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The Effect of pH to The Interaction between Folic Acid and Folate Receptor Alpha: Molecular Dynamics Study

Muhammad Yusuf[1,2], Galih Dwi Pramono[1], Zuhrotun Nafisah[2], Ade Rizqi Ridwan Firdaus[2], Ari Hardianto[1], Veronika Yulianti Susilo[3], Rustaman[1], Abdul Mutalib[1,2], Ukun MS Soedjanaatmadja[1,2]*

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Sumedang - Jawa Barat, Indonesia
[2]Research Center for Theranostics, Universitas Padjadjaran, Bandung - Jawa Barat, Indonesia
[3]Center for Radioisotopes and Radiopharmaceuticals, National Nuclear Energy Agency, Puspiptek Area Serpong, Tangerang - Banten, Indonesia

**Corresponding author email: ukun@unpad.ac.id*

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Cancer is one of the major health problems in the world. Early detection using Magnetic Resonance Imaging (MRI) for the presence of cancer cells could improve the successful rate of treatment. For this reason, a selective contrast agent is required to improve the accuracy of cancer diagnosis. Many cancer cells overexpress the folate receptor alpha (FRA) on its surface. Therefore, the substrate of FRA, folic acid, can be used to develop a selective contrast agent such as Gd-DTPA-folate. However, it is worth noting that the slightly acidic pH in cancer cell could change the conformation of ligand binding site of FRA, thus lowering the affinity of folic acid-based contrast agent. Although the crystal structure of FRA in low pH has been solved, but the mechanism of decreasing affinity of folic acid is still not clear. Therefore, this work aims to study the structural change of FRA in low pH and to investigate the molecular interactions of folic acid and Gd-DTPA-folate to the FRA at normal and acidic pH using molecular dynamics simulations. A crystal structure of folic acid in complex with FRA was used as a template for simulations. The interaction energies were calculated using MM/GBSA method. As a result, the protonated Asp81 in the ligand binding site of FRA repulsed the pterin ring of folic acid. Interestingly, Gd-DTPA-folate was predicted to stabilize its interaction with FRA in low-pH as compared to the normal pH. It is hoped that this study could provide insight into the development of selective contrast agent for cancer.

Keywords: Cancer | folate receptor alpha | folic acid | contrast agent | molecular dynamics

Cancer is one of the major health problems which causes 13% of mortality in the world. Around 70% of cancer cases was predicted to increase in the next two decades. Hundreds type cancer have been identified and each of them requires different diagnosis and treatment (1). Early detection is urgently needed to improve the successful rate of cancer therapy, thus enhancing the survival rate of cancer patient. Magnetic Resonance Imaging (MRI) is one of the diagnosis methods for cancer. By using a paramagnetic contrast agent such as gadolinium diethylenetriamine pentaacetate (Gd-DTPA, Magnevist), MRI is able to locate the presence of cancer cell in the patient body (2) Gd-DTPA has been widely used as an MRI contrast agent due to its structural stability, thus considerably safe for human use without significant side effect (3). Moreover, the selectivity of Gd-DTPA could be enhanced by conjugating it with a targeting molecule, such as folic acid. Folic acid was selected as a targeting agent due to its high affinity to the folate receptor alpha (FRA), which is known to be overexpressed in cancer cells (4). Various cancer cells, e.g. cervical, uterus, ovarium, breast, are known to specifically overexpress alpha-type of folate receptor, not the beta-type (FRB). Therefore, Gd-DTPA-folate is a promising MRI contrast agent that can selectively determine the occurrence and the specific location of cancer cells (5). FRA binds folic acid 50 times stronger than the FRB (6). The binding affinity of folic acid with the FRA was 0.1 nM (7). Also, molecular dynamics study of Gd-DTPA-folate indicated its stability inside the ligand binding site of FRA. Interestingly, Wibowo and colleagues (8) found that the affinity of folic acid with the FRA in acidic pH was decreased 2,000 times as compared to that of physiological pH. This finding had causes for concern, since the pH at the extracellular region of cancer cell was slightly acidic, around 6.5 until 6.8 ((9), (10), (11)). It is noted that folic acid interacts with the extracellular FRA. Furthermore, it is shown that the conformation of ligand binding site of FRA at pH 5.5 was changed, as confirmed with its crystal structure (8). Although the crystal structure of FRA at acidic pH has been solved, but the mechanism of the decreasing affinity of folic acid in slightly acidic pH is still not clear.

Computational methods and structural bioinformatics such as electronic calculation and molecular dynamics simulation have been used to reveal the molecular mechanism of protein behavior which is not explicitly presented by crystal structures (12)Therefore, this study aimed to investigate the structural changes of FRA and the molecular interaction between folic acid and FRA at low pH using computational methods. The protonation states of acidic and basic residues of FRA were predicted based on the pKa values from the 3D structure. The binding of folic acid inside the ligand binding site of FRA was observed and quantified by using molecular dynamics simulation and Molecular Mechanics Generalized Born Surface Area

(MM/GBSA) methods, respectively. Furthermore, similar methods were used to study the binding of Gd-DTPA-folate with FRA.

METHODS

Prediction of pKa values of FRA residues

Crystal structure of FRA with PDB (Protein Data Bank) ID 4LRH was submitted to PDB2PQR server (13). This file contained the quaternary structure of FRA in complex with folic acid (7). The pKa of each residues was calculated using PROPKA . PROPKA is a program that predicts the pKa values of ionizable group in proteins based on the three-dimensional structure. Acidic residue which are buried inside the protein structure and histidines which are exposed to the solvent were described as the protonated model. Each protonation state was determined at pH of 5.5, 6.5, and 7.4 which represented the changed conformation at low pH, extracellular cancer cell environment, and physiological pH in normal cell, respective.

Modeling the structure of Gd-DTPA-folate.

Gd-DTPA-folic is conjugate compound which Gd-DTPA conjugated to folic acid by ethylene diamine (EDA). The structure of Gd-DTPA was obtained as CIF (Crystallographic Information File) format from CCDC (The Cambridge Crystallographic Data Centre) with code of 1307236. The CIF file was converted to PDB format using BIOVIA Discovery Studio Visualizer (BDSV) (14). To prepare the structure of Gd-DTPA-folic, the carboxyl group of DTPA was connected to the ethylene group of EDA, while the amine group of EDA was linked to the ɣ-carboxyl group of folic acid.

Molecular dynamics simulation.

The minimization and molecular dynamics simulation were performed by using AMBER14. The structure of ligands was parameterized using AM1_BCC semiempirical calculation by the Antechamber program in AmberTools 15 (15). The ff14SB and GAFF force fields were used for the protein and DTPA-folic molecules, respectively, while that of Gd3+ was taken from the trivalent ionic parameters (16). The ligand-receptor's complex structure was minimised by using 1,000 and 4,000 steps of steepest descent and conjugate gradient. The system was gradually heated to 310 K for 60 ps in the NVT ensemble. Then, one ns of NPT equilibration was done. In this stage, harmonic restraints gradually reduced by 1 kcal/molÅ2 until it reached zero. Finally, a production run in the NPT ensemble was done for 50 ns. The timestep used was two fs. The cutoff value of non-bonded interactions was 10 Å. The analysis of MD trajectory was performed using the cpptraj module of AmberTools 15. The MMPBSA.py (17) program calculated the interaction energy between ligand and receptor. MMPBSA method has been widely applied as an efficient and reliable free energy simulation method to model molecular recognition.

RESULTS

Protonation state of FRA in slightly acidic pH.

The decreasing affinity of folic acid to FRA in the slightly acidic pH is interesting to be studied. Any cancer diagnostic or therapeutic agents based on folic acid should be carefully evaluated since the pH of cancer cell is lower than physiological one. PROPKA was utilized to calculate the pKa of ionizable residues in FRA, such as aspartic acid, glutamic acid, and histidine. Normally, the pKa of aspartic acid is 3.8, which make it in negatively charged at physiological pH. The similar ionization state is possessed by glutamic acid with the pKa of 4.5. Histidine is the only residues that should be carefully determined around physiological pH. When histidine is exposed to the water, it will be protonated to form a positively charged residues. In this study, the other positively charged residues such as arginine and lysine were not included in the analysis, since their pKa are much higher than the physiological pH. Hence, these two residues are always carrying positive charge in low pH. The 2000-times lower affinity of folic acid at the pH of 5.5 might be caused by the changes of protonation state of ionizable residues located at the ligand binding site of FRA. Fig. 1 shows that Asp81 is in a favorable position to form hydrogen bond with folic acid. If this residue was protonated, then it would resulted in an unfavorable electrostatic repulsion with the pterin ring of folic acid. MD simulation of protein using AMBER is able to model the pH effect by specifying the notation of amino acid in the MD system. It is noted that ASH is a protonated model of aspartic acid (ASP), while HIP is a protonated model of histidine (HID/HIE if the proton assigned to the δ-nitrogen or ε-nitrogen, respectively).

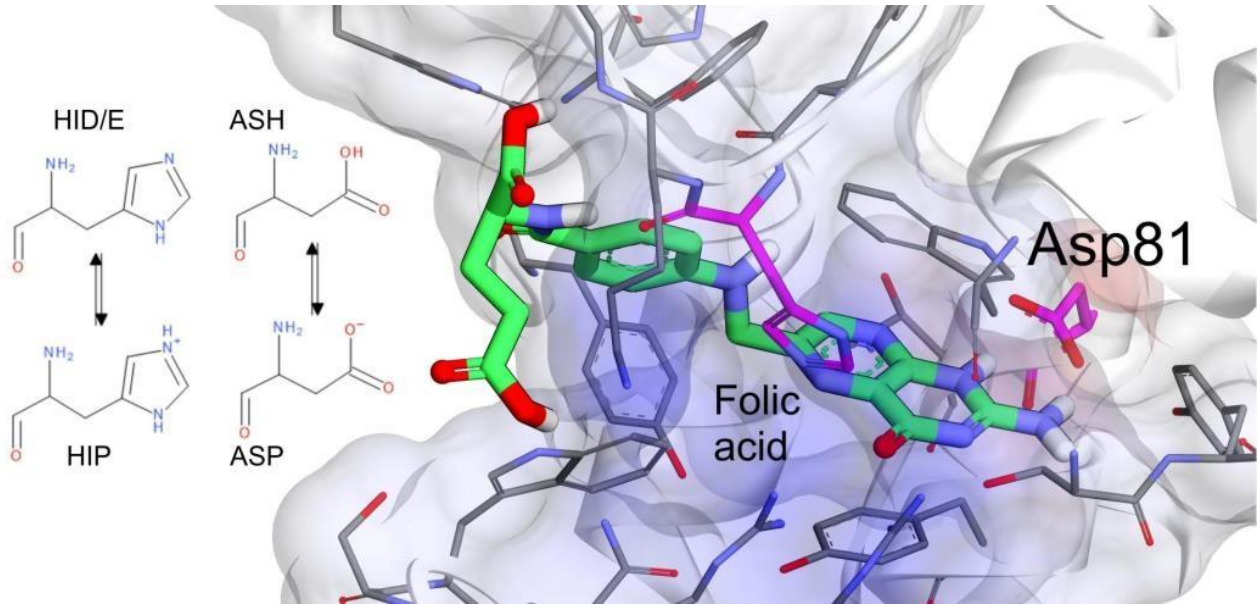

Figure 1. Ionizable residues located at the ligand binding site of FRA.

Table 1. Calculated pKa values of aspartic acid, glutamic acid, and histidine which are located at the ligand binding site of FRA.

No.	Residue	pKa	pKmodel	% Buried area	AMBER notation at pH 5.5
1	Asp81	6.82	3.80	100%	ASH
2	Asp116	5.96	3.80	86%	ASP
3	Glu69	4.57	4.50	0%	GLU
4	Glu122	3.77	4.50	0%	GLU
5	His32	6.14	6.50	7%	HIE
6	His135	2.58	6.50	87%	HIP
7	His157	5.69	6.50	25%	HIE
8	His173	6.31	6.50	9%	HIE

Folic acid is visualized in green colored stick, while the ionizable residues around the folic acid are represented in magenta. To determine the protonation state of ionizable residues, the calculated pKa for those located at the ligand binding site of FRA is listed in Table 1. Table 1 shows that Asp81, which is supposed to stabilize the pterin ring of folic acid by hydrogen bond, was predicted to be protonated. The buried percentage of Asp81 and Asp116 were high (100% and 86%, respectively), thus lowering the probability of these residues to be ionized into negatively charged carboxylate group. Therefore, the predicted pKa for Asp81 was 6.82.It is predicted that at pH of 5.5, Asp81 and Asp116 were protonated, while Glu69 and Glu122 were remained ionized. Whereas for histidines, the low percentage of buried area would increased their probability of getting protonated by water. Thus, His135 was predicted to be protonated at pH of 5.5, while His32, His157, and His 173 were not. The calculated pKa values from PROPKA were used to determine the notation of each ionizable residues in AMBER, prior to molecular dynamics simulation. It is noted that the notation for His32, His157, and His173 were HIE, due to the optimum hydrogen bond formation with the surrounding residues.

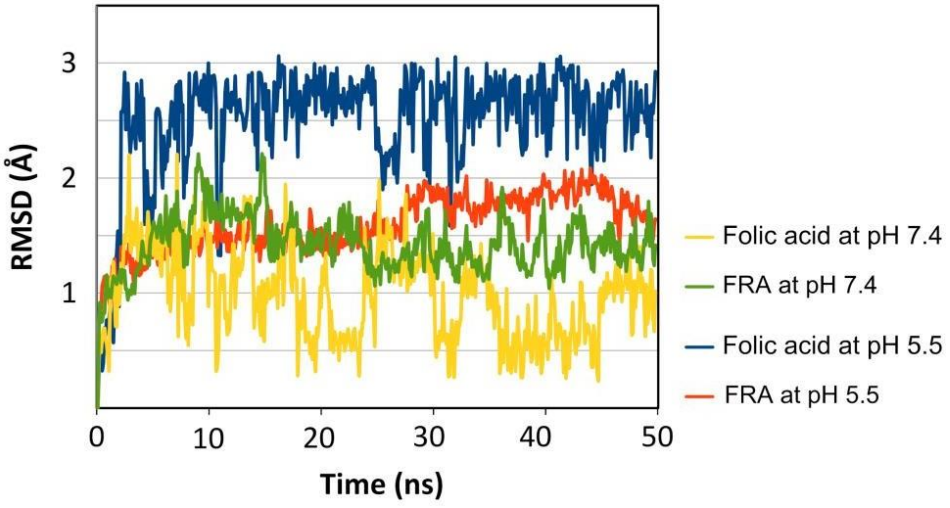

Figure 2. RMSD plot of FRA and folic acid in physiological (7.4) and acidic pH (5.5) throughout 50 ns of MD simulation.

The effect of low pH to the interaction of folic acid with FRA.

The structural deviation of FRA and folic acid throughout 50 ns of MD simulation was calculated and presented in Fig. 2. It is shown that the deviation of both FRA and folic at the pH 5.5 were higher than that of physiological pH. This result indicating the structural change of FRA and the instability of folic acid binding at low pH.Furthermore, the time evolution snapshots which are taken from the production MD trajectory was generated and presented in Fig. 3.

Interestingly, the overlaid snapshots of folic acid conformation during 50 ns of simulation suggesting its stability in forming interaction with the receptor. Whereas a notable motion of folic acid at pH 5.5 was observed by the difference of its conformation throughout simulation.

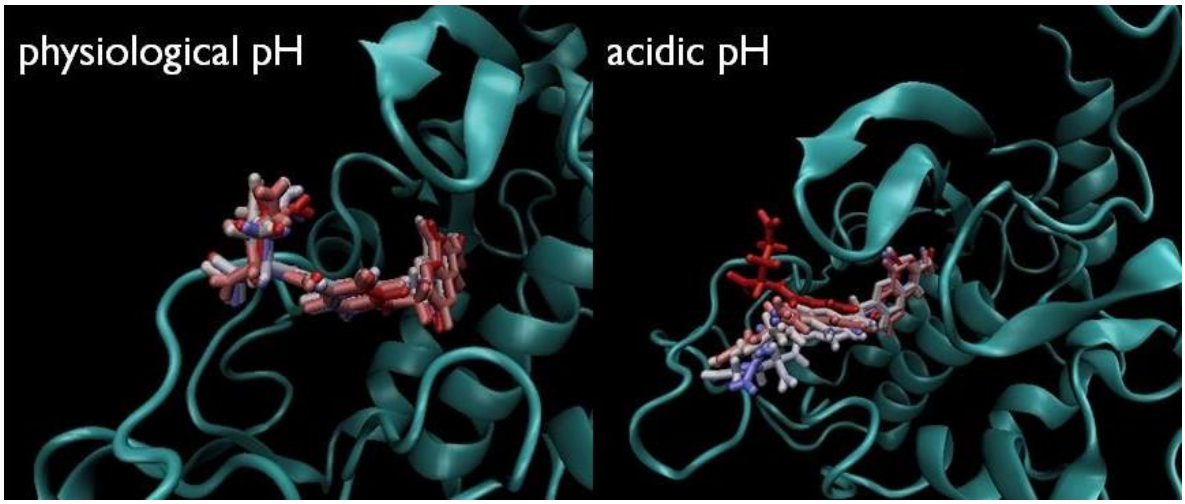

Figure 3. Time evolution snapshots of MD system at pH 7.4 (left) and pH 5.5 (right).

The affinity of folic acid to FRA in pH 7.5 and 5.5 were computed using MM/GBSA method. The calculated binding energy of folic acid to FRA in acidic pH (-47 kcal/mol) is weaker than that in physiological pH (-62 kcal/mol) (Fig. 4). This result is in correspond with the experimental Kd values that reported before, i.e. 0.01 nM and 21 nM in neutral and acidic pH, respectively (Wibowo et al., 2013).

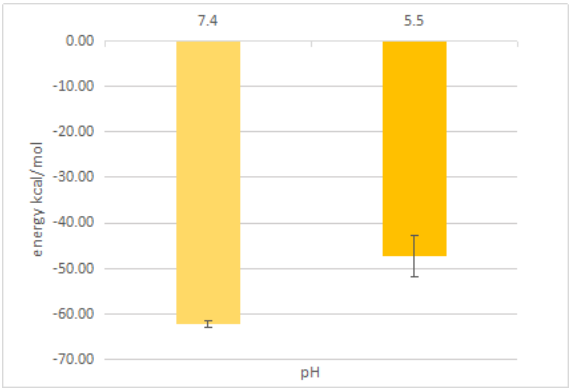

Figure 4. The computed binding energy of folic acid to FRA in pH 7.4 and 5.5

The mechanism of the decreasing affinity of folic acid to FRA in low pH could be explain by the change of protonation state of ionizable residues. Asp81 has a major role in stabilizing folic acid in the active site of FRA through hydrogen bonds (7). In acidic pH of 5.5, however, our study suggests that Asp81 in the active site of FRA is protonated and, thus, prevent the formation of hydrogen bonds with the pterin ring of folic acid (Fig. 5). As a result, binding interactions between folic acid and FRA in physiological and acidic pH are different. For example, in acidic pH, the pterin ring of folic acid is lost its interaction with His135, whereas in physiological pH, it maintains a hydrogen bond with the residue through the carbonyl moiety (Fig. 5). In total, the number of hydrogen bond formed by folic acid and FRA in physiological and acidic pH are 10 and 5, respective.

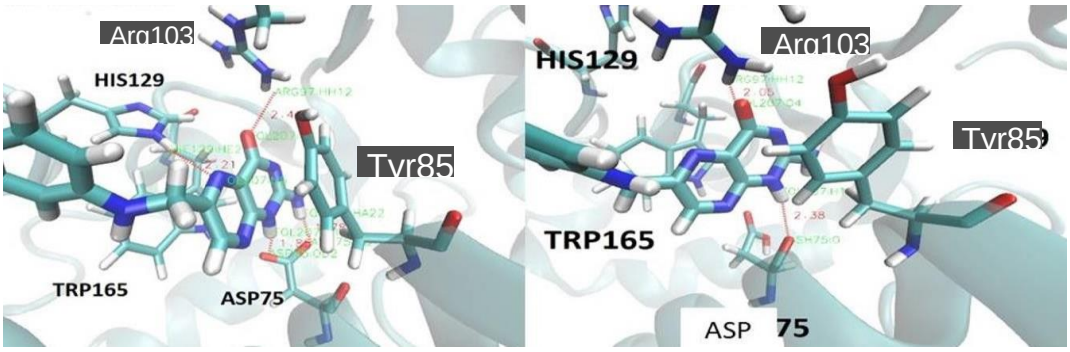

Figure 5. Molecular interaction of folic acid at the ligand binding site of FRA in physiological pH (left) and acidic pH (right).

The effect of the protonated His135 to the binding of folic acid is also observed. At low pH, a polar hydrogen at the δ-nitrogen of His135 side chain would form an unfavorable binding with the hydroxyl group of Thr172. As a result, the conformation of His135 was altered, thus disrupting the interaction with folic acid (Fig. 6). As shown in Fig. 6, the polar hydrogen at the ε-nitrogen of His135 was supposed to form hydrogen bond with the pterin ring of folic acid.

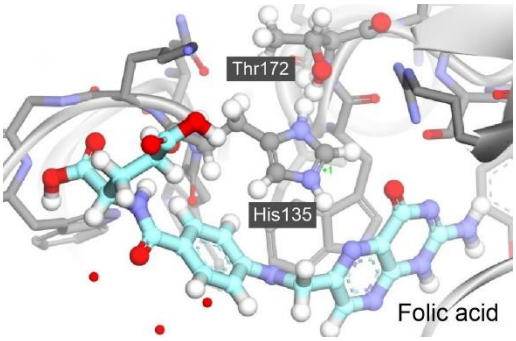

Figure 6. The interaction of the protonated His135 at pH 5.5 with surrounding residues physiological pH (left) and acidic pH (right).

The effect of low pH to the interaction of Gd-DTPA-folate with FRA

The decreasing affinity of folic acid to FRA in low pH suggested that it would also affected the binding of Gd-DTPA-folate as the MRI contrast agent for cancer. However, the calculation of binding energy between Gd-DTPA-folate and FRA at the pH 5.5, 6.8, and 7.4 showed opposite results. Unexpectedly, the affinity of Gd-DTPA-folate at the acidic pH was stronger than that of the higher pH (Fig. 7).

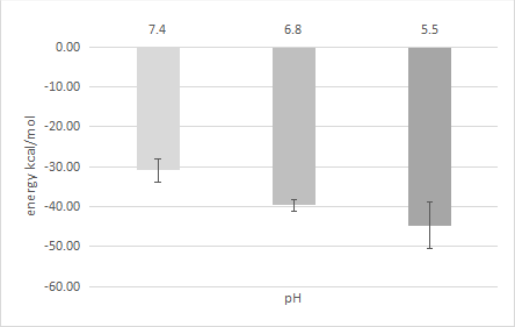

Figure 7. The computed binding energy of Gd-DTPA-folate to FRA in pH 7.4, 6.8, and 5.5

Upon inspection to the MD trajectory and molecular surface of FRA, especially at pH 5.5, a positively charged Lys136 was exposed to the solvent accessible surface. In the neutral system, this Lys136 formed salt bridge with Glu169. Due to the structural changes that occurred in acidic pH, this salt bridge was disrupted, as monitored in Fig.8.

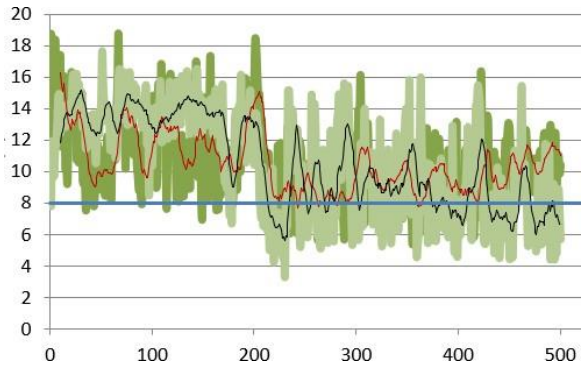

Figure 8. The time-dependent distance between Lys136 and Glu169 in physiological pH (red line) and acidic pH (black line) during 50 ns of MD simulation.

Discussion

The effect of pH on protein behaviour is always interesting to be studied. Although some of the protein structures at different states have been solved by either X-ray crystallography or cryo-EM, the molecular mechanism behind the observed experimental behavior remains unclear. Structural bioinformatics, including MD simulations, is a powerful tool to investigate the dynamic behaviour of protein at the atomic level. Many studies have been done to explain the experimental behaviour of protein using a structural bioinformatics approach. Hardianto and colleagues found that the specific protonation state of balanol is required to have good inhibitory activity towards protein kinase as a protein target for cancer therapy(18). In another study, the molecular mechanism behind the drug resistance in influenza virus was only observed in MD simulation. A specific protonation state of histidine at the ligand binding site has an important role in the whole stability of drug entrance to the active site (12).

Moreover, the effect of the mutation at the proton channel of ATPase complex on the disruption of proton hopping was explained using a structural bioinformatics approach. The altered ATP production due to the failure in proton hopping is challenging to be determined by any experimental methods. In this study, the slightly acidic pH of the cancer cell was observed to affect the affinity of folic acid to FRA. This observation should be taken seriously because folic acid has been used widely as the molecular probe for diagnostics and therapeutics for cancer. This research can give an in-depth understanding of the molecular interactions between the designed conjugate compound, and molecular target is required to evaluate the efficacy of diagnostics, therapeutics, or even theranostic agents, before further preclinical or clinical tests.

Conclusion

Amongst the ionizable residues of FRA, Asp81 and His135 were affected by the slightly acidic environment. At the pH of 5.5, these two residues were protonated. The polar hydrogen at the carboxyl group of Asp provided unfavourable interactions with the polar hydrogen at the pterin ring of folic acid. Whereas the positively charged His135 resulted in the electrostatic repulsion with the hydroxyl group of Thr172. Interestingly, although the affinity of folic acid decreases at low pH, Gd-DTPA-folate still has a stable affinity value**.** The altered conformation of FRA in the acidic pH increased the electrostatic interactions between the exposed Lys136 and the negatively charged DTPA. This study suggested that the affinity of Gd-DTPA-folate was stronger in the acidic extracellular environment than in physiological pH in a normal cell. This result is expected to be useful in the design of specific diagnostic and therapeutic agents based on the interaction between folic acid and FRA

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