software (OriginLab Corporation, Northampton, MA).

RESULTS AND DISCUSSION

Reactive Ability. For the purpose of depicting the conjugation between the ligand

and the macromolecule, steady state fluorescence of quenching of Trp residues in lysozyme at pH＝7.4 with different amounts of chloramphenicol was shown in Fig. 2. Evidently, lysozyme exhibited a fluorescence emission peak at 337 nm, following an excitation at 295 nm, and the addition of chloramphenicol aroused a gradual reducing of the fluorescence signal. Under the experimental conditions, chloramphenicol behaved no fluorescence emission in the range 300～450 nm which did not affect lysozyme intrinsic Trp fluorescence. These phenomena illustrated clearly there were reactions between protein and chloramphenicol, and chloramphenicol located in the domain where Trp residue situated within or near the fluorophore.[20] Similar finding has also been announced by Agudelo et al.[21] for the binding of chitosan nanoparticles to milk β-lactoglobulin.

Fig. 2 here about

As noted earlier, binding of ligands to proteins is specially vital as it can influence the distribution and elimination of the ligand as well as the duration and strength of its pharmacological and toxicological action, thus it is necessary to grasp the affinity of chloramphenicol to lysozyme. The inset of Fig. 2 indicates the plot of $\log(F_0-F)/F$ *versus* $\log[Q]$ according to equation (3) (Supporting Information) for the chloramphenicol-lysozyme mixture from fluorescence titration, the calculated association constant fitted from Fig. 2 was $K=2.234\times10^4$ M^{-1} ($R=0.9991$) and the value of n is approximately equal to 1. From the point of sight of Kragh-Hansen,[22] and combined with the recent publications on the theme of protein-ligand, such as alkanone flavors, α-lactalbumin, quercetin, quercitrin, retinol, retinoic acid, retinal,

β-carotene, α-carotene and β-cryptoxanthin,[23-27] it is quite obvious that the biointeraction of chloramphenicol with lysozyme belongs to moderate affinity with respect to the other strong protein-ligand complexes with affinities ranging from 10^6 to 10^8 M^{-1}. As has been argued, the reabsorptive and secretory duties of the renal tubule are operated by a variety of membrane carriers located in the basolateral and luminal membranes of the tubular epithelium. If a diminish in the binding of a drug to a transporter can yield a growth in the amount of distribution of the drug when the volume of allocation is computed as the ratio of the quantity of drug in the body to the drug concentration in the target organ.[28] Thereby we believe that the free concentration of chloramphenicol in the kidney is so large when it enters the human body through food chain. This deduction may possibly be authenticated by some much earlier *in vivo* studies that chloramphenicol is quickly absorbed, inactivated and eliminated, and in patients who have normal renal function about 75 to 90 percent of the oral or parenteral treated drug is disposed in the urine.[29,30] Moreover, the chloramphenicol residues are cleared not only by glomerular filtration, but by active tubular secretion as well. We should pay more attention to this issue, because lysozyme is one of the most important LMW proteins that have been assayed as renal transporter systems for antibiotics delivery to proximal tubular cells. Meantime, the value of $n \approx 1$, which may explain the existence of just one kind of binding site in protein for chloramphenicol. Basically, intrinsic fluorescence of lysozyme is majorly owing to the Trp-62 residue,[31] from the value of n, chloramphenicol binding site in lysozyme is most likely near the Trp residue. And, based on the thermodynamic

equation: $\Delta G° = -RT\ln K$, we can also compute the Gibbs binding free energy $\Delta G° = -24.81$ kJ mol^{-1}, which implies that the formation of lysozyme-chloramphenicol adduct was an exothermic reaction.

Reactive Properties. To explain the nature of fluorescence quenching, the raw data were analyzed according to the well-known Stern-Volmer equation (2), and the corresponding results fitted from Fig. S1 (Supporting Information) was found to be $y = 0.01839x + 0.9803$ ($R = 0.9996$), $K_{SV} = 1.839 \times 10^4$ M^{-1} and $k_q = 9.941 \times 10^{12}$ M^{-1} s^{-1}. Intuitively, a linear Stern-Volmer plot (Fig. S1) is generally indicative of a single type of fluorophore, all equivalently accessible to quencher. The value of bimolecular quenching constant k_q is about 3 orders of magnitude larger than the maximum value for diffusion-controlled quenching in water ($\sim 10^{10}$ M^{-1} s^{-1}), so it obviously declares that some type of binding interaction happened between lysozyme and chloramphenicol. It is important to recognize that observation of a linear Stern-Volmer plot does not prove that collisional quenching of fluorescence has occurred, where the measurement of fluorescence lifetimes is the most definitive method to distinguish static and dynamic quenching. As can be seen from Table S1, Trp residues are known to signify multiexponential decays, we have not tried to appoint the individual components, on the contrary, the average fluorescence lifetime has been used in order to receive a qualitative analysis. The average fluorescence lifetime of lysozyme decreases from 1.85 ns to 1.66 ns, stating patently that the quenching of lysozyme fluorescence by chloramphenicol is probably static quenching.[32]

Circular Dichroism. As is well known, binding of a ligand to proteins or catalyze typically induces alteration to their three-dimensional structure in most cases, then a realization of structure, in turn, is essential to the discussion of function joins fundamental with the protein-ligand affinity.[33] To analyze the structural changes of lysozyme quantitatively, the raw CD spectra of the protein in the absence and presence of chloramphenicol were shown in Fig. S2, and secondary structure components based on CD data were listed in Table S2. Obviously, the CD curves exhibited two negative peaks in the far-UV CD region at 208 nm and 222 nm, characteristic of a α-helical structure of protein. A reasonable explanation is that the negative peaks between 208 and 209 nm and 222 and 223 nm are both contributed by the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of amide groups, and thereby are influenced by the geometries of the polypeptide backbones.[34] Table S2 shows that free lysozyme contains 42.1% α-helix, 17.6% β-sheet, 14.4% turn and 25.9% random coil, upon complex with chloramphenicol, diminution of α-helix was observed from 42.1% α-helix free lysozyme to 34.3% complex, while increase in β-sheet, turn and random coil from 17.6%, 14.4% and 25.9% free lysozyme to 19.3%, 17.2% and 29.2% complex. The decline of α-helix with an increase in β-sheet, turn and random coil revealed that chloramphenicol complexes with the amino acid residues of the polypeptide chain, i.e. the biological interactions of amphenicol with LMW protein evoked spatial structural destabilization in lysozyme conformation, which can probably be correlated with its physiological function.[35,36]

Hydrophobic ANS Probe. The goal of executing ANS studies was to further

confirm the essence of the chloramphenicol recognition by lysozyme. According to the protocol, binding studies were executed in the presence of ANS under identical conditions, and the relative fluorescence (F/F_0) *against* ligand concentration ([Ligand]) plots is denoted in Fig. S3. Evidently, at a ligand concentration of 45 μM, both chloramphenicol and ANS had a similar quenching effect on lysozyme fluorescence, i.e. chloramphenicol could quench about 71.57%, and ANS approximately 76.86%. Nevertheless, when chloramphenicol is added to the ANS-lysozyme complex, the fluorescence of ANS-lysozyme missed nearly 21.76%. Stryer[37,38] has described that interaction of ANS with the solvent exposed hydrophobic clusters of proteins leads to a considerable augment of ANS fluorescence intensity. At present, the fluorescence intensity of ANS-lysozyme disappeared 21.76% upon the addition of chloramphenicol, indicating that chloramphenicol can compete against ANS moderately for its binding site, then the ANS-lysozyme fluorescence would decrease. This corroborates the following results of molecular modeling placing the chloramphenicol at the active site of lysozyme, which constituted by a deep crevice.

Molecular Modeling. To capture comprehensive insight into the conjugation between lysozyme and antibiotic chloramphenicol, we conduct computational studies and emphasize the important intermolecular interactions between rigid ligand and potential binding clefts on known crystal structure of lysozyme. Based on the high-resolution X-ray diffraction measurement of lysozyme, the active site of the LMW protein consists of a deep crevice, which divides the protein into two domains

linked by an α-helix. One domain (residues 40 to 85) comprises almost entirely of β-sheet structure, while the second domain (residues 89 to 99) is more helical.[39] The best energy pictures are shown in Fig. 3, it can be perceived that the chloramphenicol fills the groove on the surface of the receptor lysozyme (Fig. 3(A)), thereby the interaction of antibiotic chloramphenicol with lysozyme clearly belongs to the sphere of biochemical reaction on biomacromolecule surface. The nitrogen atom and the oxygen atom of the nitryl, the oxygen atom of the hydroxyl group-2, the hydrogen atom of the secondary amine, and the hydrogen atom of the hydroxyl group-1 in chloramphenicol can make seven hydrogen bonds with the hydrogen atom of the secondary amine in Trp-62 and Trp-63, the hydrogen atom of the amino group in Arg-73, and the oxygen atom of the carboxyl group in Asp-101 residues (Fig. 3(B)), the bond lengths are 3.11 Å, 3.06 Å, 2.96 Å, 1.99 Å, 2.50 Å, 1.90 Å and 2.00 Å, respectively. On the basis of surface modification of the active site in lysozyme (Fig. 3(C)), we discovered that the preferred conformation of chloramphenicol is located at the hydrophobic patch stably that is composed of Trp-62, Trp-63, Leu-75, Cys-76 and Ile-98 residues, indicating evident hydrophobic interactions worked between chloramphenicol and lysozyme. Furthermore, the plane surface of benzene ring in chloramphenicol is parallel to the plane of indole ring in Trp-62 residue perfectly, and the molecular distance between the heart of the benzene ring and the center of the indole ring is 3.10 Å, which illustrated the existence of tenacious π stacking between antibiotic chloramphenicol and LMW protein. In addition, the association affinity and Gibbs free energy of lysozyme-chloramphenicol adduct are $K = 2.344 \times 10^4$ M^{-1} and

$\Delta G° = -24.93$ kJ mol^{-1}, respectively, the experimental result ($K = 2.234 \times 10^4$ M^{-1} and $\Delta G° = -24.81$ kJ mol^{-1}) is immensely close to the theoretical value, authenticating the believability of the data on steady state fluorescence illumination.

Fig. 3 here about

Exploration of Lysozyme-Thiamphenicol/Florfenicol Adducts. Thiamphenicol and, more recently, florfenicol, which have molecular structures similar to that of chloramphenicol, appears to reserve the antibacterial properties, decrease strikingly the metabolism by the liver, promote kidney excretion, and eliminate the occurrence of aplastic anemia, although it is possibly more subject to evoke dose-dependent reversible depression of the bone marrow.[40-42] These properties make them preferably in certain cases to chloramphenicol. Commonly, thiamphenicol is proposed for the treatment and control of respiratory and intestinal diseases in cattle and poultry. It may also act as a reasonable replacement for other antibiotics that present long depletion times in aquaculture.[43] While florfenicol has been permitted for treatment of bovine respiratory disease in the United States, and has also been recently approved in Japan for use by the aquaculture industry to preclude yellowtail disease.[44] In order to further comprehend the binding mode between chloramphenicol and lysozyme and give some general clues to the amphenicols, thiamphenicol and florfenicol were selected and we have performed preliminarily comparative analyses regarding the recognition properties between the amphenicols and the LMW protein lysozyme by using computer-aided molecular modeling. The best docking energy results are shown in Fig. 4, and the definite noncovalent bonds and bond lengths/distances are collected

in Table 1. The binding modes of thiamphenicol (Fig. 4(A)) and florfenicol (Fig. 4(B)) are analogous to the chloramphenicol, that is, chloramphenicol and its structurally analogues, thiamphenicol and florfenicol are located within the trench, which is composed by residues 40 to 85 and residues 89 to 99. More specifically, thiamphenicol and florfenicol can yield hydrogen bonds with several central amino acid residues such as Trp-62, Trp-63, Arg-73 and Asp-101, but the intensity of hydrogen bonds is variant due to structural disparities in amphenicols. Further, the same binding location of amphenicols in LMW protein gives thiamphenicol and florfenicol and the indole ring in Trp-62 residue a guaranty to produce π stacking, and the hydrophobic interactions also existed between lysozyme and amphenicols at the same time.

Fig. 4 here about

Table 1 here about

In view of the molecular modeling described above, we could reasonably point out that there is an apparent discrepancy between amphenicols and lysozyme, and the sequence of binding free energy $\Delta G°$ in the molecular recognition was computed as follows: chloramphenicol > thiamphenicol > florfenicol, owing to the slight differences of substituting group in the molecular structure of amphenicols. The distinction can be deduced logically from both the noncovalent interactions and the substituent properties, as explained below. In contrast with the methylsulfonyl group in thiamphenicol and florfenicol, nitryl was present on benzene ring in chloramphenicol molecule. This event would allow chloramphenicol to yield more

strong hydrogen bonds with lysozyme, because the nitrogen atom of the nitryl could serve as a receptor for hydrogen bonds, and then noticeably enhances the noncovalent interactions between chloramphenicol and some amino acid residues such as Trp-62 and Trp-63. Therefore, the binding affinity of chloramphenicol to lysozyme is far stronger than thiamphenicol and florfenicol. Nevertheless, thiamphenicol and florfenicol have very similar chemical structures but have only different substituent species in the position-2; if the hydroxyl group in the position-2 of thiamphenicol is turned into fluorine atom, it changes to florfenicol. In the eyes of the number and strength of hydrogen bonds, both hydroxyl group and fluorine atom substitution had an important enhancement effects in the formation of hydrogen bonds. However, the hydroxyl group in the position-2 of thiamphenicol can produce hydrogen bond with amino acid residue Arg-73, but the intensity of hydrogen bond was partly less than that of the fluorine atom. Consequently, the most crucial factor for the toxicity of amphenicols to lysozyme is lying in the substituent kind on the benzene ring (Fig. 4(C)); in this case, chloramphenicol is the most toxic amphenicols known to human health. According to the above molecular modeling results, it is also particular to note that binding of both chloramphenicol and its analogues to lysozyme could induce change its congenital dynamics evidently. This phenomenon is obvious for grasping protein behavior better, but also has insinuations for the drug industry. Generally, drugs such as antibiotics are designed to rival their target protein, thereby convincing optimization of potential interactions between drug and protein, which makes the binding stronger. If the recognition also alters the dynamic characters of protein, this

change should be taken into account, as it influences the entropy of the protein as well.[45,46] This element is frequently disregarded in drug design process, which may generate serious side effects to human.

CONCLUSIONS

On the whole, renal transport systems play a crucial function in the defense against a vast variety of latently injurious chemicals to which we are continually exposed through drugs, environment, food and occupation. And the current story portrays an integrated experimental and computational modeling method of the biointeractions of the extensively food contaminant amphenicols with the most important carrier in the proximal tubule – lysozyme in aqueous solution at physiological pH＝7.4. Fluorescence pointed out that the quenching of Trp residues was the formation of the lysozyme-amphenicol complex, which coincides with the time-resolved fluorescence attesting the conclusion that static mechanism does appear to be predominant in this conjugation, and amphenicol binds to lysozyme reversibly through hydrogen bonds, hydrophobic and π stacking with a moderately affinity of 10^4 M^{-1}. The data of CD spectra proclaimed that the compactly spatial structure of lysozyme was obviously disturbed after the addition of amphenicol with a decrease of α-helix accompanied by an increase in β-sheet, turn and random coil, which stood for a partial disruption of protein. According to the results of extrinsic ANS displacement, one can ascertain amphenicol was located at the crevice on the surface of the protein,

this matches the molecular modeling placing amphenicol at the hydrophobic region, where Trp-62, Trp-63, Leu-75, Cys-76 and Ile-98 residues on the lysozyme and the Trp-62 and Trp-63 residues are all near to ligand. Through comparative analyses of the molecular recognition of chloramphenicol and its structurally analogues, that is thiamphenicol and florfenicol with lysozyme, we may reasonably understand that the reactivity between lysozyme and chloramphenicol was much higher than that of thiamphenicol and florfenicol. And, several key substituents, such as nitryl and methylsulfonyl group on the benzene ring in amphenicols may probably have a great influence on the toxicological action to human health.

ASSICIATED CONTENT

Supporting Information

Detailed experimental procedures for time-resolved fluorescence and circular dichroism of lysozyme and lysozyme-amphenicol, calculation of reactive ability, Stern-Volmer plot, the plot of extrinsic ANS displacement and the abbreviations used in this manuscript.

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NOTES

The authors declare no competing financial interest.

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Table 1

Noncovalent bonds analyses from the results of molecular modeling for lysozyme with amphenicols

Amphenicols	Noncovalent bonds	Ligands	Lysozyme	Distance (Å)
Chloramphenicol	Hydrogen bonds	$-\underline{N}O_2$	Trp-62: $-N\underline{H}$	3.11
		$-\underline{N}O_2$	Trp-62: $-N\underline{H}$	3.06
		$-\underline{N}O_2$	Trp-63: $-N\underline{H}$	2.96
		$-N\underline{O}_2$	Trp-63: $-N\underline{H}$	1.99
		$\underline{O}H$-2	Arg-73: $-N\underline{H}_2$	2.50
		$N\underline{H}$-3	Asp-101: $-CO\underline{O}$	1.90
		$O\underline{H}$-1	Asp-101: $-CO\underline{O}$	2.00
	π stacking	benzene ring (center)	Trp-62: indole ring (center)	3.10
Thiamphenicol	Hydrogen bonds	$-SO_2CH_3$: O-6	Trp-62: $-N\underline{H}$	3.14
		$-SO_2CH_3$: O-5	Trp-63: $-N\underline{H}$	2.01
		$\underline{O}H$-2	Arg-73: $-N\underline{H}_2$	2.50
		$N\underline{H}$-3	Asp-101: $-CO\underline{O}$	1.92
		OH-1	Asp-101: $-CO\underline{O}$	2.01
	π stacking	benzene ring (center)	Trp-62: indole ring (center)	3.10
Florfenicol	Hydrogen bonds	$-SO_2CH_3$: O-6	Trp-62: $-N\underline{H}$	3.12
		$-SO_2CH_3$: O-5	Trp-63: $-N\underline{H}$	1.97
		F-2	Arg-73: $-N\underline{H}_2$	2.47
		F-2	Arg-73: $-N\underline{H}_2$	3.05
		$N\underline{H}$-3	Asp-101: $-CO\underline{O}$	1.95
		$O\underline{H}$-1	Asp-101: $-CO\underline{O}$	2.00
	π stacking	benzene ring (center)	Trp-62: indole ring (center)	3.10

Figure Captions

Fig. 1. Molecular structures of chloramphenicol (A), thiamphenicol (B), and florfenicol (C).

Fig. 2. Fluorescence emission spectra of 4.0 μM lysozyme at λ_{ex}＝295 nm showing the quenching effect of increasing concentrations of chloramphenicol (a→h): 0, 5.0, 10, 15, 20, 25, 30 and 35 μM, (x) 35 μM chloramphenicol only. Spectra were recorded at pH＝7.4 and T＝298 K. The inset corresponds to reactivity plot describing lysozyme Trp quenching caused by chloramphenicol biointeraction. y＝1.02x＋4.349, R＝0.9991, based on equation (3). The λ_{em} maximum occurred at 337 nm and all data were corrected for quencher fluorescence. Each point was the mean of three independent observations±S.D. ranging 0.31%－3.77%.

Fig. 3. Molecular modeling of chloramphenicol docked to lysozyme (panel (A)), the colorized carbon skeleton model displays chloramphenicol, colored as per the atoms and possesses opaque surface of electron spin density. The key amino acid residues around chloramphenicol (green stick) have been implied in stick model, warm pink model exhibits hydrogen bonds (panel (B)) between Trp-62, Trp-63, Arg-73, Asp-101 residues and chloramphenicol; panel (C) illustrates hydrophobic interactions between Trp-62, Trp-63, Leu-75, Cys-76, Ile-98 residues and chloramphenicol (ball-and-stick

model); light pink model (panel (B)) expresses π stacking between Trp-62 residue and chloramphenicol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4. Molecular modeling of thiamphenicol (panel (A)) and florfenicol (panel (B)) docked to lysozyme. The critical amino acid residues around thiamphenicol (magenta stick) and florfenicol (cyan stick) have been manifested in stick model, warm pink stick model presents hydrogen bonds between Trp-62, Trp-63, Arg-73, Asp-101 residues and thiamphenicol and florfenicol, respectively; light pink stick model explains π stacking between Trp-62 residue and thiamphenicol and florfenicol, respectively; panel (C) interprets the superimposable conformations of chloramphenicol, thiamphenicol and florfenicol binding to lysozyme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

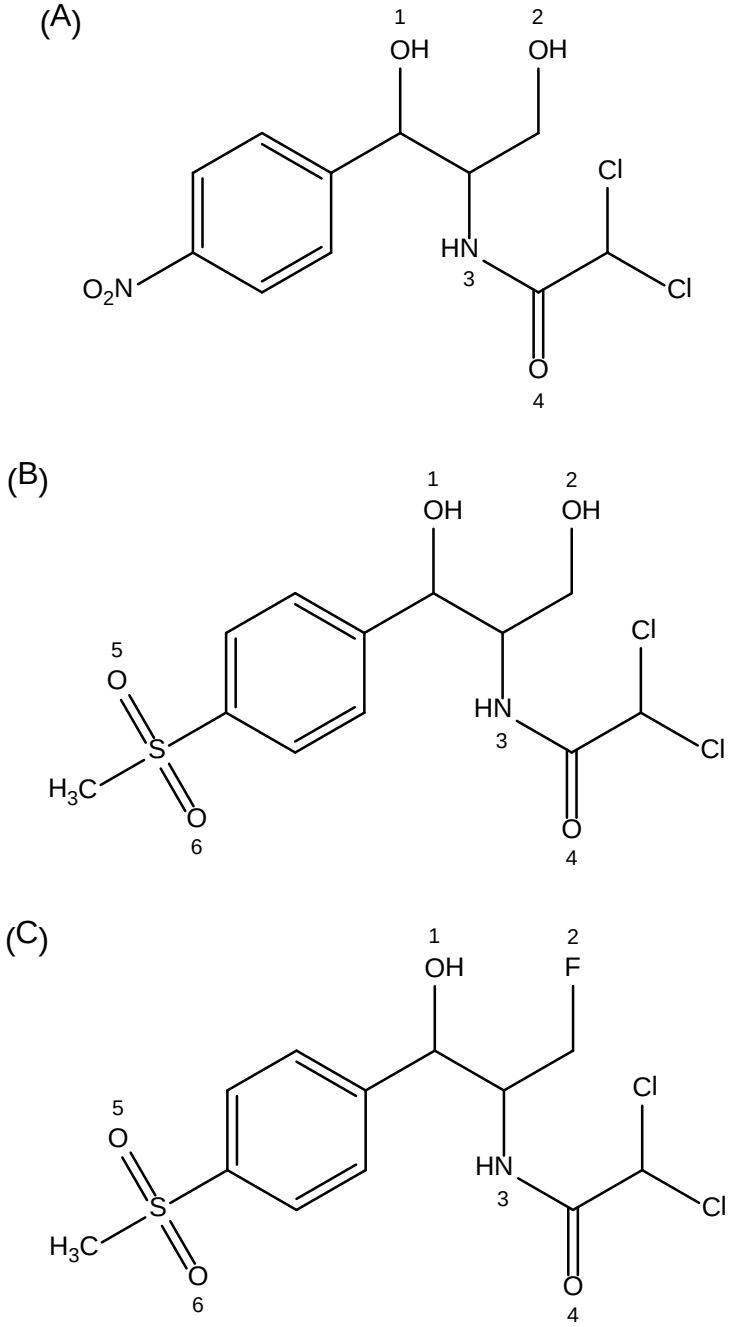

Fig. 1

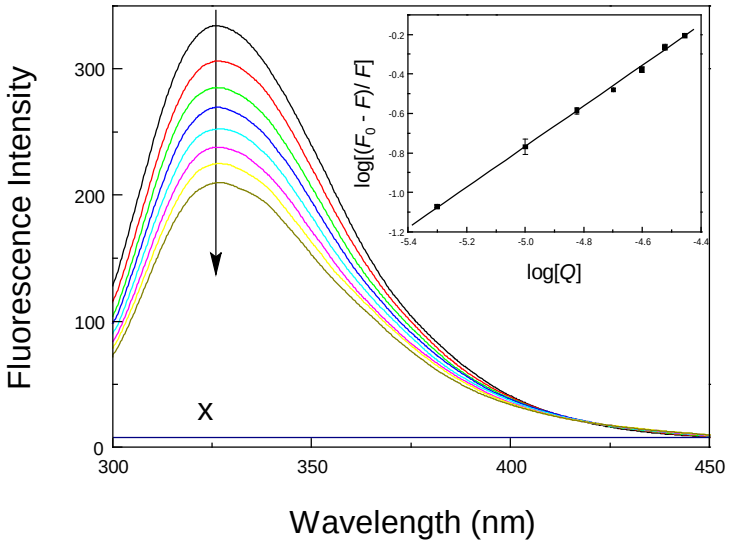

Fig. 2

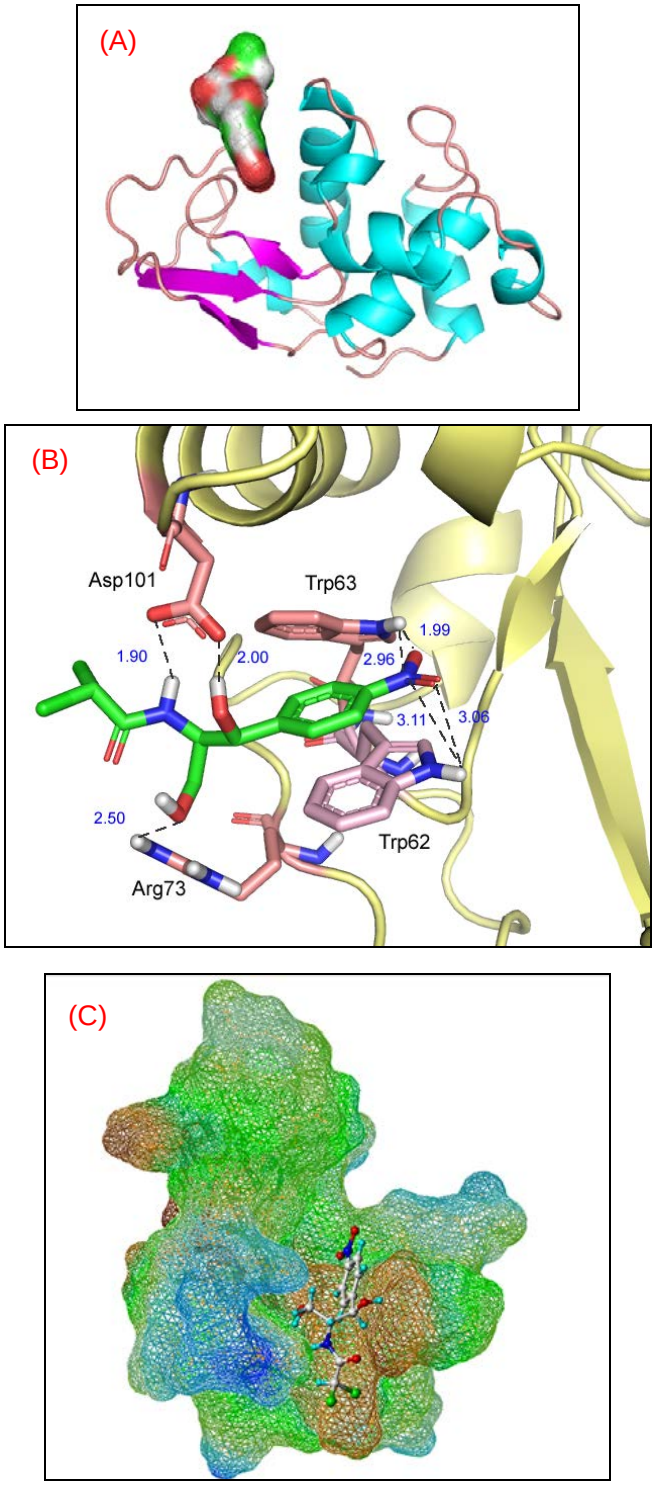

Fig. 3

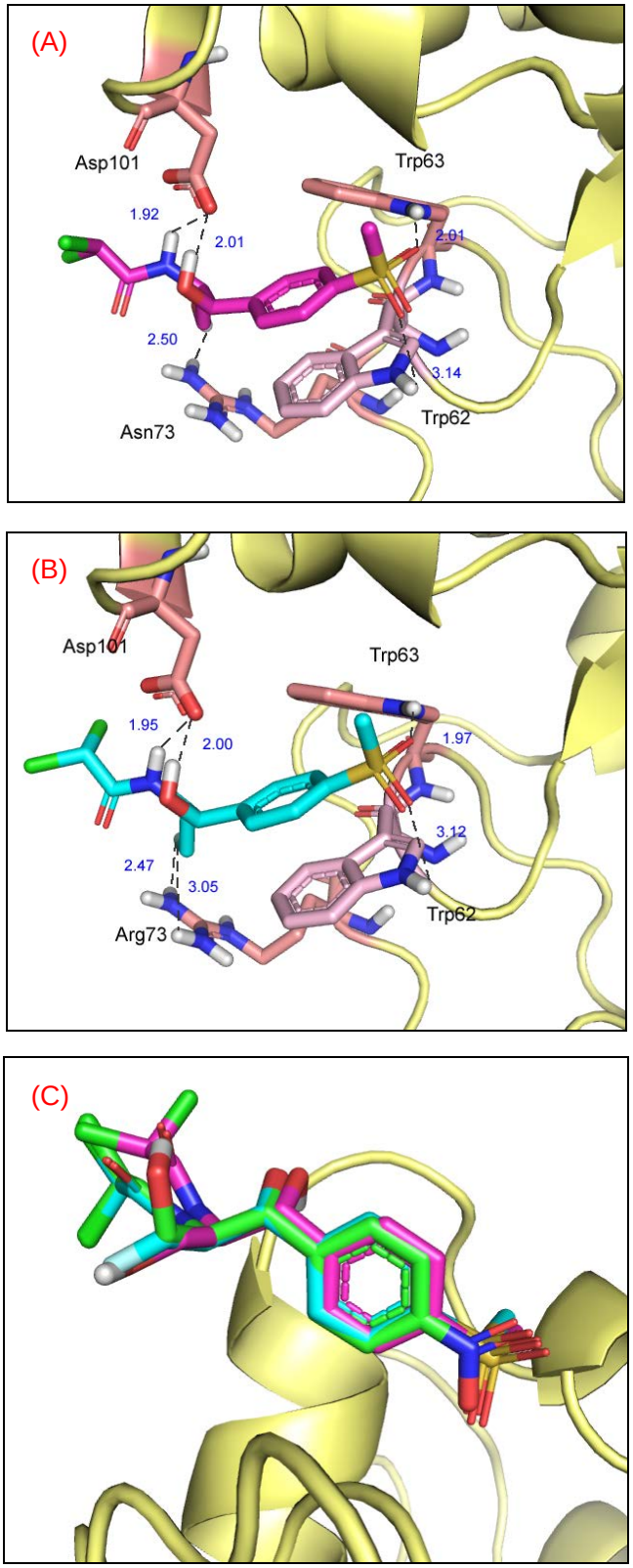

Fig. 4