



Synthesis and biological evaluation of febrifugine analogues as potential antimalarial agents

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ABSTRACT

Febrifugine is an alkaloid isolated from *Dichroa febrifuga* Lour as the active component against *Plasmodium falciparum*. Adverse side effects have precluded febrifugine as a potential clinical drug. In this study novel febrifugine analogues were designed and synthesized. Lower toxicity was achieved by reducing or eliminating the tendency of forming chemically reactive and toxic intermediates and metabolites. Synthesized compounds were evaluated for acute toxicity and in vitro and in vivo antimalarial efficacy. Some compounds are much less toxic than the natural product febrifugine and existing antimalarial drug chloroquine and are expected to possess wide therapeutic windows. These compounds, as well as the underlying design rationale, may find usefulness in the discovery and development of new antimalarial drugs.

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1. Introduction

Malaria is one of the most common infectious diseases in over 100 countries in Africa, Southeast Asia, and South America. It kills more people than any other communicable disease except for tuberculosis. Worldwide, malaria affects approximately 300 million patients, leading to more than 2 million death a year.¹ The increasing prevalence of multiple drug resistant strains of *Plasmodium falciparum* in most malaria endemic areas has significantly reduced the efficacy of current antimalarial drugs for prophylaxis and treatment of this disease. For instance, resistance to the inexpensive antimalarial mainstays, such as chloroquine, is worldwide. Similarly, resistance to mefloquine, which was proposed as the drug of choice for chloroquine-resistant malaria, has been reported from Africa and Southeast Asia.^{2,3} Therefore, medicinal agents based on novel mode of action are required to overcome the emergence of resistance and to control an ever-increasing number of epidemics caused by the malaria parasite.

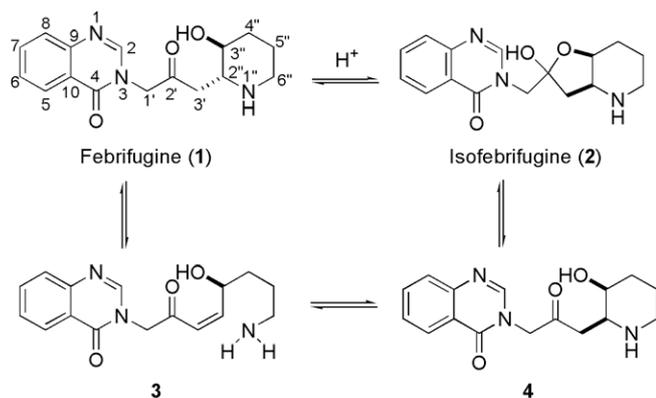
Febrifugine (**1**) and isofebrifugine (**2**) were isolated as the active components against malaria in the Chinese herb Chang Shan (*Dichroa febrifuga* Lour),^{4,5} which has been employed by the local people as medicine against fevers caused by malaria parasites for a long time. Febrifugine acts by impairing haemazoin formation required for maturation of the parasite at the trophozoite stage.⁶ The use of febrifugine as antimalarial agent is initially appealing not only because of its rapid effect and no drug resistance, but also because of

its availability. Subsequent pre-clinical researches have found that febrifugine possesses adverse side effects. Strong liver toxicity has precluded febrifugine as a clinical drug.^{7–9} Major drug toxicities can be grouped into five categories in terms of the underlying toxicity mechanism: (I) on-target, or mechanism-relayed, toxicity; (II) hypersensitivity and related immunological reactions; (III) off-target pharmacology; (IV) biological activation to toxic metabolites; (V) idiosyncratic toxicities. One concept in the study of toxicity is the so-called ‘metabolic activation’ of drugs and other chemicals. One explanation is that drugs can be transformed into reactive electrophiles, which bind covalently with proteins, DNA and low-molecular-mass nucleophiles such as glutathione (GSH).

Total synthesis and structural modifications of febrifugine have been made.^{10–16} It has been elucidated the essential role played by the 4-quinazolinone ring and 1"-amino group in the appearance of activity. It is also known that febrifugine **1** and isofebrifugine **2** are in equilibrium when they are in solution (Scheme 1). This equilibrium most likely follows a three-step sequence: (i) retro-Michael reaction (**1**→**3**); (ii) Michael addition (**3**→**4**); (iii) hemi-ketal cyclization (**4**→**2**). Intermediate **3**, as a Michael acceptor, is highly electrophilic. If half-life of **3** is sufficiently long, then it is very possible that this electrophile will non-selectively alkylate biomolecules such as proteins, peptides, DNA, or RNA inside the host cell, resulting in the observed toxicity.

Febrifugine could be metabolized to the corresponding arene oxide **5** by cytochrome P-450 enzymes (Scheme 2). When arene oxide **5** escapes deactivation process by certain enzymes such as epoxide hydrase or glutathione S-transferase, toxicity can result because this reactive electrophile will form covalent adducts with

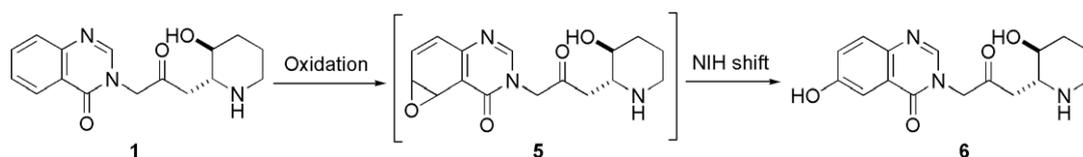
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Scheme 1.

DNA, RNA, proteins, or other biomolecules of the host. Such binding can cause mutations and result in cell damage. In a recent metabolic study of febrifugine, Oshima and co-workers isolated metabolite **6**.¹⁴ This study indicates that arene oxide **5** is most probably a short-lived reactive metabolic intermediate because metabolite **6** would have been derived from intermediate **5** through the rearrangement known as *NIH shift*.¹⁷

There are two possible ways to make this process unfavorable: (I) Blocking the C-5 or C-6 position of the quinoline ring; and (II) Increasing the oxidation potential of the molecule. As part of an ongoing malaria chemotherapy project in our laboratory, we undertook the synthesis and antimalarial activity evaluation of some febrifugine analogues (compounds **7–22**, shown in Fig. 1). Lower toxicity of these newly designed compounds will be achieved by reducing or eliminating the tendency to form chemically reactive and toxic intermediates. Formation of five-membered rings are kinetically more favored than that of six-membered rings due to entropy effect. In the case of compounds **7–22** where the original piperidine ring was replaced by a pyrrolidine ring, retro-Michael reaction would less likely occur. Also, the half-life of the corresponding Michael acceptor, once formed, would be much shorter. Thus, the host is less exposed to toxic electrophiles resulted from the retro-Michael reaction of the administered drug. For compounds **8–16**, an extra nitrogen atom or a substitution group was also introduced on the aromatic ring to block the C-5 or C-6 position of the quinazolinone ring or to increase the oxidation potential of the molecule. Compounds **17** and **18** each bear a bulky group, on C-5 or C-6 position of the quinazolinone ring. Compounds **19** and **20** each bear a benzyloxy group on C-3'' position of the quinazolinone ring. These compounds are designed to block the aforementioned unwarranted metabolic pathway. Overall, these compounds closely resemble febrifugine itself by possessing a planar aromatic ring, a 1''-amino group and C-2', C-3'' O-functionality and are therefore expected to possess same or similar mode of action. Meanwhile, they would be much less likely to produce toxic intermediates because of the replacement of the original piperidine ring by a pyrrolidine moiety, the blocking of C-5 and C-6 position or the increase of the oxidation potential of the molecule.



Scheme 2.

As a comparative study, compounds **21** and **22** were also designed. These compounds each bear an electron-donating methoxy group on the C-7 or C-8 position of the aromatic ring. These compounds have comparable or even greater tendency to undergo oxidation. Biological data from these compounds should further validate, or nullify, the hypothesis that some oxidized febrifugine metabolites have contributed to the observed toxicity.

2. Chemistry

Scheme 3 illustrated the synthesis of compound **7**. Other compounds (**8–22**) were synthesized by similar procedures. The known alcohol **23**, available from borane reduction of commercially available *trans*-4-benzyloxy-*N*-Cbz-*L*-proline, was converted to the corresponding alkyl iodide **24**. Coupling between **24** and vinyl magnesium bromide, in the presence of copper(I) iodide, furnished alkene **25** in good yield.¹⁸ Although initial oxidation of alkene **25** by *m*CPBA to give oxirane **26** was not successful, the transformation was smooth and high-yielding via a three-step sequence: (i) dihydroxylation; (ii) tosylation of the primary alcohol; and (iii) epoxide ring formation facilitated by potassium carbonate in methanol. 3*H*-Quinazolin-4-one condensed with oxirane **26**, to furnish corresponding alcohol **27**, as a pair of diastereomers, this follows the methods of Ogasawara et al.¹⁰ Compound **27** then underwent TPAP oxidation¹⁹ and hydrogenolysis, to afford compound **7**. The synthesis of compounds **8–22** follows similar route. The respective aromatic moieties are available from previously published procedures.^{20–22}

3. Results and discussion

Synthesized compounds (**7–22**) were tested against *P. falciparum* clones W-2, a chloroquine resistant cell line for in vitro efficacy. All compounds were also evaluated for antimalarial efficacy and toxicity in mouse models. The data are summarized in Table 1. Most compounds (**7–16**, **19–22**) have exhibited comparable or superior in vitro and in vivo antimalarial activity, as compared to parent natural product febrifugine. This confirms earlier observation that it is crucial to possess a planar aromatic ring, a 1''-amino group and C-2', C-3'' O-functionality in order to preserve the excellent antimalarial activity of febrifugine. Compounds **17** and **18**, bearing a bulky *tert*-butyl group at the C-5 or C-6 position of the aromatic ring, are less active. The acute toxicity study of new compounds was conducted and the MTDs (maximum tolerated dose) were determined. Compound **7**, bearing a pyrrolidine ring instead of the original piperidine, becomes 4 times less toxic as compared to febrifugine itself. Compounds **8–18**, where the aromatic ring has either an extra nitrogen atom, an electron-withdrawing group, or a bulky group, have shown drastically reduced toxicity.

Compounds **19** and **20** each bears a benzyloxy group on C-3'' position of the aromatic ring. They possess excellent in vitro antimalarial activity. The IC_{50} 's are comparable to compounds **8** and **15**, which bear a free hydroxyl group on C-3'' position of the aromatic ring. It is interesting to note that the in vivo efficacy of compounds **19** and **20** is superior to that of **8** and **15**. The toxicity level is also elevated. Compounds **19** and **20**, with a benzyloxy group on

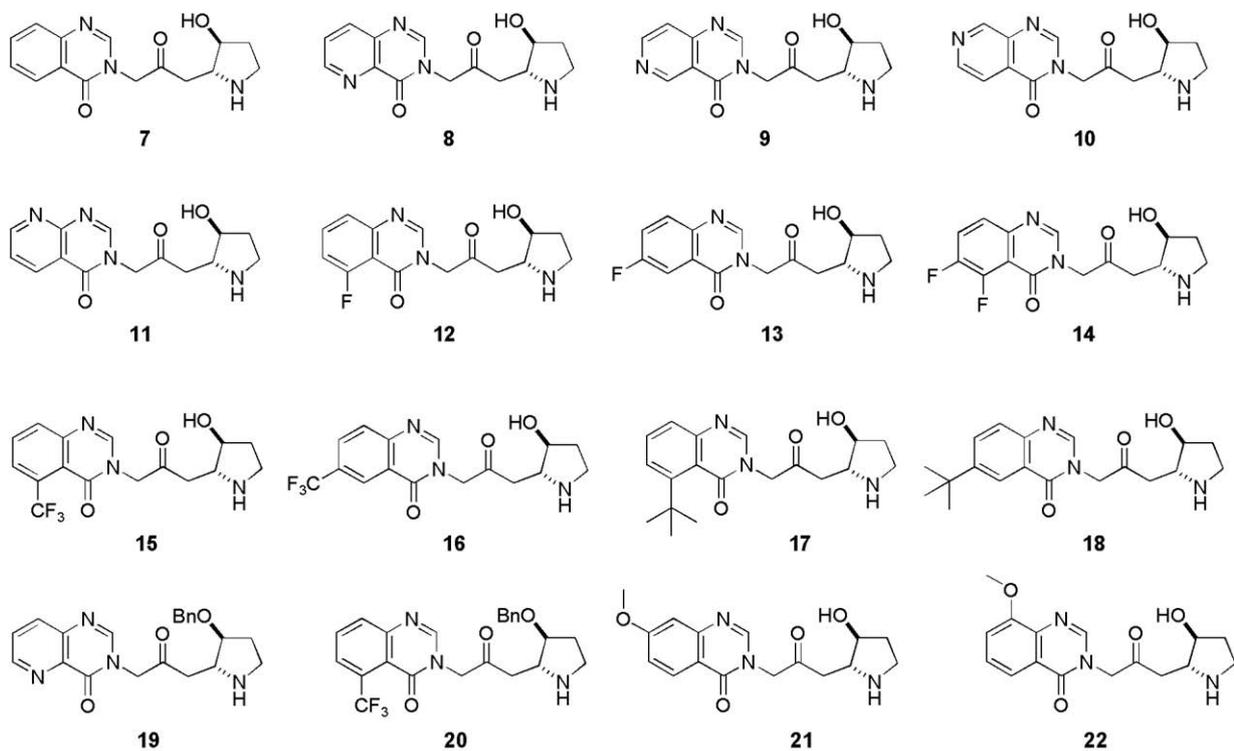
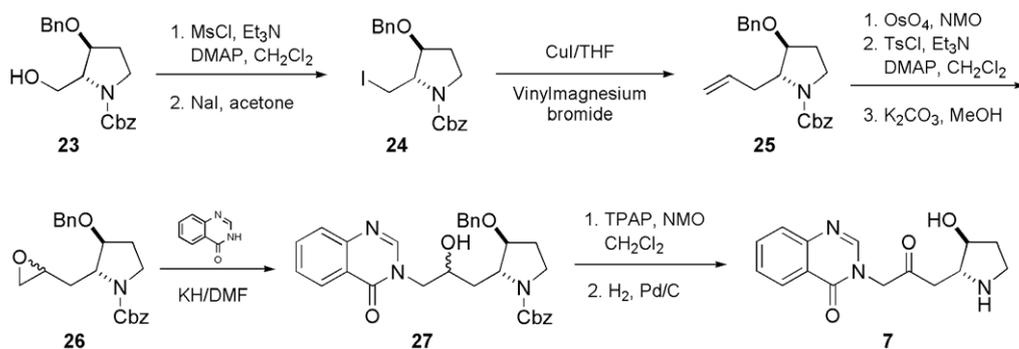


Figure 1.



Scheme 3.

C-3' position of the aromatic ring, are more lipophilic. Consequently, they may possess improved bioavailability.

Although the antimalarial efficacy of compounds **21** and **22** is about the same as that of compound **7**, these compounds are considerably more toxic. Compounds **21** and **22** each possess an electron-donating methoxy group attached to the C-7 or C-8 of the aromatic ring and possess even greater tendency to undergo oxidation than that of compound **7**. These biological data further validate the hypothesis that some oxidized febrifugine metabolites have contributed to the observed toxicity.

Antimalarial potency was also measured by determination of the MCD (minimum clearance dose), a commonly used efficacy measurement. The therapeutic indices, which were obtained by using the MCDs as the effective parameters, were calculated. Based on the therapeutic-index data, compounds **8–16**, **19**, and **20** are much less toxic than the natural product febrifugine and existing antimalarial drug chloroquine and are expected to possess wide therapeutic windows. Four compounds (**17**, **18**, **21**, and **22**) possess narrow therapeutic windows. They are either less active (compounds **17**, **18**) or quite toxic (compounds **21**, **22**).

In conclusion, new febrifugine analogues were designed and synthesized. Some of these compounds (**8**, **9**, **19**, **20**) possess a therapeutic index over ten times superior to that of the commonly used antimalarial drug chloroquine. These compounds, as well as the underlying design rationale, may find usefulness in the discovery and development of new antimalarial drugs.

4. Experimental

4.1. Chemistry

Melting points were determined on a Mettler FP62 melting point apparatus and are uncorrected. Unless otherwise noted, all nonaqueous reactions were performed under an oxygen-free atmosphere of nitrogen with rigid exclusion of moisture from reagents and glassware. Analytical thin layer chromatography (TLC) was performed using EM Reagents 0.25 mm silica gel 60-F plates. Visualization of the developed chromatogram was performed by UV absorbance, aqueous potassium permanganate, or ethanolic anisaldehyde. Liquid chromatography was performed using a force

Table 1
Antimalarial efficacy and toxicity evaluation^a

Test compounds	In vitro W2-strain (IC ₅₀ , ng/mL)	In vivo <i>P. berghei</i> (ED ₅₀ , mg/kg)	MCD (mg/kg)	MTD (mg/kg)	TI ^b
7	0.56	1.6	8.4	144	17
8	0.12	0.44	3.1	345	111
9	0.14	0.49	2.9	310	107
10	0.31	1.2	6.6	338	51
11	0.34	1.3	5.9	439	74
12	0.29	1.0	7.1	399	56
13	0.26	1.1	6.8	425	63
14	0.40	1.7	8.9	379	43
15	0.23	0.74	4.7	335	71
16	0.28	1.5	7.9	440	56
17	2.9	8.7	39	429	11
18	3.4	10	42	504	12
19	0.14	0.14	0.77	102	132
20	0.19	0.12	0.69	88	128
21	0.62	1.7	5.6	55	10
22	0.57	1.5	4.9	51	10
Febrifugine	0.76	2.3	12	35	3
Chloroquine	224	4.5	40.0	240	6

^a Antimalarial activities were determined after i.p. administration of the compounds once daily for 4 days to infected mice.

^b Therapeutic indices were calculated as MTD divided by MCD.

flow (flash chromatography) of the indicated solvent system on EM Reagents Silica Gel 60 (70–230 mesh). Preparative TLC was performed using Whatman Silica Gel C8 TLC plates (PLK5F). ¹H NMR spectra were recorded in deuteriochloroform, unless otherwise noted, on a Bruker Avance 600 spectrometer at the frequency of 600.1 MHz. Chemical shifts are reported in parts per million on the δ scale from an internal standard of tetramethylsilane. Data are reported as follows: chemical shifts, multiplicity (*s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *qn* = quintet, *m* = multiplet, and *br* = broad), coupling constant in Hertz, integration, and assignment. ¹³C NMR spectra were recorded in deuteriochloroform, unless otherwise noted, on a Bruker Avance 600 spectrometer at the frequency of 150.9 MHz. Chemical shifts are reported in parts per million on the δ scale from an internal standard of tetramethylsilane. Combustion analyses were performed by Atlantic Microlab, Inc. (Norcross, Georgia). All purified compounds possess a purity of at least 95%. When necessary, solvents and reagents were dried as follows: ether, tetrahydrofuran, benzene, and toluene were stored and distilled from sodium benzophenone ketyl; dichloromethane, triethylamine, pyridine, and hexane were distilled over calcium hydride. Unless otherwise stated, the reagents were purchased from Fisher Scientific, Aldrich Chemical Company, Lancaster, or Fluka, and used as received.

4.1.1. 3-Benzyloxy-2-iodomethyl-pyrrolidine-1-carboxylic acid benzyl ester (24)

Mesyl chloride (MsCl, 8.0 mL) was added into a stirred solution of alcohol **23** (25.3 g), triethylamine (Et₃N, 30 mL), and 4-dimethylaminopyridine (DMAP, 1.0 g) in 200 mL of methylene chloride (CH₂Cl₂) in an ice-water bath. The reaction mixture was then warmed to room temperature and stirred for additional 2 h. Solvent was evaporated. The resulting slurry was dissolved in ethyl acetate (180 mL), washed with water (150 mL), then aqueous sodium bicarbonate (80 mL), and brine (70 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the essentially pure mesylate. This mesylate (29.5 g) and sodium iodide (NaI, 23.0 g) were dissolved in 180 mL of acetone. The resulting solution was then heated at reflux for 12 h. Solvent was evaporated. The resulting slurry was dissolved in ethyl acetate (150 mL), washed with water (130 mL), then aqueous sodium bicarbonate (60 mL), and brine (50 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the crude

product. Silica gel flash chromatography (15% ethyl acetate in hexanes) furnishes **24** as viscous oil. Yield: 85% (two steps). ¹H NMR: 7.31–7.27 (m, 10H), 5.34 (m, 2H), 4.61 (m, 2H), 4.09 (m, 1H), 3.85 (m, 1H), 3.57 (m, 2H), 3.38 (dd, *J* = 11 and 5.4 Hz, 1H), 3.24 (dd, *J* = 11 and 4.2 Hz, 1H), 1.94 (m, 2H); ¹³C NMR: 159.4, 140.9, 137.9, 128.7, 128.5, 127.7, 127.6, 127.4, 127.3, 80.2, 76.2, 70.8, 59.0, 39.1, 28.5, 13.5. HRESIMS *m/z* 474.0531 [M+Na]⁺ (calcd for C₂₀H₂₂INNaO₃⁺, 474.0542) (100%). Anal. Calcd for C₂₀H₂₂INO₃: C, 53.23; H, 4.91; I, 28.12; N, 3.10. Found: C, 53.29; H, 4.88; I, 28.21; N, 3.04.

4.1.2. 2-Allyl-3-benzyloxy-pyrrolidine-1-carboxylic acid benzyl ester (25)

Vinyl magnesium bromide [1.0 M in tetrahydrofuran (THF), 53.0 mL] was added into a stirred solution of alkyl iodide **24** (11.0 g) and copper(I) iodide (CuI, 2.5 g) in 100 mL of THF at –78 °C under nitrogen atmosphere. The reaction mixture was kept at –78 °C for 1 h, then slowly warmed to room temperature over a period of 2 h. The reaction mixture was quenched with aqueous sodium bicarbonate (60 mL). After partition between ethyl acetate (120 mL) and water (120 mL), the separated organic layer was washed with brine (55 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the crude product. Silica gel flash chromatography (15% ethyl acetate in hexanes) furnished **25** as viscous oil. Yield: 94%. ¹H NMR: 7.31–7.27 (m, 10H), 5.73 (m, 1H), 5.34 (m, 2H), 5.07 (m, 1H), 4.99 (m, 1H), 4.61 (m, 2H), 4.09 (m, 1H), 3.85 (m, 1H), 3.57 (m, 2H), 2.38 (m, 2H), 1.94 (m, 2H); ¹³C NMR: 158.6, 142.1, 140.5, 138.2, 128.6, 128.4, 127.8, 127.7, 127.5, 127.2, 115.5, 79.2, 75.8, 71.2, 57.6, 38.7, 31.4, 27.5. HRESIMS *m/z* 374.1739 [M+Na]⁺ (calcd for C₂₂H₂₅NNaO₃⁺, 374.1732) (100%). Anal. Calcd for C₂₂H₂₅NO₃: C, 75.19; H, 7.17; N, 3.99. Found: C, 75.24; H, 7.15; N, 3.92.

4.1.3. 3-Benzyloxy-2-oxiranylmethyl-pyrrolidine-1-carboxylic acid benzyl ester (26)

Alkene **25** (9.0 g), osmium tetroxide (OsO₄, 4% in water, 1.0 mL), and *N*-methyl morpholine *N*-oxide (NMO, 10.5 g) were dissolved in 80 mL of water and 80 mL of tetrahydrofuran (THF). The resulting heterogeneous solution is stirred vigorously for 4 days at room temperature. After partition between ethyl acetate (150 mL) and water (100 mL), the separated organic layer was washed with brine (70 mL), dried over anhydrous sodium sulfate,

and evaporated in a rotary evaporator under reduced pressure to furnish the essentially pure diol. Tosyl chloride (TsCl, 4.6 g) was added into a stirred solution of this diol (8.9 g), triethylamine (Et₃N, 7.0 mL), and 4-dimethylaminopyridine (DMAP, 300 mg) in 100 mL of methylene chloride (CH₂Cl₂) in an ice-water bath. The reaction mixture was then warmed to room temperature and stirred for additional 2 h. Solvent was evaporated. The resulting slurry was dissolved in ethyl acetate (100 mL), washed with water (80 mL), then aqueous sodium bicarbonate (80 mL), and brine (50 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the essentially pure mono-tosylate. Potassium carbonate (K₂CO₃, 1.0 g) was added into a stirred solution of this mono-tosylate (11.4 g) in 25 mL of methanol (MeOH) at room temperature. After 4 h, reaction mixture was partitioned between ethyl acetate (80 mL) and water (80 mL), the separated organic layer was then washed with brine (40 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the crude product. Silica gel flash chromatography (25% ethyl acetate in hexanes) furnishes **26** as viscous oil. Yield: 76% (three steps). ¹H NMR: 7.30–7.22 (m, 10H), 5.37 (m, 2H), 4.65 (m, 2H), 3.85 (m, 1H), 3.57 (m, 2H), 3.34 (m, 1H), 3.05 (m, 1H), 2.79 (m, 1H), 2.52 (m, 1H), 1.94 (m, 2H), 1.87–1.82 (m, 2H); ¹³C NMR: 158.8, 141.7, 139.3, 128.5, 128.2, 127.9, 127.6, 127.4, 126.8, 76.2, 73.8, 71.9, 51.4, 47.5, 43.3, 35.8, 34.8, 28.4. HRESIMS *m/z* 390.1677 [M+Na]⁺ (calcd for C₂₂H₂₅NNaO₄⁺, 390.1681) (100%). Anal. Calcd for C₂₂H₂₅NO₄: C, 71.91; H, 6.86; N, 3.81. Found: C, 71.84; H, 6.91; N, 3.85.

4.1.4. 3-Benzoyloxy-2-[2-hydroxy-3-(4-oxo-4H-quinazolin-3-yl)-propyl]-pyrrolidine-1-carboxylic acid benzyl ester (**27**)

Potassium hydride (KH, 30% in mineral oil, 3.6 g) was suspended in 50 mL of dimethylformamide (DMF). It was cooled in an ice-water bath, and solid 3H-quinazolin-4-one (4.0 g) was added in. After 30 min, a solution of oxirane **26** (2.2 g) in 10 mL of DMF was added in. The reaction mixture was then heated at 80 °C for 12 h under nitrogen atmosphere. It was partitioned between ethyl acetate (80 mL) and water (80 mL), separated organic layer was washed with water (3 × 60 mL), then brine (40 mL), dried over anhydrous sodium sulfate, and evaporated in a rotary evaporator under reduced pressure to furnish the crude product. Silica gel flash chromatography (80% ethyl acetate in hexanes) furnished **27** as off white solid. Yield: 77%. Mp: 187 °C. ¹H NMR: 8.28 (d, *J* = 7.5 Hz, 1H), 8.24 (s, 1H), 7.73 (m, 2H), 7.48 (m, 1H), 7.31–7.27 (m, 10H), 5.45 (m, 2H), 5.19 (br, 1H), 4.81 (m, 2H), 4.24 (m, 1H), 4.16 (m, 1H), 3.85 (m, 2H), 3.62 (m, 2H), 3.23 (m, 1H), 1.89 (m, 2H), 1.57 (m, 2H); ¹³C NMR: 168.4, 163.1, 158.7, 145.3, 141.2, 138.2, 134.1, 129.3, 128.9, 128.6, 127.8, 127.6, 127.4, 127.1, 127.0, 126.7, 123.8, 80.8, 77.4, 71.3, 66.4, 55.1, 51.9, 39.4, 32.4, 26.6. HRESIMS *m/z* 536.2170 [M+Na]⁺ (calcd for C₃₀H₃₁N₃NaO₅⁺, 536.2161) (100%). Anal. Calcd for C₃₀H₃₁N₃O₅·HCl: C, 65.51; H, 5.86; Cl, 6.45; N, 7.64. Found: C, 65.56; H, 5.83; Cl, 6.51; N, 7.62.

4.1.5. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (**7**)

A solution of alcohol **27** (2.1 g) in 5 mL of CH₂Cl₂ was added into a stirred slurry of tetrapropylammonium perruthenate (TPAP, 70 mg), *N*-methyl morpholine *N*-oxide (NMO, 936 mg), and grounded molecular sieve (800 mg) in 10 mL of CH₂Cl₂ at room temperature. After 1 h, the reaction mixture was loaded directly into a short column of silica gel and eluted with 5% MeOH/EtOAc. Concentration of the eluant under reduced pressure afforded the corresponding ketone. This ketone (1.89 g) was dissolved in 15 mL of 95% EtOH/H₂O. Three hundred milligrams of 10% Pd on carbon was added in. It was then treated with hydrogen (60 psi)

in a Parr apparatus for 12 h. Solid was filtered off and the solution was evaporated under vacuum to dryness. Recrystallization from ethanol–water (with addition of dilute aqueous HCl solution, 4–5 equiv of HCl) furnishes compound **7** (HCl salt) as off-white crystals. Yield: 901 mg, 76% (two steps). Mp: 217–218 °C. [α]_D²⁵ +24.9 (c 0.51, EtOH). ¹H NMR (CD₃OD): 8.27 (dd, *J* = 7.9, 1.0 Hz, 1H), 7.92 (s, 1H), 7.77 (ddd, *J* = 8.1, 7.9, 1.0 Hz, 1H), 7.71 (dd, *J* = 8.1, 0.9 Hz, 1H), 7.50 (dt, *J* = 7.9, 0.9 Hz, 1H), 5.11 (d, *J* = 17.1 Hz, 1H), 4.96 (d, *J* = 17.1 Hz, 1H), 4.41 (m, 1H), 4.14 (m, 1H), 3.49–3.42 (m, 2H), 2.89 (dd, *J* = 15.9, 4.2 Hz, 1H), 2.81 (dd, *J* = 15.9, 6.1 Hz, 1H), 2.24 (m, 1H), 1.90 (m, 1H); ¹³C NMR (CD₃OD): 204.1, 169.2, 165.2, 147.9, 133.2, 128.1, 127.4, 124.3, 121.9, 75.1, 57.9, 56.4, 39.2, 33.4, 31.7. HRESIMS *m/z* 310.1159 [M+Na]⁺ (calcd for C₁₅H₁₇N₃NaO₃⁺, 310.1168) (100%). Anal. Calcd for C₁₅H₁₇N₃O₃·HCl: C, 55.64; H, 5.60; Cl, 10.95; N, 12.98. Found: C, 55.60; H, 5.63; Cl, 10.99; N, 12.89.

4.1.6. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-pyrido[3,2-*d*]pyrimidin-4-one (**8**)

Yellow crystal. Mp: 261 °C. [α]_D²⁵ +21.8 (c 0.5, EtOH). ¹H NMR (CD₃OD): 9.71 (d, *J* = 7.2 Hz, 1H), 9.24 (d, *J* = 7.2 Hz, 1H), 8.80 (s, 1H), 8.21 (t, *J* = 7.2 Hz, 1H), 5.08 (d, *J* = 17.3 Hz, 1H), 4.92 (d, *J* = 17.3 Hz, 1H), 4.41 (m, 1H), 4.14 (m, 1H), 3.49–3.42 (m, 2H), 2.89 (m, 1H), 2.81 (dd, *J* = 15.1 and 6.1 Hz, 1H), 2.24 (m, 1H), 1.90 (m, 1H); ¹³C NMR (CD₃OD): 202.1, 163.7, 159.2, 151.5, 149.6, 146.4, 132.9, 131.2, 81.0, 57.9, 54.1, 51.9, 44.7, 36.7. HRESIMS *m/z* 311.1116 [M+Na]⁺ (calcd for C₁₄H₁₆N₄NaO₃⁺, 311.1120) (100%). Anal. Calcd for C₁₄H₁₆N₄O₃·HCl: C, 51.78; H, 5.28; Cl, 10.92; N, 17.25. Found: C, 51.82; H, 5.26; Cl, 10.88; N, 17.28.

4.1.7. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-pyrido[4,3-*d*]pyrimidin-4-one (**9**)

Pale-yellow crystal. Mp: 251 °C. [α]_D²⁵ –31.4 (c 0.5, EtOH). ¹H NMR (CD₃OD): 9.54 (s, 1H), 9.19 (d, *J* = 7.5 Hz, 1H), 8.71 (s, 1H), 8.13 (d, *J* = 7.5 Hz, 1H), 5.07 (d, *J* = 17.4 Hz, 1H), 4.90 (d, *J* = 17.4 Hz, 1H), 4.39 (m, 1H), 4.18 (m, 1H), 3.46–3.41 (m, 2H), 2.94 (m, 1H), 2.85 (dd, *J* = 15.1 and 6.1 Hz, 1H), 2.23 (m, 1H), 1.87 (m, 1H); ¹³C NMR (CD₃OD): 202.6, 166.6, 163.6, 159.1, 154.5, 149.0, 147.1, 135.2, 80.3, 56.9, 55.3, 50.9, 44.3, 35.6. HRESIMS *m/z* 311.1128 [M+Na]⁺ (calcd for C₁₄H₁₆N₄NaO₃⁺, 311.1120) (100%). Anal. Calcd for C₁₄H₁₆N₄O₃·HCl: C, 51.78; H, 5.28; Cl, 10.92; N, 17.25. Found: C, 51.74; H, 5.31; Cl, 10.90; N, 17.22.

4.1.8. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-pyrido[3,4-*d*]pyrimidin-4-one (**10**)

Yellow crystal. Mp: 221–222 °C. [α]_D²⁵ +27.4 (c 0.43, EtOH). ¹H NMR (CD₃OD): 9.24 (s, 1H), 8.74 (d, *J* = 7.3 Hz, 1H), 8.49 (d, *J* = 7.3 Hz, 1H), 8.25 (s, 1H), 5.16 (d, *J* = 17.1 Hz, 1H), 5.03 (d, *J* = 17.1 Hz, 1H), 4.37 (m, 1H), 4.23 (m, 1H), 3.44–3.38 (m, 2H), 2.91 (m, 1H), 2.89 (dd, *J* = 15.4 and 6.2 Hz, 1H), 2.27 (m, 1H), 1.92 (m, 1H); ¹³C NMR (CD₃OD): 202.6, 167.8, 164.6, 159.2, 155.2, 154.7, 139.9, 134.8, 79.2, 56.1, 54.9, 51.2, 45.1, 34.8. HRESIMS *m/z* 311.1114 [M+Na]⁺ (calcd for C₁₄H₁₆N₄NaO₃⁺, 311.1120) (100%). Anal. Calcd for C₁₄H₁₆N₄O₃·HCl: C, 51.78; H, 5.28; Cl, 10.92; N, 17.25. Found: C, 51.84; H, 5.24; Cl, 10.99; N, 17.31.

4.1.9. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-pyrido[2,3-*d*]pyrimidin-4-one (**11**)

Yellow crystal. Mp: 213–214 °C. [α]_D²⁵ –19.6 (c 0.42, EtOH). ¹H NMR (CD₃OD): 8.88 (d, *J* = 6.8 Hz, 1H), 8.75 (d, *J* = 6.8 Hz, 1H), 8.28 (s, 1H), 8.19 (t, *J* = 6.8 Hz, 1H), 5.03 (d, *J* = 16.8 Hz, 1H), 4.92 (d, *J* = 16.8 Hz, 1H), 4.37 (m, 1H), 4.24 (m, 1H), 3.45–3.40 (m, 2H), 2.82 (dd, *J* = 15.5, 4.6 Hz, 1H), 2.76 (dd, *J* = 15.5, 6.2 Hz, 1H), 2.28 (m, 1H), 1.84 (m, 1H); ¹³C NMR (CD₃OD): 204.1, 171.8, 167.3, 162.8, 152.1, 142.6, 133.1, 130.9, 77.4, 58.5, 56.8, 38.7, 33.9, 32.1. HRESIMS *m/z* 311.1122 [M+Na]⁺ (calcd for C₁₄H₁₆N₄NaO₃⁺,

311.1120) (100%). Anal. Calcd for $C_{14}H_{16}N_4O_3 \cdot HCl$: C, 51.78; H, 5.28; Cl, 10.92; N, 17.25. Found: C, 51.83; H, 5.25; Cl, 10.81; N, 17.34.

4.1.10. (2R,3S)-5-Fluoro-3-[3-(3-hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (12)

Pale-yellow crystals. Mp: 242 °C. $[\alpha]_D^{25} -33.8$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.32 (s, 1H), 8.20 (d, $J = 7.1$ Hz, 1H), 7.73 (d, $J = 7.2$ Hz, 1H), 7.55 (t, $J = 7.1$ Hz, 1H), 5.10 (d, $J = 17.4$ Hz, 1H), 4.93 (d, $J = 17.4$ Hz, 1H), 4.43 (m, 1H), 4.10 (m, 1H), 3.48–3.40 (m, 2H), 2.82 (m, 1H), 2.77 (dd, $J = 15.2$ and 5.8 Hz, 1H), 2.28 (m, 1H), 1.93 (m, 1H); ^{13}C NMR (CD_3OD): 203.1, 164.1, 158.5, 152.7, 149.3, 147.5, 132.8, 132.2, 129.6, 80.2, 58.6, 53.2, 50.9, 43.8, 35.9. HRESIMS m/z 328.1082 $[M+Na]^+$ (calcd for $C_{15}H_{16}FN_3NaO_3^+$, 328.1073) (100%). Anal. Calcd for $C_{15}H_{16}FN_3O_3 \cdot HCl$: C, 52.71; H, 5.01; Cl, 10.37; F, 5.56; N, 12.30. Found: C, 52.80; H, 5.00; Cl, 10.26; F, 5.51; N, 12.34.

4.1.11. (2R,3S)-6-Fluoro-3-[3-(3-hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (13)

Pale-yellow crystal. Mp: 196–197 °C. $[\alpha]_D^{25} +26.3$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.24 (s, 1H), 7.84 (d, $J = 7.1$ Hz, 1H), 7.53 (s, 1H), 7.46 (d, $J = 7.1$ Hz, 1H), 5.08 (d, $J = 17.3$ Hz, 1H), 4.95 (d, $J = 17.3$ Hz, 1H), 4.48 (m, 1H), 4.15 (m, 1H), 3.47–3.39 (m, 2H), 2.86 (m, 1H), 2.79 (dd, $J = 15.1$ and 5.6 Hz, 1H), 2.32 (m, 1H), 1.89 (m, 1H); ^{13}C NMR (CD_3OD): 202.9, 167.9, 163.6, 147.1, 133.2, 129.7, 128.9, 128.0, 127.7, 78.4, 60.4, 54.0, 51.1, 42.5, 34.5. HRESIMS m/z 328.1066 $[M+Na]^+$ (calcd for $C_{15}H_{16}FN_3NaO_3^+$, 328.1073) (100%). Anal. Calcd for $C_{15}H_{16}FN_3O_3 \cdot HCl$: C, 52.71; H, 5.01; Cl, 10.37; F, 5.56; N, 12.30. Found: C, 52.64; H, 5.05; Cl, 10.42; F, 5.52; N, 12.24.

4.1.12. (2R,3S)-5,6-Difluoro-3-[3-(3-hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (14)

Pale-yellow crystal. Mp: 241 °C. $[\alpha]_D^{25} -11.8$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.31 (s, 1H), 7.80 (d, $J = 6.9$ Hz, 1H), 7.74 (d, $J = 6.9$ Hz, 1H), 5.06 (d, $J = 17.4$ Hz, 1H), 4.92 (d, $J = 17.4$ Hz, 1H), 4.39 (m, 1H), 4.12 (m, 1H), 3.48–3.40 (m, 2H), 2.92 (m, 1H), 2.78 (dd, $J = 14.9$ and 5.8 Hz, 1H), 2.28 (m, 1H), 1.87 (m, 1H); ^{13}C NMR (CD_3OD): 202.8, 162.8, 161.6, 158.5, 152.1, 149.4, 147.2, 132.4, 130.5, 80.7, 58.5, 53.8, 51.4, 43.6, 37.2. HRESIMS m/z 346.0984 $[M+Na]^+$ (calcd for $C_{15}H_{15}F_2N_3NaO_3^+$, 346.0979) (100%). Anal. Calcd for $C_{15}H_{15}F_2N_3O_3 \cdot HCl$: C, 50.08; H, 4.48; Cl, 9.85; F, 10.56; N, 11.68. Found: C, 50.14; H, 4.44; Cl, 9.91; F, 10.49; N, 11.75.

4.1.13. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-5-trifluoromethyl-3H-quinazolin-4-one (15)

Off-white crystals. Mp: 262 °C. $[\alpha]_D^{25} +27.5$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.28 (s, 1H), 7.87 (d, $J = 7.2$ Hz, 1H), 7.59 (d, $J = 7.2$ Hz, 1H), 7.50 (t, $J = 7.2$ Hz, 1H), 5.05 (d, $J = 16.8$ Hz, 1H), 4.84 (d, $J = 16.8$ Hz, 1H), 4.40 (m, 1H), 4.19 (m, 1H), 3.47–3.41 (m, 2H), 2.85 (m, 1H), 2.78 (dd, $J = 15.3$ and 6.2 Hz, 1H), 2.27 (m, 1H), 1.93 (m, 1H); ^{13}C NMR (CD_3OD): 202.8, 162.7, 158.2, 150.5, 148.3, 141.4, 133.3, 132.7, 131.7, 116.8, 80.5, 57.6, 53.6, 50.5, 42.4, 36.5. HRESIMS m/z 378.1050 $[M+Na]^+$ (calcd for $C_{16}H_{16}F_3N_3NaO_3^+$, 378.1041) (100%). Anal. Calcd for $C_{16}H_{16}F_3N_3O_3 \cdot HCl$: C, 49.05; H, 4.37; Cl, 9.05; F, 14.55; N, 10.73. Found: C, 49.12; H, 4.33; Cl, 9.01; F, 14.62; N, 10.82.

4.1.14. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-6-trifluoromethyl-3H-quinazolin-4-one (16)

Off-white crystal. Mp: 206–207 °C. $[\alpha]_D^{25} +16.7$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.26 (s, 1H), 7.81 (d, $J = 7.2$ Hz, 1H), 7.75 (s, 1H), 7.58 (d, $J = 7.2$ Hz, 1H), 5.09 (d, $J = 17.4$ Hz, 1H), 4.98 (d, $J = 17.4$ Hz,

1H), 4.39 (m, 1H), 4.24 (m, 1H), 3.47–3.41 (m, 2H), 2.85 (dd, $J = 15.7$, 4.4 Hz, 1H), 2.77 (dd, $J = 15.7$, 5.9 Hz, 1H), 2.20 (m, 1H), 1.92 (m, 1H); ^{13}C NMR (CD_3OD): 203.3, 167.8, 162.6, 147.3, 137.1, 133.5, 129.8, 128.1, 127.4, 119.2, 76.9, 58.2, 57.1, 38.8, 33.1, 30.9. HRESIMS m/z 378.1035 $[M+Na]^+$ (calcd for $C_{16}H_{16}F_3N_3NaO_3^+$, 378.1041) (100%). Anal. Calcd for $C_{16}H_{16}F_3N_3O_3 \cdot HCl$: C, 49.05; H, 4.37; Cl, 9.05; F, 14.55; N, 10.73. Found: C, 48.98; H, 4.41; Cl, 9.11; F, 14.49; N, 10.67.

4.1.15. (2R,3S)-5-tert-Butyl-3-[3-(3-hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (17)

Off-white crystal. Mp: 193–194 °C. $[\alpha]_D^{25} +12.4$ (c 0.51, EtOH). 1H NMR (CD_3OD): 8.25 (d, $J = 7.2$ Hz, 1H), 8.17 (s, 1H), 7.74 (d, $J = 7.2$ Hz, 1H), 7.63 (t, $J = 7.2$ Hz, 1H), 5.12 (d, $J = 16.9$ Hz, 1H), 5.00 (d, $J = 16.9$ Hz, 1H), 4.34 (m, 1H), 4.12 (m, 1H), 3.49–3.42 (m, 2H), 2.91 (dd, $J = 15.5$, 4.5 Hz, 1H), 2.82 (dd, $J = 15.5$, 5.8 Hz, 1H), 2.22 (m, 1H), 1.89 (m, 1H), 1.31 (s, 9H); ^{13}C NMR (CD_3OD): 202.3, 169.5, 165.1, 152.9, 147.2, 143.1, 133.9, 130.1, 129.9, 77.3, 58.6, 56.4, 39.4, 35.6, 33.6, 31.9, 30.5. HRESIMS m/z 366.1782 $[M+Na]^+$ (calcd for $C_{19}H_{25}N_3NaO_3^+$, 366.1794) (100%). Anal. Calcd for $C_{19}H_{25}N_3O_3 \cdot HCl$: C, 60.07; H, 6.90; Cl, 9.33; N, 11.06. Found: C, 60.13; H, 6.87; Cl, 9.29; N, 11.12.

4.1.16. (2R,3S)-6-tert-Butyl-3-[3-(3-hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-quinazolin-4-one (18)

Off-white crystal. Mp: 190–191 °C. $[\alpha]_D^{25} -10.3$ (c 0.45, EtOH). 1H NMR (CD_3OD): 8.20 (s, 1H), 7.94 (d, $J = 7.1$ Hz, 1H), 7.69 (d, $J = 7.1$ Hz, 1H), 7.56 (s, 1H), 5.09 (d, $J = 17.1$ Hz, 1H), 4.97 (d, $J = 17.1$ Hz, 1H), 4.30 (m, 1H), 4.18 (m, 1H), 3.48–3.40 (m, 2H), 2.93 (dd, $J = 15.9$, 4.6 Hz, 1H), 2.84 (dd, $J = 15.9$, 5.9 Hz, 1H), 2.29 (m, 1H), 1.92 (m, 1H), 1.34 (s, 9H); ^{13}C NMR (CD_3OD): 202.9, 167.9, 164.6, 154.1, 143.2, 134.7, 129.9, 128.8, 128.4, 79.2, 59.1, 56.9, 38.8, 36.2, 34.2, 31.7, 30.2. HRESIMS m/z 366.1803 $[M+Na]^+$ (calcd for $C_{19}H_{25}N_3NaO_3^+$, 366.1794) (100%). Anal. Calcd for $C_{19}H_{25}N_3O_3 \cdot HCl$: C, 60.07; H, 6.90; Cl, 9.33; N, 11.06. Found: C, 60.01; H, 6.93; Cl, 9.37; N, 10.98.

4.1.17. (2R,3S)-3-[3-(3-Benzoyloxy-pyrrolidin-2-yl)-2-oxo-propyl]-3H-pyrido[3,2-d]pyrimidin-4-one (19)

Yellow crystal. Mp: 232–233 °C. $[\alpha]_D^{25} +25.7$ (c 0.5, EtOH). 1H NMR (CD_3OD): 9.61 (d, $J = 7.2$ Hz, 1H), 9.19 (d, $J = 7.2$ Hz, 1H), 8.76 (s, 1H), 8.31 (t, $J = 7.2$ Hz, 1H), 7.22–7.18 (m, 5H), 5.08 (d, $J = 17.2$ Hz, 1H), 4.89 (d, $J = 17.2$ Hz, 1H), 4.69 (m, 2H), 4.40 (m, 1H), 4.12 (m, 1H), 3.48–3.42 (m, 2H), 2.88 (m, 1H), 2.78 (dd, $J = 15.1$ and 6.0 Hz, 1H), 2.26 (m, 1H), 1.92 (m, 1H); ^{13}C NMR (CD_3OD): 204.3, 163.2, 159.9, 151.1, 149.3, 146.1, 137.9, 133.8, 131.6, 128.9, 127.8, 127.5, 81.1, 75.1, 57.3, 54.8, 51.3, 44.2, 36.2. HRESIMS m/z 401.1586 $[M+Na]^+$ (calcd for $C_{21}H_{22}N_4NaO_3^+$, 401.1590) (100%). Anal. Calcd for $C_{21}H_{22}N_4O_3 \cdot HCl$: C, 60.79; H, 5.59; Cl, 8.55; N, 13.50. Found: C, 60.83; H, 5.55; Cl, 8.62; N, 13.44.

4.1.18. (2R,3S)-3-[3-(3-Benzoyloxy-pyrrolidin-2-yl)-2-oxo-propyl]-5-trifluoromethyl-3H-quinazolin-4-one (20)

Pale yellow crystals. Mp: 229–230 °C. $[\alpha]_D^{25} -17.9$ (c 0.5, EtOH). 1H NMR (CD_3OD): 8.22 (s, 1H), 7.84 (d, $J = 7.1$ Hz, 1H), 7.62 (d, $J = 7.1$ Hz, 1H), 7.55 (t, $J = 7.1$ Hz, 1H), 7.23–7.19 (m, 5H), 5.09 (d, $J = 16.9$ Hz, 1H), 4.82 (d, $J = 16.9$ Hz, 1H), 4.71 (m, 2H), 4.39 (m, 1H), 4.13 (m, 1H), 3.46–3.40 (m, 2H), 2.89 (m, 1H), 2.79 (dd, $J = 15.4$ and 6.0 Hz, 1H), 2.29 (m, 1H), 1.91 (m, 1H); ^{13}C NMR (CD_3OD): 203.7, 162.1, 158.9, 151.2, 149.1, 140.7, 139.2, 133.5, 132.4, 131.9, 128.9, 127.8, 127.7, 116.8, 80.1, 76.2, 57.9, 52.9, 51.5, 42.1, 33.8. HRESIMS m/z 468.1519 $[M+Na]^+$ (calcd for $C_{23}H_{22}F_3N_3NaO_3^+$, 468.1511) (100%). Anal. Calcd for $C_{23}H_{22}F_3N_3O_3 \cdot HCl$: C, 57.32; H, 4.81; Cl, 7.36; F, 11.83; N, 8.72. Found: C, 57.39; H, 4.76; Cl, 7.42; F, 11.74; N, 8.80.

4.1.19. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-7-methoxy-3H-quinazolin-4-one (21)

Off-white crystal. Mp: 181–182 °C. $[\alpha]_D^{25} +18.3$ (c 0.5, EtOH). ^1H NMR (CD_3OD): 8.13 (s, 1H), 7.72 (s, 1H), 7.43 (d, $J = 7.2$ Hz, 1H), 7.33 (d, $J = 7.2$ Hz, 1H), 5.08 (d, $J = 17.3$ Hz, 1H), 4.92 (d, $J = 17.3$ Hz, 1H), 4.38 (m, 1H), 4.11 (m, 1H), 3.81 (s, 3H), 3.43–3.36 (m, 2H), 2.91 (dd, $J = 15.4, 4.3$ Hz, 1H), 2.79 (dd, $J = 15.4, 6.2$ Hz, 1H), 2.29 (m, 1H), 1.81 (m, 1H); ^{13}C NMR (CD_3OD): 202.5, 168.3, 165.5, 161.3, 188.5, 138.4, 134.5, 130.6, 128.9, 76.4, 58.7, 57.2, 55.9, 38.8, 34.1, 31.4. HRESIMS m/z 340.1285 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{NaO}_4^+$, 340.1273) (100%). Anal. Calcd for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_4\cdot\text{HCl}$: C, 54.32; H, 5.70; Cl, 10.02; N, 11.88. Found: C, 54.41; H, 5.67; Cl, 10.11; N, 11.79.

4.1.20. (2R,3S)-3-[3-(3-Hydroxy-pyrrolidin-2-yl)-2-oxo-propyl]-8-methoxy-3H-quinazolin-4-one (22)

Off-white crystal. Mp: 184–185 °C. $[\alpha]_D^{25} -15.6$ (c 0.5, EtOH). ^1H NMR (CD_3OD): 8.18 (s, 1H), 7.55 (t, $J = 7.3$ Hz, 1H), 7.36 (d, $J = 7.3$ Hz, 1H), 7.19 (d, $J = 7.3$ Hz, 1H), 5.09 (d, $J = 17.5$ Hz, 1H), 5.01 (d, $J = 17.5$ Hz, 1H), 4.43 (m, 1H), 4.08 (m, 1H), 3.77 (s, 3H), 3.51–3.44 (m, 2H), 2.81 (dd, $J = 16.1, 4.3$ Hz, 1H), 2.75 (dd, $J = 16.1, 6.0$ Hz, 1H), 2.27 (m, 1H), 1.92 (m, 1H); ^{13}C NMR (CD_3OD): 203.1, 166.2, 162.3, 160.5, 147.3, 137.4, 132.9, 129.9, 128.7, 80.1, 58.9, 57.3, 56.9, 39.7, 34.8, 30.8. HRESIMS m/z 340.1266 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{NaO}_4^+$, 340.1273) (100%). Anal. Calcd for $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_4\cdot\text{HCl}$: C, 54.32; H, 5.70; Cl, 10.02; N, 11.88. Found: C, 54.25; H, 5.74; Cl, 9.98; N, 11.96.

4.2. Parasite culture

Chloroquine-resistant W2-strain of *P. falciparum* were cultivated in RPMI 1640 media with 6% human erythrocytes supplemented with 10% of human serum. The parasites were cultured in an atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 at 37 °C.

4.3. In vitro drug susceptibility assay^{23,24}

Febrifugine and analogs were tested in a cell-based in vitro drug susceptibility assay to determine if they were capable of interrupting *Plasmodium* metabolism and growth. The semi-automated micro-dilution technique was used to assess the sensitivity of the parasites to the selected compounds. The incorporation of [^3H]hypoxanthine into the parasites was measured as a function of compound concentration to determine IC_{50} values.

4.4. In vivo efficacy test²⁵

In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council. Typically, 4–5 weeks old IRC mice were housed in plastic cages with free access to water and food. *Plasmodium berghei* parasite-infected erythrocytes were obtained from donor mice. On experiment day 0, the donor mice were anesthetized and exsanguinated via cardiac puncture. The pooled blood from

the donor mice was then diluted with normal mouse serum to a concentration of 1×10^6 *P. berghei*-infected erythrocytes per inoculum (0.1 mL). The groups of experimental and control mice were inoculated with this parasitized blood on day 0. The tested mice were treated with either candidate antimalarial drugs or with vehicle alone to serve as negative controls from day 3 to day 10 orally (PO) or subcutaneously (SC) once a day for eight days. Each experimental group received a different dose level, with up to five different dose groups per compound. Blood films and body weights were taken on the third and sixth days post-infection, then at weekly intervals through day 60. Films were Giemsa stained and examined microscopically to determine parasitemia. All mice were observed twice a day to assess their clinical signs that were recorded. All treated mice with negative smear on day 60 were considered cured.

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