http://dx.doi.org/10.5012/bkcs.2012.33.8.2731

Solubilization of Pyrimethamine, Antibacterial Drug, by Low-Molecular-Weight Succinoglycan Dimers Isolated from *Shinorhizobium meliloti*

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Received May 17, 2012, Accepted May 23, 2012

The use of pyrimethamine as antibacterial drug is limited by the poor solubility. To enhance its solubility, we prepared complexes of pyrimethamine with low-molecular-weight succinoglycan isolated from *Sinorhizobium meliloti*. Low-molecular-weight succinoglycans are monomers, dimers, and trimers of the succinoglycan repeating unit. The monomers and dimers were separated into their three species (M1, M2, and M3) and four fractions (D1 to D4) using chromatographic techniques, which were shown to be nontoxic. The solubility of pyrimethamine was markedly increased up to 42 fold by succinoglycan D3, where the level of its solubility enhancement was even 8-20 fold higher comparing with cyclodextrin or its derivatives. The complex formation of succinoglycan D3 with pyrimethamine was confirmed by ^{1}H nuclear magnetic resonance spectroscopy, Fourier-transform infrared spectroscopy, differential scanning calorimetry, scanning electron microscopy, and molecular modeling studies. Herein, we suggest that the low-molecular-weight succinoglycans may be utilized as highly effective solubilizers of pyrimethamine for pharmaceutical purposes.

Key Words : Pyrimethamine, Solubilization, Complexation, Succinoglycan

Introduction

Pyrimethamine, 5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidineamine is a common medication used for protozoan infection, such as malaria by *Plasmodium falciparum* (Fig. 1(a)).[1] It is a dihydrofolate reductase (DHFR) inhibitor of protozoan, and DHFR is an essential enzyme responsible for the conversion of folic acid into folinic acid during nucleic acid biosynthesis.[2,3] However, because of the low aqueous solubility of pyrimethamine, high doses are needed in order to sufficiently inhibit parasite proliferation.[4] Thus, methods to improve the aqueous solubility of pyrimethamine are needed. A well-known approach to increase the solubility of an insoluble drug is complexation with cyclodextrins (CD) and its derivatives.[5-10] In a previous study, pyrimethamine aqueous solubility was enhanced by the complexation with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) and α-cyclodextrin (α-CD).[4,9,10]

Succinoglycan, a symbiotically important exopolysaccharide, is required for establishment of the nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and its host plant *Medicago sativa* (alfalfa).[11] *Sinorhizobium meliloti* 1021 produces both high-molecular-weight (HMW) and low-molecular-weight (LMW) succinoglycan.[12] The LMW succinoglycan has several advantages over HMW succinoglycan, including its low viscosity and simpler structure.[13] The LMW succinoglycan is composed of monomers, dimers, and trimers of the octasaccharide unit.[14] It consists of a β-1,3, β-1,4, and β-1,6 linked octasaccharide subunit containing one galactose at the reducing end and seven glucose residues with succinyl, acetyl, and pyruvyl modifications (Fig. 1(b)).[12,14] Depending on the number of succinyl moieties in their structure, the monomers are purified into M1, M2, and M3 fractions, and the dimers are purified into D1, D2, D3, and D4 fractions.[12,15,16] In addition, we previously

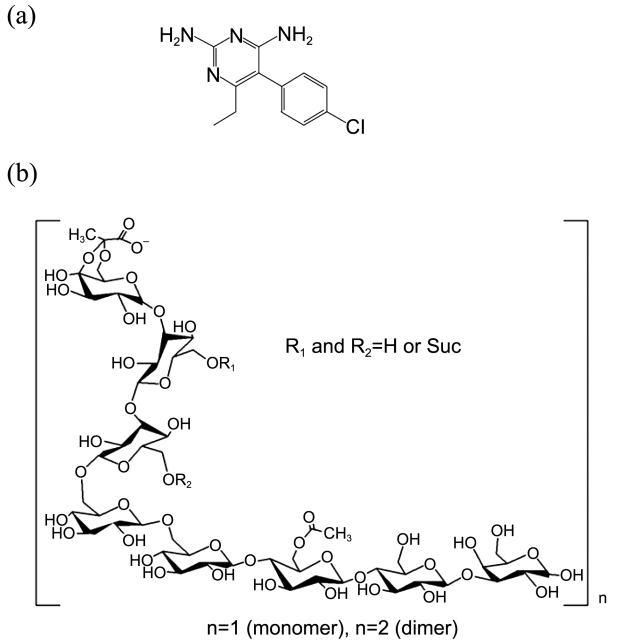


Figure 1. Chemical structures of pyrimethamine (a) and succinoglycan monomers and dimers (b).

demonstrated that succinoglycan dimers did not cause any significant cytotoxicity by the colorimetric MTS test (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) using a human cell line, HEK 293.[17]

Here, we first prepared the complexes of pyrimethamine with various types of succinoglycan monomers or dimers as solubilizers in order to see whether there is any succinoglycan that may improves the aqueous solubility of the drug. Two CD molecules, α-CD and HP-β-CD, were used as a control for complexation. Upon the comparison of their levels of solubility, it was demonstrated that among succinoglycan dimers (D1 to D4), D3 is the most effective solubilizer for pyrimethamine. It is possible that succinoglycan dimers form an effective complex with the hydrophobic pyrimethamine.[13] Furthermore, the complexation of pyrimethamine with D3 was confirmed by ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR), differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The results obtained using the above physicochemical characterization techniques suggest the successful complexation between pyrimethamine and succinoglycan D3.

Experimental

Chemicals. Pyrimethamine, α-cyclodextrin (α-CD) and 2-hydropropyl-β-cyclodextrin (HP-β-CD) were purchased from Sigma-Aldrich Chemicals Co (St. Louis, Mo, USA). Deuterium oxide (99.96 atom %D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Ethanol (HPLC grade) was purchased from Sigma-Aldrich Chemicals Co (St. Louis, Mo, USA). All aqueous solutions were prepared using ultra pure water (MILLI Q).

Bacterial Cultures and the Purification of LMW Succinoglycan. Isolation and purification of LMW succinoglycan produced by *Sinorhizobium meliloti* 1021 were carried out as described previously.[12,15] *Sinorhizobium meliloti* 1021 was grown in a glutamate-D-mannitol-salts (GMS) medium at 30 °C for 5 days. Cells were centrifuged at 8000 rpm for 15 min, and the supernatant containing the secreted exopolysaccharide was concentrated fivefold by rotary evaporation. After adding 3 volumes of ice-cold ethanol, the precipitate containing HMW succinoglycan was removed by centrifugation. The LMW succinoglycan in the supernatant was concentrated again and 7 volumes of ethanol was added. The precipitate was collected by centrifugation, and the sample succinoglycan monomers, dimers, and trimers were separated through size exclusion chromatography (Bio-Gel P6). The monomers (M1, M2, and M3) and dimers (D1, D2, D3, and D4) were further fractionated on a column (1.5 × 48 cm) of DEAE Sephadex A-25 (Sigma). Finally, the collected fractions were desalted and lyophilized. The structure of the obtained LMW succinoglycan was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) as described previously.[15,16]

Phase-Solubility Study. Phase-solubility assay was performed according to the method of Higuchi and Connors.[18] Pyrimethamine was dissolved in ethanol, and the ethanolic solution was added to aqueous solutions of M1 to M3 and D1 to D4 (0.0 to 1.2 mM). These vials were screw-capped, then sonicated for 10 min. The suspensions were magnetically stirred at 25 °C for 24 h, shielded from light to prevent degradation of the molecules. After equilibrium was reached, ethanol was evaporated using N_2 gas, and the mixture was lyophilized, to which 0.6 mL water was added with filtering (PVDF 0.2 μm filter, Whatman). The aliquot from each vial was analyzed using a spectrophotometer (UV 2450, Shimadzu Corporation) at 272 nm to evaluate the concentration of dissolved pyrimethamine.

The apparent binding constants ($K_{1:1}$) of the pyrimethamine /host complexes were calculated from the straight line portion of the phase solubility diagram using the following equation:

$$K_{1:1}\ (M^{-1}) = \text{slope}/S_0\ (1\text{-slope})$$

where S_0 is the inherent solubility of the drug in the aqueous complexation medium. In this case, the slope is always less than unity.[19] If 2:1 drug/host complex is formed, then the binding constant ($K_{2:1}$) of the complex will be determined by the equation[20]

$$K_{2:1}\ (M^{-2}) = \text{slope}/S_0^2\ (2\text{-slope})$$

In this equation, the slope of the linear phase solubility diagram is always less than two.

Preparation of Pyrimethamine/D3 Complex. Pyrimethamine dissolved in ethanol and D3 dissolved in water were mixed and stirred at 25 °C for 24 h. After the contact period was complete, ethanol was evaporated using N_2 gas, and then the mixture was lyophilized. The sample was dissolved in 0.5 mL water, filtered and freeze-dried.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectroscopic analysis was carried out on a Burker 500MHz spectrometer (AMX, Germany) at a temperature of 298 K. The samples were dissolved in deutrated water (D_2O, 99.96%). The chemical shift displacements were calculated according to the formula: $\Delta\delta = \delta_{(complex)} - \delta_{(free)}$, where $\delta_{(free)}$ is the chemical shift of pyrimethamine without D3, and $\delta_{(complex)}$ is the chemical shift of pyrimethamine with D3.

Fourier-Transform Infrared Spectroscopy (FTIR). FTIR spectroscopy (Bruker IFS-66/Spectrometer, AMX, Germany) was performed to investigate the interaction of pyrimethamine with D3 in the range of 4000-500 cm^{-1} in a KBr matrix. Spectra were obtained for pyrimethamine, D3, their physical mixture, and the pyrimethamine/D3 complex.

Differential Scanning Calorimetry (DSC). Thermal analysis of the sample (pyrimethamine, D3, and their complex) was carried out using a DSC 7020 (SEICO INST.) All accurately weighed samples (5 mg) were placed in sealed aluminum pans, before heating under nitrogen flow (40 mL min^{-1}) at a scanning rate of 10 °C min^{-1}, over the temperature range of 30 °C to 380 °C. An indium standard was used to calibrate the temperature scale.

Scanning Electron Microscopy (SEM). The surfaces

morphology of materials was examined using a scanning electron microscope JSM-6380. The samples were fixed on a brass stub using two-sided adhesive carbon tape and then gold coated in vacuum by a sputter coater. Images were then taken at an excitation voltage of 5 kV.

Computational Method. The molecular model for the D3 succinoglycan was built using Conformation Search module in MacroModel software (Schrodinger Inc.). Global energy-minimum structure for the D3 was calculated using mixed torsional/low-mode sampling method with OPLS2001 force field. Molecular docking simulation of pyrimethamine to the D3 was carried out using GOLD 5.1 program from Cambridge Crystallographic Data Centre. A Lamarckian genetic algorithm (GA) was applied to conformational sampling with a maximum number of 1×10^6 energy evaluations and 100 individual populations. Other parameters were used with a mutation frequency of 95, a crossover frequency of 95, and a migration frequency of 10. Binding affinities were calculated from resultant docked poses using a ChemPLP[1] fitness function. This scoring function uses hybridized piecewise linear potential with Chemscore terms.[30]

Results and Discussion

The determination of the phase-solubility diagram is a widely accepted method for evaluation of the effect of CD complexation on drug solubility.[20-23] The solubility diagram shown in Figure 2 indicates that the solubility of pyrimethamine is greatly with the increasing amounts of D3, D4, D2, or M3 when compared to those with HP-β-CD or α-CD. The aqueous solubility of pyrimethamine in the presence of 1.2 mM of the dimers, D3 and D4, were increased 42 and 33-fold, respectively. On the other hand, in the case of HP-β-CD, the solubility of pyrimethamine was increased only 5-fold by its addition. Pyrimethamine with M3 and α-CD

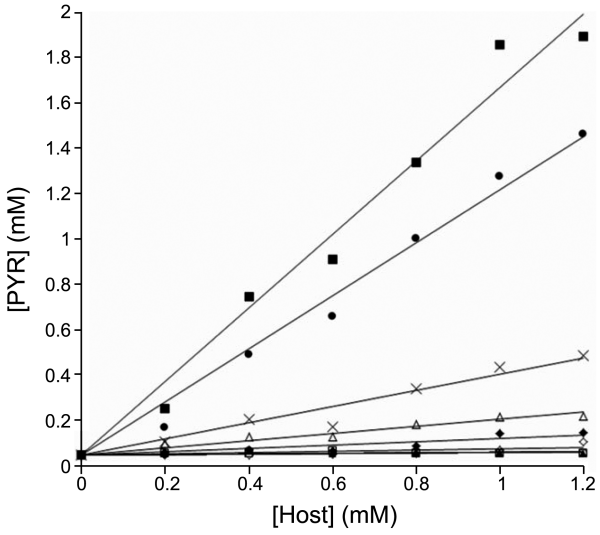


Figure 2. Phase solubility diagram of pyrimethamine in solutions with host molecules. 2 mM solution of pyrimethamine was mixed with 0.2, 0.4, 0.6, 0.8, 1, and 1.2 mM solutions of the host molecule. α-CD (◇); HP-β-CD (△); D1 (▲); D2 (×); D3 (■); D4 (●); M1 (□); M2 (○); M3 (◆).

Table 1. Binding constants for pyrimethamine/succinoglycan monomers and pyrimethamine/succinoglycan dimers

Complex	$K_{1:1}$	$K_{2:1}$
PYR/α-CD	$6.50 \times 10^2\ M^{-1}$	
PYR/HP-β-CD	$4.21 \times 10^3\ M^{-1}$	
PYR/M1	$2.77 \times 10^2\ M^{-1}$	
PYR/M2	$2.75 \times 10^2\ M^{-1}$	
PYR/M3	$1.80 \times 10^3\ M^{-1}$	
PYR/D1	$3.16 \times 10^2\ M^{-1}$	
PYR/D2	$1.24 \times 10^4\ M^{-1}$	
PYR/D3		$2.13 \times 10^9\ M^{-2}$
PYR/D4		$7.02 \times 10^8\ M^{-2}$

resulted in a 3 and 2-fold increase in solubility. According to Higuchi and Conners, all of the complexes formed an A_L-type curve, indicating that the complex is first order with respect to host and first or higher order with respect to guest.[19] When the slope of an A_L-type system is greater than one, the higher order complexes of guest are indicated.[20] Figure 2 shows that pyrimethamine formed a 2:1 complex with D3 and D4. Also, the lower slope was indicative of a 1:1 stoichiometry and thus the other carbohydrates except, D3 and D4, formed a 1:1 complex with pyrimethamine.[21] From the slope of the linear fit shown in Figure 2, the binding constants (K_b) were calculated and summarized in Table 1. The binding constants ($K_{2:1}$) of D3 and D4 with pyrimethamine were determined to be $2.13 \times 10^9\ M^{-2}$ and $7.02 \times 10^8\ M^{-2}$, respectively, and that of D2 was $1.24 \times 10^4\ M^{-1}$. The overall order binding constants were in the following order: D3 > D4 > D2 > HP-β-CD > M3 > α-CD > D1 > M1 > M2. These results show that the solubilizing effect of D3 was the greatest among the nine complexes.

To determine the stoichiometry of the pyrimethamine/D3 complex that displayed in the highest binding constant from phase solubility study, job plots were obtained using the continuous variation method.[24,25] Job developed the continuous variation technique, which can reliably determine complex stoichiometries, based on the difference in a physical parameter, for example, absorbance ΔA ($\Delta A = A - A_0$) of D3 in the presence of the pyrimethamine.[27] ΔA values were calculated by measuring the absorbance of the D3 solutions in the absence (A_0) and presence (A) of the corresponding concentration of the pyrimethamine. As shown in Figure 3, the results shows the absorbance was maximal at a molar fraction of 0.4, which corresponds to a stoichometry of 2:1. These results are in agreement with the stoichiometry suggested from the phase solubility study.[24,25]

To confirm the interaction between pyrimethamine and D3, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopic analyses were carried out. ^{1}H NMR spectroscopy is a suitable method for the evaluation of non-covalent interactions at the molecular level.[26] The chemical shift assignments of the pyrimethamine in the presence and absence of D3 are given in Table 2. By the complexation with D3, the peaks of pyrimethamine were shifted upfield. The chemical shift change of the protons was 0.07 ppm in the chlorophenyl

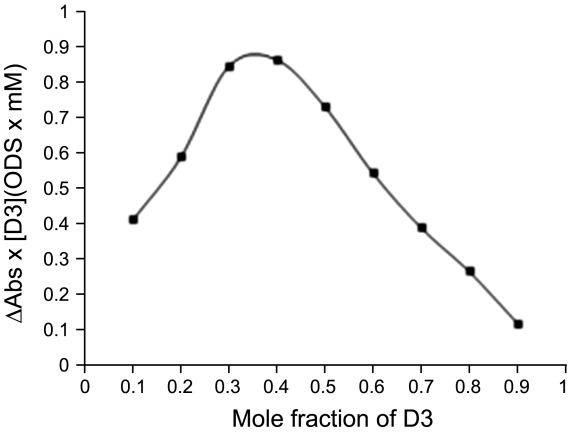
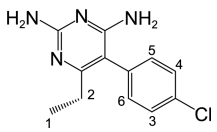


Figure 3. Continuous variation plot (Job's plot) of the pyrimethamine/D3 system.

Table 2. ^{1}H chemical shifts of PYR in the free state and in the presence of D3. ll were expressed as $|\Delta\delta| = \delta_{complex} - \delta_{free}$



PYR proton	Chemical shift (ppm)		$\|\Delta\delta\|$ (ppm)
	PYR	PYR/D3	
H-1	1.04	1.01	0.03
H-2	2.29	2.26	0.03
H-3 and H-4	7.35	7.28	0.07
H-5 and H-6	7.60	7.53	0.07

moiety and 0.03 ppm in the ethyl group, respectively. Those results indicate that there were some hydrophobic interactions between the nonpolar groups of pyrimethamine and D3.

In order to investigate the vibrational changes upon the pyrimethamine/D3 interaction, the samples were analyzed using FTIR spectroscopy. Figure 4 shows the FTIR spectra of pyrimethamine, D3, their physical mixture, and their complex. The spectrum of pyrimethamine contained the characteristic peaks at 3466 and 3145 cm^{-1}. These peaks are indicatives of stretching vibrations of N-H and aromatic ring C-H of pyrimethamine, respectively. The spectrum also displayed absorption bands between 1400 and 1649 cm^{-1}, which correspond to the stretching vibrations of C=C and C=N from the aromatic rings in PYR (Fig. 4(a)).[10] The FTIR spectrum of D3 showed a broad absorption band at 3429 cm^{-1} for O-H stretching vibrations and absorption at 2918 cm^{-1}, which was attributed to C-H stretching.[28] The peaks shown at 1730 cm^{-1} and 1070 cm^{-1} corresponded to the symmetric stretching of carboxyl groups and the asymmetric C-O stretch, respectively (Fig. 4(b)). The spectrum of the physical mixture displayed the characteristic pyrimethamine peaks at 3466, 1627, and 1576 cm^{-1} (Fig. 4(c)). However, the FTIR spectrum of the pyrimethamine/D3 complex showed the absence of the characteristic absorption peaks of

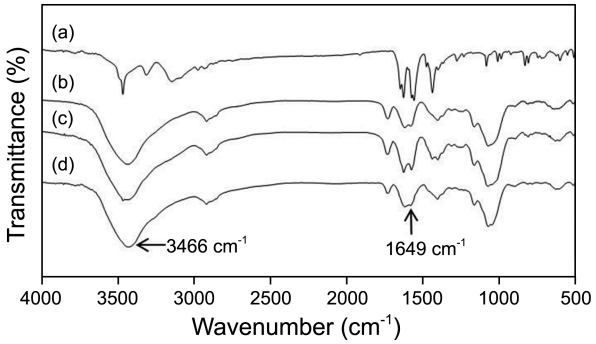


Figure 4. FTIR spectrum of pyrimethamine alone (a), D3 alone (b), pyrimethamine/D3 physical mixture (c), and pyrimethamine/D3 complex (d).

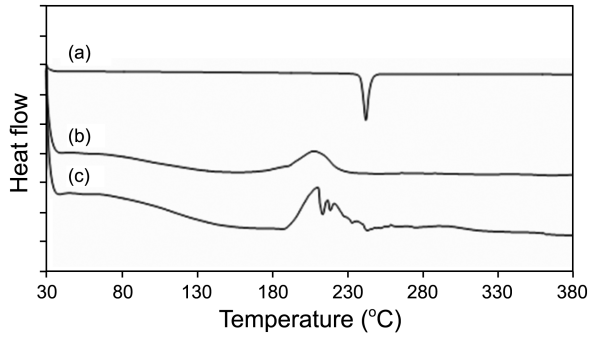


Figure 5. DSC curves of pyrimethamine alone (a), D3 alone (b), and pyrimethamine/D3 complex (c).

pyrimethamine at 3466, 1649 and 1400 cm^{-1} (Fig. 4(d)). These results provide further evidence of the effective complexation between D3 and pyrimethamine.

The DSC thermograms for pyrimethamine alone, D3 alone, the pyrimethamine/D3 complex are shown in Figure 5. The DSC curve of pyrimethamine (Fig. 5(a)) contained a single sharp endothermic peak at 242 °C, corresponding to the melting point, whereas that of D3 alone exhibited a boarder exothermic event at 184.1-232 °C (Fig. 5(b)). After complexation, the pyrimethamine endothermic peak disappeared, indicating that the pyrimethamine was completely included in the D3 molecule (Fig. 5(c)).[4,9,10] Also, the presence of multiple peaks of exothermic event around 184.1-239 °C were probably due to various structural characteristics of 1:2 complex between D3 and pyrimethamines.[17]

SEM was used to image the pyrimethamine, the succinoglycan D3, and pyrimethamine/D3 complex (Fig. 6). Although this method cannot be used to conclusively confirm the complex formation, it helps to assess the existence of a single component in the preparation products. Pyrimethamine was characterized by the presence of a cube particle with a regular size (Fig. 6(a)), whereas D3 appeared as amorphous particles without a definite shape (Fig. 6(b)). After complexation, the original morphologies of pyrimethamine or D3 disappeared (Fig. 6(c)). In addition, the pyrimethamine/D3 complex was irregular-shaped and adopted a more complex shape than the D3 particle, which indicates maximum or complete complex formation.

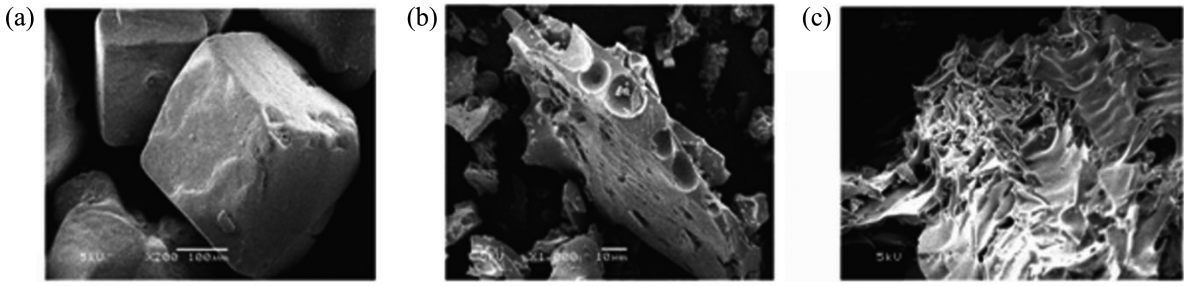


Figure 6. SEM images of pyrimethamine alone (a), D3 alone (b), pyrimethamine/D3 complex (c).

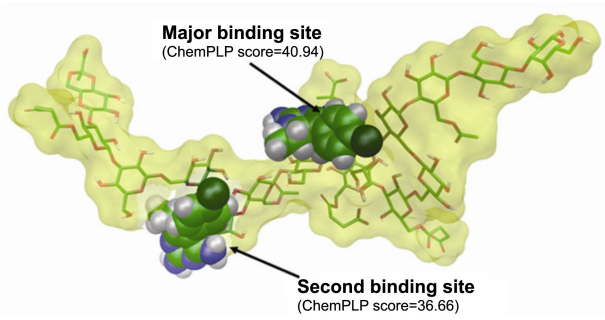


Figure 7. Global energy-minimum docking configuration of pyrimethamine /D3 complex.

In the molecular docking simulations, most poses of pyrimethamine were located on two different sites of D3 through 100 GA-runs. Figure 7 is a representative image for the docked poses of two pyrimethamine molecules upon succinoglycan D3. The pyrimethamine was bound to a major binding site of D3 through intermolecular contacts. The other pyrimethamine was located on a second binding site of the D3. The docking score of pyrimethamine for the each major and second binding site was 40.94 and 36.66, respectively. The major binding site provided stable molecular environment for the association of pyrimethamine. In both cases, a chlorophenyl residue of pyrimethamine showed close contacts with the sugar rings of D3. An ethyl group attached to pyrimidinyl ring of pyrimethamine also made a contact with the succinyl group of D3. Two NH_2 groups of the pyrimidinyl moiety were located on the outward position of the binding surface; they exhibited weak interatomic contacts with the D3-sugar residues. Overall docked features were reasonably corresponding to the NMR-derived structural characteristics of 1:2 complex between D3 and pyrimethamines.

Conclusion

In the present report, we attempted to demonstrate that succinoglycan dimers showed better solubility enhancement for complexation with pyrimethamine than both α-CD and HP-β-CD.[4,9,10] This is an important finding since pyrimethamine is widely used for the treatment of malaria disease. Phase solubility studies have indicated that pyrimethamine complex with D3 is more stable than other pyrimethamine/host complexes in terms of the binding constant. In addition, the continuous variation plot of pyrimethamine/D3 confirmed that the complex between pyrimethamine and D3 shows a 2:1 stoichiometry. Finally, the complex formation of pyrimethamine/D3 was analyzed by ^{1}H NMR, FTIR, DSC, SEM, and Molecular Dynamics. According to the Molecular Dynamics, the chlorophenyl residue of the pyrimethamine showed close contacts with the sugar rings of D3. The complexation of succinoglycan D3 with pyrimethamine was demonstrated by the various non-covalent interactions, such as *van der Waals* interactions, hydrogen bonding and dipole interactions between pyrimethamine, and the D3 and three dimensional structure derived from helical succinoglycan.[29] Since the cytotoxicity of succinoglycan dimers is not detected upon the treatment of human cells,[17] based on the combined results of the present study, we suggest that low-molecular-weight succinoglycan may be used as an effective solubilizer in therapeutical purposes.

Acknowledgments. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF 20110026022 and NRF-2011-619-E0002). SDG.

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