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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MAY 2014

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Original article

Aromatic diacylhydrazine derivatives as a new class of polo-like kinase 1 (PLK1) inhibitors

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ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
8 April 2014
Accepted 6 May 2014
Available online 9 May 2014

Keywords:
Polo-like kinase 1
Aromatic diacylhydrazine
Computer simulation

ABSTRACT

A novel class of aromatic diacylhydrazine derivatives was designed as PLK1 inhibitors. All the 19 new synthesized compounds were assayed for antitumor activity against the respective cervical cancer cells. In which, nine compounds with better antitumor activities were further tested for their PLK1 inhibitory activity. Last, we have successfully found that compound **7k** showed both the promising antitumor activity with IC_{50} of 0.17 μM against the cervical cancer cells, and also processed the most potent PLK1 inhibitory activity with IC_{50} of 0.03 μM. In addition, docking simulation also carried out in this study to give a potent prediction binding mode between the small molecule and PKL1 (PDB code: 1umw) protein.

1. Introduction

Polo-like kinases (PLKs), a type of Ser/Thr protein kinases, have gathered much attention as important elements that regulate cell cycle progression, particularly mitosis [1]. Human Polo-like kinase subfamily includes four closely related members, that is, PLK1, PLK2, PLK3, and PLK4. Recently, PLK5 has been identified; however, it lacks a kinase domain and does not seem to function in cell cycle regulation [2]. Among them, PLK1 participates in multiple steps in mitosis by phosphorylating various substrates, but not in normal diploid cells or nondividing cells [3–6]. Inhibition of PLK1 activity in cancer cell causes mitotic arrest and finally induces strong cell-killing effect [5,7–11]. It is also reported that PLK1 is overexpressed in many clinical cancer samples, such as lung, colon, prostate, ovary, breast, head, neck squamous cell carcinoma, melanoma and others [12–15]. All of these indicate its involvement in carcinogenesis and its potential as a therapeutic target.

PLK1 offers two functionally crucial target sites within one molecule-the N-terminal kinase domain and the C-terminal polo-box domain. Several crystal structures of PLK1 kinase domain have been determined and they are complex with an ATP pocket-binding ligand such as a nonhydrolyzable ATP analogue adenylyl-limidodiphosphate [16], a pyrrolo-pyrazole inhibitor (PHA-680626) [17], a purine mimetic inhibitor [18], a quinazoline mimetic inhibitor [19], a pyrazolopyridine inhibitor [20] and so on. Besides, polo-box domain is essential for the subcellular localization and mitotic functions of PLK1, it is ideally suited for the development of anti-PLK1 inhibitors that interfere with polo-box domain-dependent protein–protein interactions [13,21,22]. These provide a good basis to elucidate the structure–activity relationship of these diverse compounds and further design novel PLK1 inhibitors. For this purpose, diacylhydrazine derivatives disclosed by Lu et al. [23] were selected as a starting point for our efforts because of its unique structure and strong enzyme potency (Fig. 1). Besides, a number of 1,4-benzodioxan template-containing compounds have ability as potential anticancer drugs with excellent bioavailability and low cytotoxicity [24–26]. Antitumor activities of various cinnamic acid derivatives were also explored by many research groups. Particularly, cinnamic acid ester derivatives have shown the potential antitumor activity [27,28]. Therefore, we endeavor to explore a new structure class based on known PLK1 inhibitors by chemical modifications according to bioisosteric replacement. Here we report the design and SAR study of novel diacylhydrazine PLK1 inhibitors.

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http://dx.doi.org/10.1016/j.ejmech.2014.05.026
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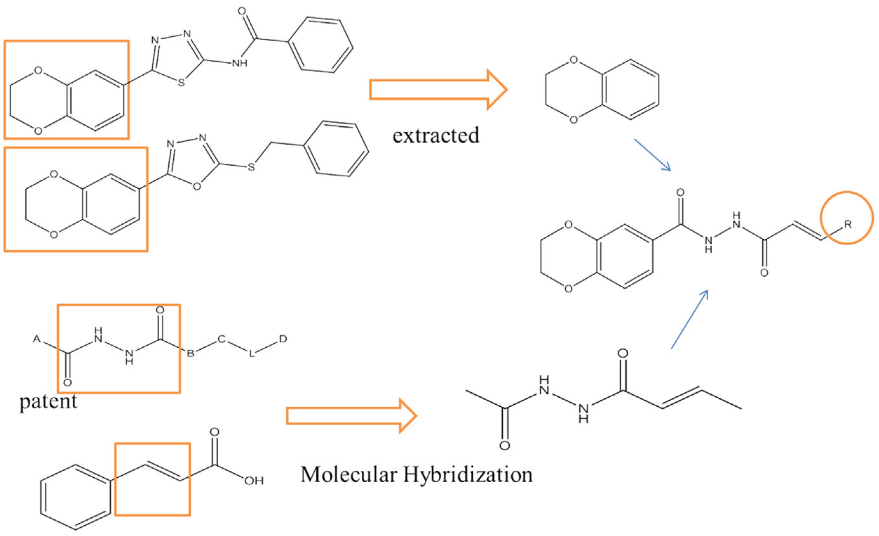


Fig. 1. The general formula of Chinese patent and our design solution of aromatic diacylhydrazine derivatives. (R means substituted benzene group)

2. Results and discussion

2.1. Chemistry

Methyl 2,3-dihydrobenzo[b][1,4]dioxine-6-carboxylate **3** was prepared in two steps as shown in Scheme 1. It was prepared in two steps. Firstly, 3,4-dihydroxybenzoic acid **1** gave methyl 3,4-dihydroxybenzoate **2**, catalyzed by concentrated sulfuric acid in methanol. Secondly, compound **3** was prepared by treatment of **2** with dibromoethane in acetone. The synthetic route to target compounds (**7a**–**7s**) is shown in Scheme 1. Firstly, 2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide was prepared by treatment of **3** with hydrazine hydrate (85%) in ethanol. Then, compound **6** gave diacylhydrazine derivatives by using carbodiimide hydrochloride and N-hydroxybenzotriazole in anhydrous CH_2Cl_2 with different substituted cinnamic acids.

All of the synthetic compounds gave satisfactory analytical and spectroscopic data, which were full accordance with their depicted structures.

2.2. Pharmacology

Generally speaking, the inhibitory activity of the compounds is due to cell apoptosis or toxic effect, so we firstly performed cytotoxicity test before detecting biological activity. The diacylhydrazine compounds were detected for their cytotoxicity on macrophages cells. The pharmacological results of these compounds were summarized in Table 1. What we can see from the data is that most of the compounds were low toxic.

2.2.1. Cell proliferation assay (MTT method)

All the synthesized derivatives (**7a**–**7q**) were tested in vitro for the inhibition activity on cervical cancer cells. The results were summarized in Table 1. The table showed that most of the synthesized compounds exhibited potent inhibitory activity with the IC_{50} value at low micromolar.

Based on the data obtained, we surveyed a variety of substituents at different positions on the phenyl ring of these diacylhydrazine derivatives, and found that various substituents of such as halogen, methyl, methoxyl and nitro group led to distinct anticancer activities of these target compounds. Compound **7k** with a methoxyl group on the 3-position of phenyl ring manifested the best antitumor activity with IC_{50} value of 0.17 μM than other compounds, while the simplest compound **7e** of this series with a methoxyl group on the 2-position of phenyl ring displayed the worst anticancer activity. Substitutions with electron-withdrawing group on the 2-position of phenyl ring did not improve the activity markedly, nor did halogen substitutions on the phenyl ring, except compound **7c** and **7f** with IC_{50} value of 2.11 and 0.89 μM, respectively. Compound **7r** with a nitro group on the 4-position of phenyl ring displayed higher antibacterial activity with IC_{50} value of 0.61 μM than compounds with a nitro group on the 2-position (0.89 μM) and 3-position (1.56 μM) of phenyl ring, while the activity gradient of substituent group on the phenyl ring in *m*-position was $-OCH_3 > -NO_2 > -CH_3 > -Br > -Cl > -F$. Together, these results illustrate that diacylhydrazine derivatives bearing 4-position substitutions of phenyl ring have potent antitumor activities.

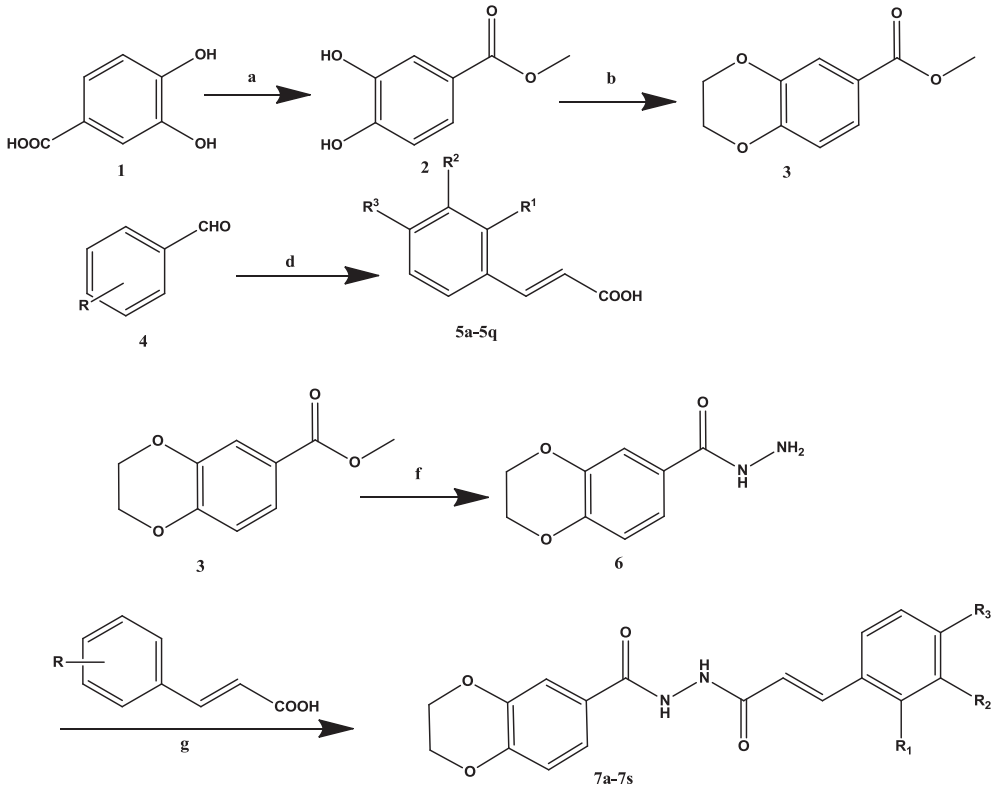
From the above-mentioned analysis, it could be concluded that the compounds with substitutions on the 4-position of phenyl ring and nitro substituted benzene ring were found to be the most favorable for the antitumor activity. More importantly, most of the analogues possess low cytotoxicity. Perhaps the electron withdrawing groups could reduce the electron density of the benzene ring and make it difficult to form $\pi-\pi$ bonds with amino acids containing aromatic groups. This makes the steric hindrance of the active pocket smaller for benzene ring part to get in. Therefore, these compounds are easy access to the active site.

2.2.2. PLK1 inhibitory assay

The PLK1 inhibitory potency of the diacylhydrazine derivatives was examined and the results were summarized in Table 2. Most of the tested compounds displayed potent PLK1 inhibiting activity. Among them, compound **7k** showed the most potent inhibitory with IC_{50} of 0.03 μM, prior to the positive control poloxin. The results of PLK1 inhibitory activity of the tested compounds were in agreement to the structure relationships (SAR) of their antitumor activities. This agreement suggested that antitumor activities of the synthesized compounds would derive from the inhibition of PLK1 enzymatic activities.

2.3. Binding model of compounds into PLK1 structure

In an effort to elucidate the possible mechanism by which the title compounds can induce anticancer activity and guide further



Scheme 1. Synthesis of compounds **3**, **5a**–**5q** and **7a**–**7s**. Reagents and conditions. (a) methanol, concentrated sulfuric acid, 90°C, 8 h; (b) dibromoethane, potassium carbonate, acetone, 70°C, 12 h; (d) pyridine, piperidine, 85°C, 24 h; (f) hydrazine hydrate, ethanol, 90°C, 4 h; (g) EDC·HCl, HoBt, rt, 8-10 h.

SAR studies, molecular docking of the potent inhibitor**s** into N-terminal kinase and C-terminal polo-box domain were performed on the binding model based on the PLK1 (1umw.pdb) separately. And preprocessed using the Schrodinger Protein Preparation Guide [29]. In the N-terminal kinase domain binding model, all of the compounds couldn't enter the active pocket except compound **7s** with docking calculation score of 8.0265, much lower than the polo-box domain docking scores. Therefore, we suspect that our compounds are small molecule inhibitors that may bind to the PLK1 PBD. The binding models of compound **7k** and PLK1 PBD were depicted in Fig. 2(A) and Fig. 3. The amino acid residues which had interaction with PLK1 as well as bond length were labeled. The docking calculations of the other compounds were also depicted in Table 3.

In the binding model, the carbonyl and imino of the diacylhydrazine skeleton forms a hydrogen bond with the backbone NH and carbonyl of Trp414, respectively. And the oxygen atom of the benzodioxan forms a hydrogen bond with the backbone NH of Asp416. In addition, compound **7k** was also nicely bound to PLK1 via one charge interaction (Lys540), one π-sigma interaction (Leu491) and one $\pi-\pi$ interaction (His538). Fig. 2B displayed 2D interactional maps between the small molecule ligand poloxin and 1umw protein crystal structure. Insight into this picture, we can see that amino acid residues Trp414, Lys540 located in the binding pocket also seemed very important for the active conformation of compound poloxin. These results could provide a molecular level foundation to illustrate compound **7k** can bind well at the active site of PLK1 kinase and was a potential inhibitor of PLK1.

3. Conclusion

In conclusion, a series of diacylhydrazine derivatives have been synthesized and evaluated for their antitumor activities. Compound **7k** demonstrated the most potent inhibitory activity that inhibited the growth of cervical cancer cells with IC_{50} of 0.17 μM and inhibited the activity of PLK1 with IC_{50} of 0.03 μM. Docking simulation was performed to position compound **7k** into the PLK1 active site to determine the probable binding conformation and the result indicated that compound **7k** was a potent inhibitor of PLK1. Given the unforeseen structural differences within the active site of some pathogenic enzymes, the key to discovering inhibitors with antitumor activity lies in a detailed understanding of the PLK1 active sites. Further studies on the PLK1 inhibition ability of this compound, new structural data were guiding further modifications of the current series with the hopes of improving both enzymatic inhibition and physical properties.

4. Experimental results

4.1. Chemistry

All chemicals and reagents used in the current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates. Melting points (uncorrected) were determined on a XT4MP apparatus (Taike Corp., Beijing, China). ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR spectra were collected on a Bruker DPX300 spectrometer at room temperature with TMS and solvent signals allotted as internal standards. Chemical shifts are reported in ppm (δ). Elemental analyses were performed on a CHN-O-Rapid instrument, and were within ±0.4% of the theoretical values.

4.1.1. General procedure for the preparation of compound *2*

Protocatechuic acid (1 mmol) in methanol (30 mL) was treated with concentrated sulfuric acid (0.5 mL) under 90 °C overnight. The

Table 1
Cytotoxicity assay of the compounds on macrophages cells and cell proliferation assay on cervical cancer cells.

Compound	R	CC_{50} ± SD (μM)	IC_{50} (μM)
7a	2-F	302.00 ± 20.12	4.51
7b	2-Cl	285.27 ± 18.00	4.88
7c	2-Br	315.17 ± 31.11	2.11
7d	2-CH_3	267.82 ± 18.11	7.71
7e	2-CH_3O	312.00 ± 21.12	8.06
7f	2-NO_2	257.45 ± 11.95	0.89
7g	3-F	221.25 ± 12.11	4.11
7h	3-Cl	391.23 ± 19.76	3.56
7i	3-Br	351.22 ± 11.09	3.33
7j	3-CH_3	280.98 ± 13.02	2.89
7k	3-CH_3O	398.23 ± 22.01	0.17
7l	3-NO_2	323.15 ± 21.23	1.56
7m	4-F	319.13 ± 12.45	0.99
7n	4-Cl	308.78 ± 21.20	1.13
7o	4-Br	259.22 ± 18.36	2.33
7p	4-CH_3	332.18 ± 24.12	1.05
7q	4-CH_3O	319.22 ± 12.36	0.93
7r	4-NO_2	295.26 ± 21.08	0.61
7s	_	312.00 ± 21.12	0.55

sovlent was removed leaving oil which was dissolved in ethyl acetate (20 mL) and extracted with water (40 mL). After drying the organic layer with anhydrous Na_2SO_4 and evaporating the solvent under reduced pressure a solid appeared. The solid was recrystallized from ethanol to obtain the compound **2**.

*4.1.2. General procedure for the preparation of compounds **3***

In the three-necked flask under a nitrogen atmosphere, compound **2**(1 mmol) was dissolved in dry acetone (10 mL), and then added anhydrous potassium carbonate (2 mmol). After that, the acetone solution containing dibromomethane (1 mmol) was added dropwise then refluxed for 24 h. The reaction solution was evaporated reduced pressure distillation. The appropriate amount of water was added in the residue, and extracted with ethyl acetate (3 × 40 mL). Combined organic layer and dried with anhydrous magnesium sulfate. Then the solvent was removed by reduced pressure steam to give the crude product, the crude product is purified by column chromatography [eluant: V (petroleum ether): V (ethyl acetate) = 4: 1] to give white solid (compounds **3**).

*4.1.3. General procedure for the preparation of cinnamic acids (compounds **5a**–**5o**)*

A mixture of aromatic aldehydes (3.2 mmol), malonic acid (3.87 mmol), piperidine (0.387 mmol) was dissolved in pyridine and stirred on 85 °C for 24 h. The pyridine was removed at the vacuum. The reaction mixture was poured into water and washed with HCl. And the precipitate was filtered and washed with hexane for three times, and dried under vacuum to afford the cinnamic acids.

Table 2
PLK1 inhibitory activity of the selected compounds.

Compound	PLK1 (IC_{50}, μM)
7f	0.19
7k	0.03
7l	1.55
7m	0.59
7n	1.55
7p	1.05
7q	0.19
7r	0.12
7s	0.09
poloxin	2.31

*4.1.4. General procedure for the preparation of compounds **6***

A stirred solution of compound 3 (0.1 mol) in ethanol (50 mL) was treated with the hydrazine hydrate (85%), under 90 °C for 5 d. The sovlent was removed leaving oil which was dissolved in ethyl acetate (20 mL) and extracted with water (40 mL). After drying the organic layer with anhydrous Na_2SO_4 and evaporating the solvent under reduced pressure a solid appeared. The solid was recrystallized from ethanol to obtain the compound **6**.

*4.1.5. General procedure for the preparation of target compounds **7a**–**7s***

An equimolar compound **6** (0.001 mol) and substituted cinnamic acid in anhydrous CH_2Cl_2 was stirred for 8 h with carbodiimide hydrochloride (0.0015 mmol) and N-hydroxybenzotriazole (0.0005 mmol). Then reaction mixture was extracted twice using ethyl acetate and water. The organic layer was evaporated and recrystallized from ethanol to give the title compounds.

*4.1.5.1. (E)-N′-(3-(2-fluorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (**7a**).* White powder, mp: 186–188 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.323–4.375 (m, 4H), 6.890–6.912 (d, J = 6.6 Hz, 1H), 6.993 (s, 1H), 7.101–7.117 (m, 2H), 7.273–7.285 (m, 2H), 7.357–7.363 (m, 1H), 7.506 (s, 1H), 7.737 (s, 1H). 9.231 (s, 1H), 9.312 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 60.38, 61.59, 109.77, 112.58, 115.37, 119.77, 120.55, 122.50, 123.81, 124.11, 126.88, 128.03, 134.21, 149.32, 151.55, 158.05, 165.77, 166.92. MS (ESI): 343 ($C_{18}H_{16}FN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}FN_2O_4$: C, 63.15; H, 4.42; N, 8.18%. Found: C, 63.32; H, 4.35; N, 8.07%.

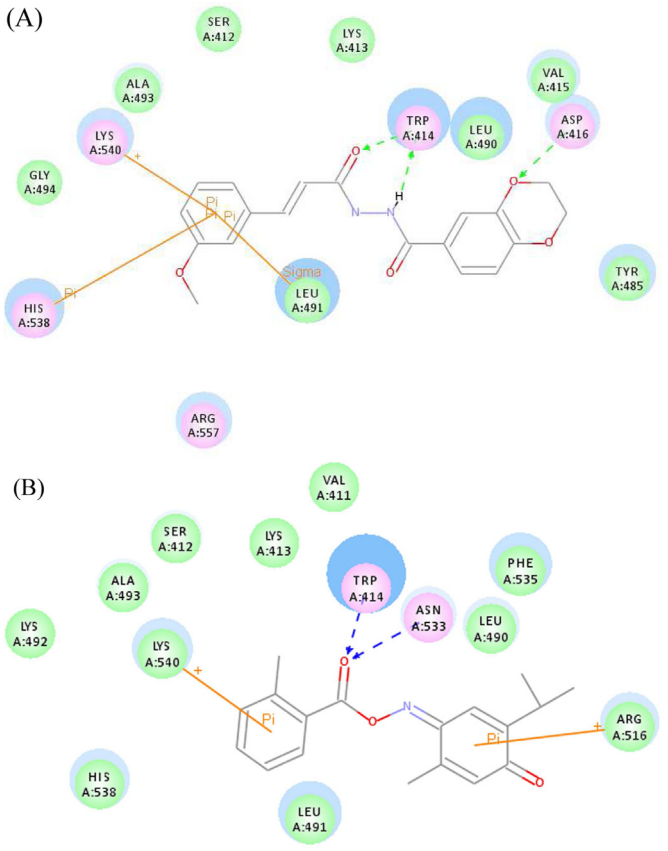


Fig. 2. 2D Ligand interaction diagram of compound **7k** with PLK1. For clarity, only interacting residues are displayed.

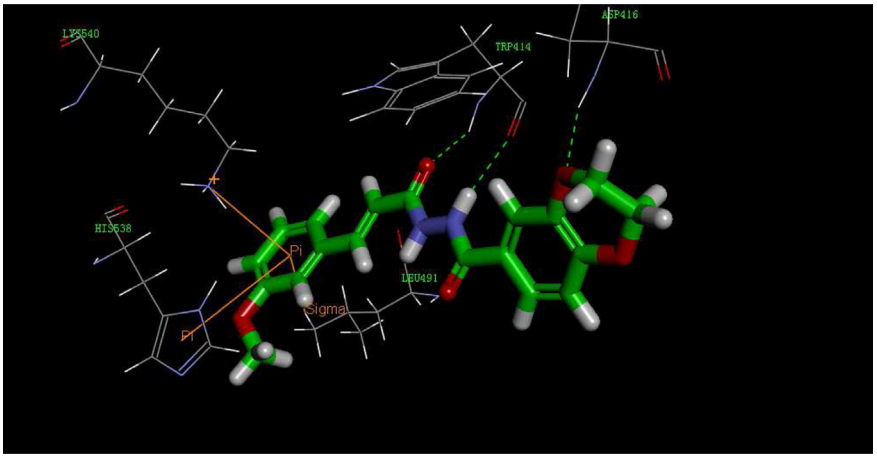


Fig. 3. 3D model of the interaction between compound **7k** and PLK1 binding site.

*4.1.5.2. (E)-N′-(3-(2-chlorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (**7b**).* White powder, mp: 229–230 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.223–4.256 (m, 4H), 6.891 (s, 1H), 7.072–7.226 (m, 2H), 7.356–7.368 (d, J = 3.6 Hz, 1H), 7.502–7.711 (m, 2H), 7.805–7.876 (m, 1H), 7.906–7.998 (m, 2H), 9.135 (s, 1H), 9.235 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.23, 62.18, 108.90, 113.06, 118.33, 122.19, 125.05, 126.74, 128.11, 129.23, 130.24, 132.02, 132.88, 135.71, 148.97, 150.04, 163.54, 166.02. MS (ESI): 359 ($C_{18}H_{16}ClN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}ClN_2O_4$: C, 60.26; H, 4.21; N, 7.81%. Found: C, 60.19; H, 4.33; N, 7.76%.

*4.1.5.3. (E)-N′-(3-(2-bromophenyl)acryloyl)-2,3-dihydrobenzo[b] [1,4]dioxine-6-carbohydrazide (**7c**).* White powder, mp: 251–252 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.481–4.553 (m, 4H), 6.669–6.721 (m, 3H), 6.910–7.111 (m, 3H), 7.275–7.288 (m, 1H), 7.505 (s, 1H), 7.736–7.812 (m, 1H), 9.013 (s, 1H), 9.123 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 60.24, 60.87, 110.06, 111.41, 119.33, 121.02, 123.71, 124.93, 127.26, 127.39, 129.22, 131.44, 138.07, 141.62, 150.08, 151.66, 165.08, 167.11. MS (ESI): 403 ($C_{18}H_{16}BrN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}BrN_2O_4$: C, 53.62; H, 3.75; N, 6.95%. Found: C, 53.81; H, 3.66; N, 7.15%.

*4.1.5.4. (E)-N′-(3-(o-tolyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7d**).* White powder, mp: 250–251 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.556 (s, 3H), 4.532–4.637 (m, 4H), 6.590–6.650 (d, J = 18.0 Hz, 1H), 6.894 (s, 1H), 7.101–7.211 (m, 4H), 7.256–7.278 (m, 2H), 7.359–7.506 (m, 1H), 9.250 (s, 1H), 9.733 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 20.18, 59.98, 60.47, 109.36, 111.46, 118.34, 120.42, 125.47, 126.51, 127.21, 128.28, 130.72, 133.11, 135.74, 140.38, 149.29, 151.63, 164.54, 166.83. MS (ESI): 339 ($C_{19}H_{19}N_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28%. Found: C, 67.31; H, 5.50; N, 8.36%.

*4.1.5.5. (E)-N′-(3-(2-methoxyphenyl)acryloyl)-2,3-dihydrobenzo[b] [1,4]dioxine-6-carbohydrazide (**7e**).* White powder, mp: 247–248 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.557 (s, 3H), 4.612–4.678 (m, 4H), 6.99 (s, 1H), 7.10–7.11 (m, 2H), 7.267–7.328 (m, 2H), 7.589–7.695 (d, J = 31.8 Hz, 1H), 7.855–7.936 (m, 3H), 9.005 (s, 1H), 9.336 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 54.82, 61.28, 62.17, 109.06, 112.36, 113.51, 116.38, 121.02, 122.38, 124.60, 125.27, 128.28, 131.55, 140.03, 150.08, 151.63, 158.67, 163.54, 165.82. MS (ESI): 355 ($C_{19}H_{19}N_2O_5$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91%. Found: C, 64.32; H, 5.21; N, 8.03%.

*4.1.5.6. (E)-N′-(3-(2-nitrophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (**7f**).* White powder, mp: 204–206 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.32–4.37 (m, 4H), 6.589–6.690 (d, J = 30.3 Hz, 1H), 6.709–6.781 (d, J = 21.6 Hz, 1H), 6.991–7.012 (m, 2H), 7.150–7.179 (m, 2H), 7.257–7.322 (m, 2H), 7.605–7.830 (m, 1H), 9.250 (s, 1H), 9.373 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.08, 62.83, 108.96, 112.61, 117.59, 122.04, 127.02, 128.11, 129.44, 131.03, 132.17, 136.08, 140.15, 147.95, 150.48, 151.66, 164.81, 165.91. MS (ESI): 370 ($C_{18}H_{16}N_3O_6$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}N_3O_6$: C, 58.54; H, 4.09; N, 11.38%. Found: C, 58.49; H, 4.15; N, 11.43%.

*4.1.5.7. (E)-N′-(3-(3-fluorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (**7g**).* White powder, mp: 229–230 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.133–4.178 (m, 4H), 6.675–6.697 (m, 1H), 6.897–6.921 (m, 2H), 6.989–7.002 (d, J = 3.9 Hz, 1H), 7.159 (s, 1H), 7.352 (s, 2H), 7.527–7.612 (m, 2H), 9.203 (s, 1H), 9.510 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 60.02, 60.96, 109.06, 112.14, 113.26, 115.70, 117.04, 121.02, 125.90, 128.24, 130.15, 132.49, 136.56, 151.43, 152.08, 159.52, 163.05, 167.12. MS (ESI): 343 ($C_{18}H_{16}FN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}FN_2O_4$: C, 63.15; H, 4.42; N, 8.18%. Found: C, 63.32; H, 4.20; N, 8.36%.

*4.1.5.8. (E)-N′-(3-(3-chlorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4] dioxine-6-carbohydrazide (**7h**).* White powder, mp: 221–223 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.298–4.311 (m, 4H), 6.893–6.906 (d, J = 3.9 Hz, 1H), 7.003 (s, 1H), 7.106–7.118 (m, 2H), 7.279–7.311 (m, 2H), 7.553–7.596 (m, 1H), 7.670–7.698 (m, 2H), 9.731 (s, 1H), 9.838 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.11, 62.37, 107.66, 111.46, 118.04, 122.74, 124.43, 125.16, 126.75, 127.23, 131.60, 134.44, 136.19, 138.82, 149.42, 150.61, 165.89, 167.08. MS (ESI): 359 ($C_{18}H_{16}ClN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}ClN_2O_4$: C, 60.26; H, 4.21; N, 7.81%. Found: C, 60.33; H, 4.35; N, 7.69%.

*4.1.5.9. (E)-N′-(3-(3-bromophenyl)acryloyl)-2,3-dihydrobenzo[b] [1,4]dioxine-6-carbohydrazide (**7i**).* White powder, mp: 228–230 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.232–4.267 (m, 4H), 6.710–6.781 (m, 2H), 6.899 (s, 1H), 7.176–7.215 (m, 2H), 7.356–7.379 (m, 1H), 7.507–7.587 (m, 1H), 7.733–7.789 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 60.88, 61.25, 108.83, 112.49, 117.14, 122.76, 125.24, 128.61, 128.98, 130.07, 131.13, 132.61, 135.19, 136.82, 150.41, 151.66, 164.53, 166.83. MS (ESI): 403 ($C_{18}H_{16}BrN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}BrN_2O_4$: C, 53.62; H, 3.75; N, 6.95%. Found: C, 53.81; H, 3.66; N, 7.03%.

Table 3
The docking calculation of the synthesized compounds (**7a**–**7s**).

Compound	-CDOCKER_ENERGY
7a	30.4561
7b	29.5392
7c	34.6617
7d	28.6713
7e	25.8179
7f	37.1536
7g	31.8337
7h	31.9411
7i	33.1001
7j	33.6739
7k	37.7233
7l	35.0481
7m	36.2040
7n	35.1197
7o	34.1645
3p	35.7668
7q	36.1167
7r	37.1974
7s	37.3372

*4.1.5.10. (E)-N′-(3-(m-tolyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7j**).* White powder, mp: 251–253 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.789 (s, 3H), 4.322–4.373 (m, 4H), 6.659 (s, 1H), 6.872–6.890 (d, J = 5.4 Hz, 1H), 7.005 (s, 1H), 7.230–7.261 (m, 2H), 7.572–7.628 (m, 3H), 7.775–7.798 (m, 1H), 9.311 (s, 1H), 9.556 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 21.15, 60.26, 61.94, 110.34, 112.37, 116.38, 120.39, 121.93, 124.21, 126.83, 128.27, 129.46, 135.20, 136.74, 139.49, 151.44, 152.01, 163.75, 165.96. MS (ESI): 339 ($C_{19}H_{19}N_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28%. Found: C, 67.58; H, 5.43; N, 8.19%.

*4.1.5.11. (E)-N′-(3-(3-methoxyphenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7k**).* White powder, mp: 171–172 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.275–1.384 (m, 3H), 4.277–4.296 (m, 4H), 6.679 (s, 1H), 6.731 (s, 1H), 6.866–6.916 (m, 3H), 7.211–7.238 (m, 2H), 7.637 (s, 1H), 7.741 (s, 1H), 9.158 (s, 1H), 9.613 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 54.21, 61.36, 62.22, 108.06, 110.43, 113.13, 115.28, 117.08, 120.21, 122.82, 128.21, 131.33, 136.73, 138.47, 150.41, 151.37, 159.38, 163.51, 165.93. MS (ESI): 355 ($C_{19}H_{19}N_2O_5$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91%. Found: C, 64.56; H, 5.09; N, 8.02%.

*4.1.5.12. (E)-N′-(3-(3-nitrophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7l**).* White powder, mp: 155–156 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.132–4.157 (m, 4H), 6.829–6.890 (d, J = 18.3 Hz, 1H), 6.910–6.992 (m, 2H), 6.999 (s, 1H), 7.310–7.351 (m, 3H), 7.662–7.696 (m, 2H), 9.567 (s, 1H), 9.738 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.20, 62.75, 109.56, 112.44, 117.85, 120.61, 121.72, 123.83, 129.05, 131.04, 134.47, 138.93, 139.51, 151.06, 151.49, 151.88, 162.04, 165.09. MS (ESI): 370 ($C_{18}H_{16}N_3O_6$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}N_3O_6$: C, 58.54; H, 4.09; N, 11.38%. Found: C, 58.67; H, 3.98; N, 11.43%.

*4.1.5.13. (E)-N′-(3-(4-fluorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7m**).* White powder, mp: 202–203 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.032–4.102 (m, 4H), 6.992–7.021 (m, 1H), 7.275–7.338 (m, 2H), 7.635–7.716 (m, 3H), 7.750–7.798 (m, 3H), 9.223 (s, 1H), 9.351 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: MS (ESI): 343 ($C_{18}H_{16}FN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}FN_2O_4$: C, 63.15; H, 4.42; N, 8.18%. Found: C, 63.08; H, 4.55; N, 8.20%.

*4.1.5.14. (E)-N′-(3-(4-chlorophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7n**).* White powder, mp: 251–252 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.321–4.371 (m, 4H), 6.892–6.910 (d, J = 5.4 Hz, 1H), 7.103–7.125 (m, 3H), 7.335 (s, 1H), 7.510–7.571 (m, 2H), 7.627–7.688 (m, 2H), 9.350 (s, 1H), 9.673 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.83, 62.35, 107.92, 112.44, 118.18, 120.83, 126.34, 127.06, 129.08, 130.71, 130.94, 133.73, 134.62, 137.56, 151.08, 151.92, 164.83, 167.37. MS (ESI): 359 ($C_{18}H_{16}ClN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}ClN_2O_4$: C, 60.26; H, 4.21; N, 7.81%. Found: C, 60.33; H, 4.29; N, 7.66%.

*4.1.5.15. (E)-N′-(3-(4-bromophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7o**).* White powder, mp: 237–238 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.155–4.172 (m, 4H), 6.679 (s, 1H), 6.870–6.911 (m, 2H), 7.094–7.150 (d, J = 16.8 Hz, 1H), 7.274–7.281 (m, 2H), 7.456–7.493 (m, 2H), 7.589–7.630 (d, J = 12.3 Hz, 1H), 9.56 (s, 1H), 9.73 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 61.58, 62.43, 108.06, 110.67, 118.32, 122.33, 124.65, 129.21, 129.54, 129.95, 132.62, 133.78, 134.53, 138.48, 151.73, 151.92, 165.04, 165.92. MS (ESI): 403 ($C_{18}H_{16}BrN_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}BrN_2O_4$: C, 53.62; H, 3.75; N, 6.95%. Found: C, 53.55; H, 3.67; N, 7.11%.

*4.1.5.16. (E)-N′-(3-(p-tolyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7p**).* White powder, mp: 253–255 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.660 (s, 3H), 3.996 (s, 4H), 6.448–6.501 (d, J = 15.9 Hz, 1H), 6.567–6.595 (d, J = 8.4 Hz, 1H), 6.984–7.013 (m, 2H), 7.090–7.144 (m, 1H), 7.144–7.172 (d, J = 8.4 Hz, 1H), 7.208 (s, 1H), 7.330–7.354 (m, 1H), 7.678–7.731 (d, J = 15.9 Hz, 1H), 9.763 (s, 1H), 9.964 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 22.03, 62.28, 62.83, 107.06, 111.43, 118.14, 122.54, 127.50, 128.06, 128.86, 129.03, 130.11, 133.72, 136.17, 137.92, 149.28, 151.08, 163.51, 167.55. MS (ESI): 339 ($C_{19}H_{19}N_2O_4$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28%. Found: C, 67.56; H, 5.28; N, 8.19%.

*4.1.5.17. (E)-N′-(3-(4-methoxyphenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7q**).* White powder, mp: 223–225 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.275 (s, 3H), 4.312–4.324 (m, 4H), 6.693–6.747 (d, J = 16.2 Hz, 1H), 6.929–6.957 (m, 1H), 7.090–7.197 (m, 2H), 7.380–7.410 (m, 2H), 7.440 (s, 1H), 7.551–7.560 (m, 1H), 7.624–7.649 (m, 1H), 9.245 (s, 1H), 9.387 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 55.25, 61.28, 61.28, 108.96, 111.46, 114.15, 114.15, 127.48, 117.04, 121.02, 126.73, 127.48, 128.13, 136.15, 150.07, 150.99, 161.67, 163.17, 165.93. MS (ESI): 355 ($C_{19}H_{19}N_2O_5$, $[M + H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91%. Found: C, 64.29; H, 5.32; N, 7.81%.

*4.1.5.18. (E)-N′-(3-(4-nitrophenyl)acryloyl)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7r**).* White powder, mp: 199–200 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 4.342–4.387 (m, 4H), 6.894–6.912 (d, J = 5.4 Hz, 1H), 6.993 (s, 1H), 7.150–7.181 (m, 2H), 7.277–7.289 (m, 2H), 7.385–7.416 (m, 1H), 7.672–7.726 (m, 2H), 9.254 (s, 1H), 9.478 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 62.11, 62.76, 110.06, 112.33, 116.94, 122.92, 126.05, 126.83, 127.11, 127.85, 129.62, 138.15, 140.58, 148.73, 150.18, 151.09, 164.77, 167.32. MS (ESI): 370 ($C_{18}H_{16}N_3O_6$, $[M + H]^+$). Anal. Calcd for $C_{18}H_{15}N_3O_6$: C, 58.54; H, 4.09; N, 11.38%. Found: C, 58.72; H, 4.15; N, 11.19%.

*4.1.5.19. (E)-N′-cinnamoyl-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (**7s**).* White powder, mp: 220–221 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 2.660 (s, 3H), 6.538–6.589 (d, J = 18.3 Hz, 1H), 6.939–6.967 (m, 1H), 7.403–7.443 (m, 4H), 7.538 (s, 1H), 7.541–7.626 (m, 2H), 7.741–7.793 (d, J = 15.6 Hz, 1H), 9.130 (m, 2H). ^{13}C NMR (400 MHz, $CDCl_3$) δ: 60.15, 61.22, 107.36, 112.14, 118.35, 122.73, 126.58, 127.08, 127.22, 128.03, 129.55, 130.24, 136.18, 139.06, 150.01, 151.66, 163.96, 165.92. MS (ESI): 325 ($C_{18}H_{17}N_2O_4$, $[M + H]^+$). Anal.

Calcd for $C_{18}H_{16}N_2O_4$: C, 66.66; H, 4.97; N, 8.64%. Found: C, 66.81; H, 4.73; N, 8.52%.

4.2. Cytotoxicity test

The cytotoxic activity in vitro was measured using the MTT assay. Cells were cultured in a 96-well plate at a density of $5*10^5$ cells and different concentrations of compounds were respectively added to each well. The incubation was permitted at 37 °C, 5% CO_2 atmosphere for 24 h before the cytotoxicity assessments. 20 μL MTT reagent (4 mg/mL) was added per well 4 h before the end of the incubation. Four hours later, the plate was centrifugaled at 1200 rcf for 5 min and the supernatants were removed, each well was added with 200 μL DMSO. The absorbance was measured at a wave length of 570 nm (OD570 nm) on an ELISA microplate reader. Three replicate wells were used for each concentration and each assay was measured three times, after which the average of CC_{50} and SD were calculated. The cytotoxicity of each compound was expressed as the concentration of compound that reduced cell viability to 50% (CC_{50}). The results were summarized in Table 1.

4.3. Cell proliferation assay

The antitumor activities of compounds **7a**–**7q** were determined using a standard (MTT)-based colorimetric assay (Sigma). Seed 10^4 cells per well into 96-well plates, incubate at 37 °C, 5% CO_2 for 24 h. Then add 100 μL a series concentration of drug-containing medium into wells to maintain the final concentration of drug as 40, 20, 6.67, 2.22, 0.74, 0.25 and 0.082 μg/mL. One concentration should be triplicated. After 48 h, cell survival was determined by the addition of an MTT solution (25 μL of 5 mg/mL MTT in PBS). After 4 h, discard the medium and add 100 μL DMSO; the plates were votexed for 10 min to make completely dissolution. Optical absorbance was measured at 570 nm.

4.4. PLK1 enzyme assay

Purified PLK1 protein was incubated with 150 μM of a substrate peptide (RRRDELMEASFADQEAKV), 10 μM ATP, and 2.0 μCi [γ-33P]-ATP (GE Healthcare) in reaction buffer (20 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT) at 25 °C for 20 min in 96 well plates containing serially diluted compound-DMSO solutions. As the reaction was terminated by the addition of 15 μL of 41.7-fold diluted H_3PO_4 solution, the ASFA peptide (DELMEASFADQEAKV) was trapped on 96 well MultiScreen-PH plate (Millipore) and each well was washed with 133-fold diluted H_3PO_4 solution. The radioactivity of each well was monitored with a liquid scintillation counter (TOPCOUNT HTS, PerkinElmer (Packard)). The IC_{50} values were calculated using Microsoft Excel. The IC_{50} values are the means of at least 2 or more assays.

4.5. Experimental protocol of docking study

The pdb file about the crystal structure of the PLK1 (1umw.pdb) was obtained from the RCSB protein data bank (http://www.pdb.org). The molecular docking procedure was performed by using CDOCKER protocol for receptor–ligand interactions section of Discovery Studio 3.1 (Accelrys Software Inc, San Diego, CA). Initially both the ligands and the receptor were pretreated. For ligand preparation, the 3D structures of all our synthesized compounds were generated with ChemBioOffice 2010 and optimized with MMFF94 method. For enzyme preparation, the hydrogen atoms were added with the pH of the protein in the range of 7.25–7.65.

Acknowledgment

The work was financed by a grant (No. J1103512) from National Natural Science Foundation of China and a grant (No. CXLX13_038) from Jiangsu Ordinary University Graduate Students Scientific Research Innovation Plan of China.

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