Evaluation of the anti-malarial activity and cytotoxicity of 2,4-diamino-pyrimidine-based kinase inhibitors

Oraphan Phuangsawai a, Paul Beswick b, Siriluk Ratanabunyong c, Lueacha Tabtimmai c,

Praphasri Suphakun c, Phongphat Obounchoey c, d, Pimonwan Srisook c,

Natharinee Horata e, Irina Chuckowree b, Supa Hannongbua a, Simon E. Ward b, **,

Kiattawee Choowongkomon c, M. Paul Gleeson a, *

a Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

b School of Life Sciences, University of Sussex, Brighton BN1 9QJ, United Kingdom

c Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

d Interdisciplinary Program in Genetic Engineering, Graduate School, Kasetsart University, Chatuchak, Bangkok 10900, Thailand

e Faculty of Medicinal Technology, Huachiew Chalermprakiet University, Samut Prakarn 10540, Thailand

abstract

A series of 2, 4 diamino-pyrimidines have been identified from an analysis of open access high throughput anti-malarial screening data reported by GlaxoSmithKline at the 3D7 and resistant Dd2 strains. SAR expansion has been performed using structural knowledge of the most plausible parasite target. Seventeen new analogs have been synthesized and tested against the resistant K1 strain of Plasmodium falciparum (Pf). The cytotoxicity of the compounds was assessed in Vero and A549 cells and their selectivity towards human kinases including JAK2 and EGFR were undertaken. We identified compound 5n and 5m as submicromolar inhibitors, with equivalent anti-malarial activity to Chloroquine (CQ). Compounds 5d and 5k, mM inhibitors of Pf, displayed improved cytotoxicity with weak inhibition of the human kinases.

1. Introduction

Malaria is tropical infectious disease of humans and other animals caused by mosquito-borne parasites that include Plasmodium falciparum (Pf) and Plasmodium vivax (Pv). The former causes the greatest percentage of deaths due to its more severe symptoms. The disease is prevalent in tropical climates in the African and Asian sub-continents, affecting countries with less developed economies [1,2]. In 2015 the World Health Organization (WHO) reported that approximately 5.9 million of children under five years of age died from this infectious disease [3]. To reduce the ongoing issue of drug resistance, the current frontline treatment artemisinin is given as a combination only, yet there are signs that its efficacy is under threat [4e6]. New antimalarial drugs that target novel modes of action are therefore highly desirable to tackle this disease [7e11].

The malaria parasite has a complex life cycle in which multiple kinase enzymes play an important role [12,13]. The target of the artemisinin has recently been suggested to be phosphoinositide 3-kinase (PfPI3K) [14], while the target of the MMV development compound MMV390048 was proposed to be phosphoinositide 4-kinase (PfPI4K) [15]. Additional protein kinases have also been implicated as possible targets [16e18]. A number of studies aimed at improving our understanding of the approximately 65 Pf specific kinases have been reported, many of which share considerable

homology with human kinases [10, 16, 19]. This has led to a kinase screening campaign [20] and SAR studies on specific protein kinases, including PfCDPK1 [21, 22], PfPK5 [23] and PfPK7 [24]. Nevertheless, it is not yet established which parasite kinase rep-resents the most valid target [10], or whether polypharmacology approaches could represent a useful approach, as found for cancer [25].

In 2010, GlaxoSmithKline reported the results of screening of almost 2 million compounds to identify anti-malarial hits. Approximately 13,500 compounds with activity at P. falciparum (Pf) 3D7, multi-drug resistant Dd2 Pf strain and for cytotoxicity in the HepG2 cell line were released in the public domain [26].In2016, these hits were re-assessed at five Pf kinases (PfCDPK1, PfCDPK4, PfPK6, PfPK7 and PfMAPK2) and led to the identification of twelve series with potential for optimization [20].

Our separate analysis of the GSK screening data led to the identification of a cluster of 2,4-diaminopyrimidine compounds with good activity, efficiency, physical properties and synthetic tractability (Table 1). The compounds display activity at the multidrug resistant Dd2 strain, whilst the HepG2 SAR suggests an anti-malarial vs. cytotoxicity window of 50 fold is achievable (Table 1). 1b is a confirmed inhibitor of PfCDPK1 (0.012 mM), CDPK4 (0.089 mM) and PfPK6 (0.054 mM). Host kinase interactions also require consideration as these could either lead to cytotoxicity or also potentially facilitate parasite eradication [17,27].

The most probable human kinases targeted by 1aed include Janus kinase 2 (hJAK2) [28] and Aurora A hAURKA [29,30]. Indeed, other hJAK and hAUR inhibitor classes have shown anti-malarial activity from repurposing studies [10,31]. The 2,4-diaminopyrimidine chemotype also appears in Epidermal growth factor (hEGFR) [32], and c-Jun N-terminal kinase (hJNK) [33] publications, as well as in patents targeting cancer-related pathways via hJAK [34] and hAURK (Fig. 1) [35]. Kinase inhibitors offer great potential as antimalarials due to the wealth of chemical, biological and safety data amassed over many years to treat diseases in humans, potentially reducing the development burden.

In this study we report the design, synthesis and biological evaluation of new 2, 4-diaminopyrimidine compounds. Our goal is to improve our SAR understanding of this series by screening additional analogs at the drug-resistant K1 Pf strain in the belief that molecules which target either individual, or a defined subset of kinases, offer potential new approaches to kill resistant strains. We also assess the cytotoxicity of the series using monkey kidney epithelial cell line (Vero), and the adenocarcinomic human alveolar basal epithelial cell line (A549). The human kinase activity of a subset are also evaluated for their potential to inhibit the human protein kinases: JAK2, JAK3 and EGFR.

2. Results

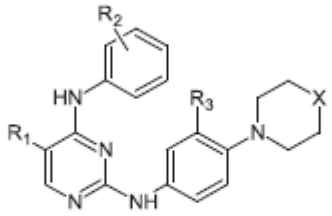
2.1. Design

The mode of action of compounds 1aed is unconfirmed, how-ever PfCDPK1, hAUR, hJAK and EGFR are probable targets. A focused library of 2, 4-diamino-pyrimidines derivatives were prepared using established synthetic methods and directed towards improving our understanding the link between Pf activity, cytotoxicity and activity at key kinases in the hosts.

Crystal structures of the Plasmodium Berghei (Pb) CDPK1 homolog and the principal human targets were downloaded for analysis (Table 2). Despite dramatic differences in sequence identity, the conserved ATP binding site means inhibitors can have considerably cross reactivity [10]. A key difference between these targets is the moderately sized, non-polar gatekeeper for the human proteins, compared to the smaller, more polar Thr sidechain [37].

We have undertaken modification to the chemotype at four different positions as highlighted in Fig. 2. Region 1 exploits the difference at the gatekeeper while region 2 (pyrimidine 4 position) targets differences within the kinase backpocket [38].

Table 1
Representative 2,4-diamino-pyrimidine hits identified from GSK HTS data.26 Pf 3D7 (IC50, μM) and HepG2 cytotoxicity (% at 10 μM) are reported for: TCMDC-134115 (**1a**), TCMDC-134116 (**1b**), TCMDC-141384 (**1c**) and TCMDC-141383 (**1d**).



ID	R_1	R_2	R_3	X	3D7 IC_{50} (μM)	HepG2 (%)
1a	CN	2-$CONH_2$	Me	NCH_3	0.009	99
1b	CN	2-$CONH_2$	H	NCH_3	0.040	43
1c	$CONHCH_3$	3-NO_2	H	NCH_3	0.138	16
1d	$CONHCH_3$	3-O^{i}Pr	H	O	0.759	19

Modifications at the latter position have resulted in dramatic changes in the hAURA protein conformation though the flipping of the DFG-loop despite only subtle changes in substitution of the 4-position aniline [39]. Modifications at region 3 will induce subtle changes in ring conformation, which can benefit selectivity, while region 4 can be exploited to improve compound solubility [40]. Anti-malarial SAR shows that compounds with R1 ¼ -CN have higher anti-malarial activity, however, this is mirrored by increases in the % cytotoxicity. We therefore evaluated the effect of incorporation of -H, -CN, -OMe and -CONH2. A range of substituted anilines at the 2- and 4- positions were selected to exploit interaction toward the kinase solvent pocket and backpocket, respectively, and maintain reasonable molecule properties (Table 3). We attempted to bias the reagent selection to focus on lower MWT (mean ¼ 405 da) and clogP (mean ¼ 4.04). This was with the goal of increasing the likelihood of achieving better solubility and permeability and solubility, a pre-requisite for reliable phenotypic testing.

2.2. Synthesis

Intermediates were synthesized using the methodology described in Scheme 1. 2a was produced from the reaction of p-fluoronitrobenzene and piperazine in DMSO with the assistance of K2CO3. The amine product was hydrogenated to 3a using Pd/C and H2. Intermediates 3b & 3c were produced in the same manner.

Compound 1a was synthesized following Scheme 2. The Cl at the 4-position of 5-cyano-2,4-dichloropyrimidine was substituted by p-aminobenzamide with the assistance of DIPEA giving intermediate 4a (Scheme 2). Intermediate 4a was reacted at with 3a assisted of DIPEA, leading to substitution at the 2-position. Com-pounds 5aee were synthesized in an analogous manner to 1a. Compound 5e was oxidized using hydrogen peroxide to yield compound 5f. Compound 5g, having an -OMe substituent at the 5-position was synthesized using the same protocol as 1a [35]. Compounds 5her, with H at the 5-position, were synthesized as follows; nucleophilic substitution at the 4-position of 2,4-dichloro pyrimidine was achieved using DIPEA base for R2 ¼ 3-SO2NH2 and 3-CH3, a

HCl promoted reaction for R2 ¼ 2-CONH2. Nucleophilic attack at the corresponding 2-position was achieved using TFA to promote the reaction.

Nineteen compounds were synthesized (Table 3) with reaction yields varied from low (<30%) to excellent (>90%). Among the 19 compounds synthesized, compounds 1a and 5j. 1a was originally reported in the study of Gamo et al. [26] and in the recent paper of Crothers et al. [20] 5j was reported as a JNK2 inhibitor by Song et al.

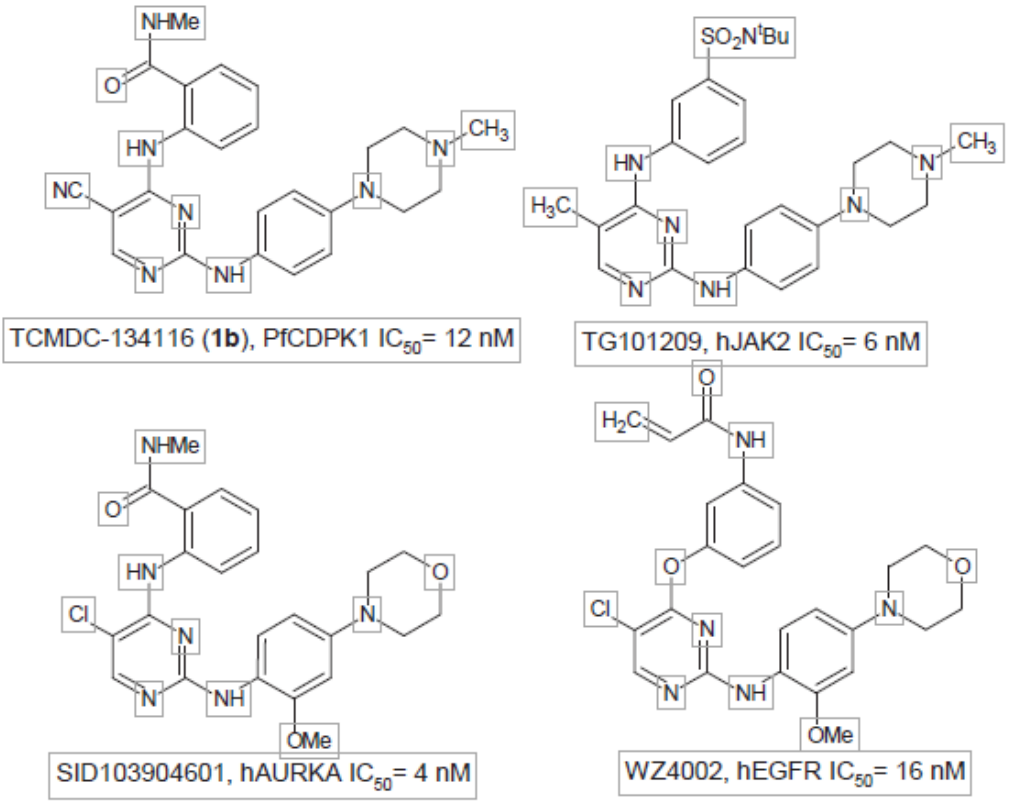


Fig. 1. 2,4-Diamino-pyrimidines kinase inhibitors; TCMDC 134116 [20], TG101209, [28] SID103904601 (www.pubchem.com) and covalent inhibitor WZ4002 [32,36].

Table 2
Sequence similarity, identity to probable Pf and host kinases.

ID	PDB ID	% Similarity to *Pf*CDPK1	% Identity to *Pf*CDPK1	Gatekeeper residue
*Pf*CDPK1	–	100	100	Thr
PbCDPK1	3Q5I	91.8	87.0	Thr
hAUR	3UOL	45.1	25.3	Leu
hJAK2	4JI9	36.1	18.6	Met
hEGFR	3IKA	33.9	15.4	Met

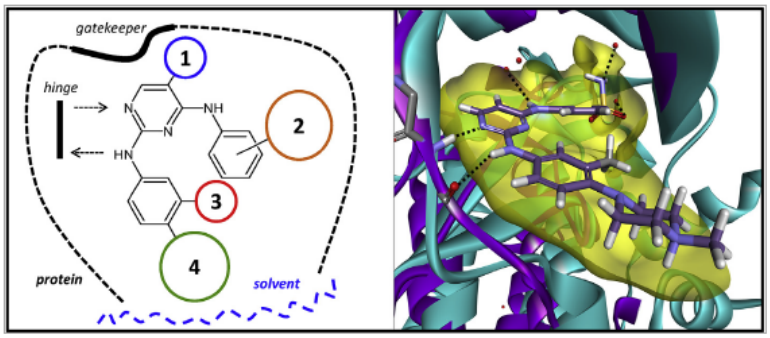


Fig. 2. Schematic (left) and docked model of 2,4-diamino-pyrimidines to PbCDPK1. The binding model of **5k** (carbons in grey) bound to the X-ray crystal structure of PbCDPK1 (3Q5I, magenta). The model was constructed using the JAK2- TG101209 complex (4JIA, cyan) [41]. Hydrogen bonds to the hinge and structural waters are indicated by dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Biological testing

2.3.1. Anti-malarial activity

The titled compounds 1a, 5aer were evaluated for their in vitro anti-malarial activity at the Pf K1 strain (EC50) using a hypoxanthine incorporation assay [42,43]. The compounds show a range in potency from 0.43 to 8.97 mM. Compound 1a, 0.009 mM against the 3D7 strain, was found to be 3.9 mM at the resistant K1 strain. Compounds 5n and 5o were 0.65 and 0.43 mM respectively. In comparison, standards chloroquine displayed an activity 0.57 mM, Mefloquine 0.07 mM and artemisinin at 0.002 mM.

Systematic analysis of the results presented in Table 3 suggests that compounds with eH at R1 are preferred for anti-malarial activity (as exemplified by 5a,r). R2 position shows a preference for anilines with R2 ¼ 3-Me (5a,r) or 2-CONH2 (5k,m). The R3 position does not display a clear preference towards the substituents employed. R4 shows a general preference towards Me-piperazine, compared to morpholine or the simpler substituted anilines.

Table 3
Activities and yields of compounds **a1**, **5a–r**.

ID	R_1	R_2	R_3	R_4	%yield[a]	clogP[d]	MWT[d]	P.f. K1 IC_{50} (μM)	Vero cytotox (%/50 μM)
1a	CN	o-$CONH_2$	Me	Me-piperazine	25	4.7	443	3.93	94
5a	CN	m-SO_2NH_2	Me	Me-piperazine	23	3.1	479	5.56	91
5b	CN	3-Me & 4-Methylpiperazine	SO_2NH_2	H	15	3.1	479	*NA*[e]	33
5c	CN	m-SO_2CH_3	Me	Me-piperazine	26	4.4	478	4.72	92
5d	CN	m-SO_2NH^tBu	Me	Me-piperazine	39	5.4	535	4.56	31
5e	CN	m-Me	Me	H	50[b]	5.1	315	*NA*[e]	40
5f	$CONH_2$	m-Me	Me	H	48[c]	3.4	333	*NA*[e]	96
5g	OMe	m-SO_2NH_2	Me	Me-piperazine	91	3.1	484	5.26	88
5h	H	o-$CONH_2$	Me	Me-piperazine	67	4.8	418	1.06	92
5i	H	o-$CONH_2$	H	Me-piperazine	56	4.3	403	1.50	91
5j	H	o-$CONH_2$	H	morpholine	11	4.2	390	4.39	68
5k	H	m-SO_2NH_2	Me	Me-piperazine	70	3.3	454	3.86	66
5l	H	m-SO_2NH_2	H	Me-piperazine	62	2.8	440	7.25	80
5m	H	m-SO_2NH_2	H	morpholine	75	2.7	427	8.97	35
5n	H	m-Me	Me	Me-piperazine	80	5.2	389	0.647	94
5o	H	m-Me	H	Me-piperazine	82	4.7	374	0.431	94
5p	H	m-Me	H	morpholine	79	4.6	361	*NA*[e]	74
5q	H	m-Me	Me	H	1.5	5.2	290	*NA*[e]	94
5r	H	4-phenylmorpholine	H	morpholine	20	4.0	433	*NA*[e]	25
Chloroquine					–	3.9	320	0.57	45
Mefloquine					–	4.1	378	0.07	–
Dihydroartemisinine					–	2.8	284	0.00254	–

[a] %yield obtained from di-substitution reaction at 2- and 4- position using condition.
[b] obtained as a by-product of the reaction to produce intermediate **5e**.
[c] %yield obtained from oxidation reaction of compound 7.
[d] Calculated logarithm of the octanol/water distribution coefficient using JChem Version 14.9.100.707.
[e] Compound not active.

2.3.2. Cytotoxicity

Compounds 1a, 5aer were evaluated for their cytotoxicity at 10 mM against the Vero cell line (ATCC CCL-81) using the method described by Hunt and co-workers [44]. The compounds show a range in potency from 25 to 96%. A degree of correlation between activity and cytotoxicity, with the most potent compounds (5n,o) also displaying high (94%) cytotoxicity. This compares to 50% for chloroquine, a standard anti-malarial treatment. Compounds 5d, 5k and 5m display mM antimalarial activity and low to moderate cytotoxicity at 31, 66 and 35%, respectively.

Five compounds were taken forward for further assessment; potent but cytotoxic compounds (5n,o), moderately potent and cytotoxic compounds (5k), and moderately potent and weakly cytotoxic compounds (5d,m). EC50s against the Vero (ATCC CCL-81) and A549 (ATCC CCL-185) cells using an MTT based method (Table 4) [45]. The EC50s are in good agreement with the % cyto-toxicity data, with 5d,k & m demonstrating better activity than compounds 5n,o. The A549 cytotoxicity data broadly mirrors those obtained in the Vero cell line. The lower EC50 values would be consistent with the higher expression of certain kinases in this disease tissue [46e50].

No clear SAR is apparent at the R1 and R2 positions. Hydrogen is preferred at the R3 position and -H and morpholine are preferred at the R4 position. Compounds 5k and 5m show a reasonable balance of anti-malarial activity and cytotoxicity, approaching the desired 50-fold window.

2.3.3. Protein kinase activity

Compounds 1a, 5aer were evaluated for their in vitro inhibition of human kinases JAK2, JAK3 and EGFR (Table 4). Inhibition was determined by using an Antibody Beacon™ Tyrosine Kinase Assay Kit (A-35725; Invitrogen, USA). Compounds 5k,meo display sub mM inhibition at hJAK2 kinase, mM inhibition at hJAK3 and all compounds display >10 mM inhibition at hEGFR.

Compounds 5m and 5o, the most potent anti-malarials, but also the most cytotoxic of compounds are more active at the human kinases assessed. 5o is a confirmed inhibitor of all three protein kinases while compound 5n inhibits JAK2 at 0.05 mM. In contrast, 5d shows minimal inhibition of the kinases and also displays good cytotoxicity at both the Vero and A549 cell lines.

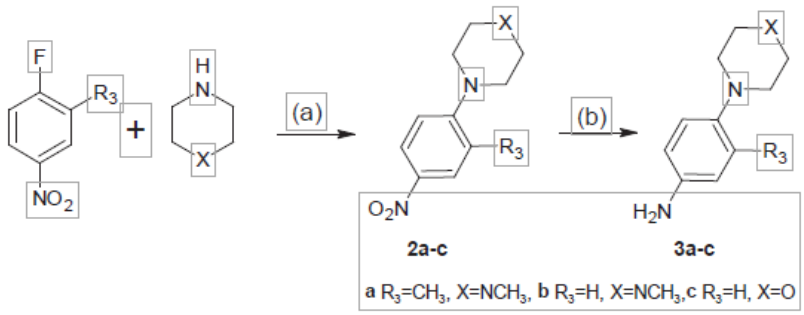
Inhibiting multiple Pf kinase targets without harming human cells will be a challenge, particularly since many kinase compounds come from human anti-cancer projects [20]. Yet, there is considerable safety and tolerability information in relation to kinase inhibition profiles that could be leveraged to develop molecules with a more targeted pharmacology. Furthermore, tackling host-parasite target interactions is also a feature of established DHFR anti-malarials. Methotrexate, a hDHFR inhibitor used in cancer treatment, also has potential as a PfDHFR anti-malarial if used at lower doses [51].

3. Conclusions

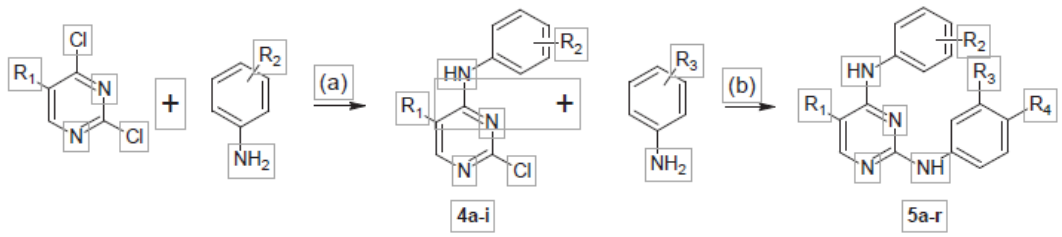
We identified a series of diamino-pyrimidines anti-malarial from mining the screening data reported by GSK [26]. We per-formed the design, synthesis and testing of new analogs to reveal additional SAR with respect to the K1 strain, cytotoxicity at the Vero and A549 cell lines as well as inhibition at representative human kinases.

Sixteen new compounds have been synthesized and evaluated against the K1 resistant Pf strain. The compounds displayed activity comparable to chloroquine. We identified 5n and 5o as having submM anti-malarial activity. These compounds were also identified as being potent human kinase inhibitors as well as being cytotoxic. Compounds 5d and 5k were identified as mM inhibitors with improved cytotoxicity and human kinase selectivity.

Results suggest series has potential but further work needed to explore the strain dependence and maximize the anti-malarial/potency window. Additional work is required to establish the molecular target(s) of this compound class.



Scheme 1. General scheme for the synthesis of anilines 5a-c. Reagents and conditions for **3a**: (a) K_2CO_3, DMSO, rt, 4–6 h, (b) Pd/C, H_2, MeOH, rt, overnight.



Scheme 2. Synthetic approach used for the preparation of compounds **1a**, **5a–r**. Reagents and conditions for; **5a**: (a) DIPEA, *n*-BuOH, 110 °C overnight, (b) DIPEA, DMF, rt, 4 h; **5i**, (a) DIPEA, *n*-BuOH, 110 °C overnight, (b) TFA, isopropanol, overnight.

Table 4
Activity of compounds selected for kinase selectivity and further cytotoxicity assessment.

ID	IC_{50}, μM			EC_{50}, μM	
	JAK2	JAK3	EGFR	Vero	A549
5d	>100	>100	>150	45.4	>100
5k	0.126	4.1	>150	>100	4.2
5m	0.129	7.1	>100	37.0	9.4
5n	0.05	1.8	>150	2.0	3.5
5o	0.14	2.1	11.0	1.0	7.1

Results suggest series has potential but further work needed to explore the strain dependence and maximize the anti-malarial/potency window. Additional work is required to establish the molecular target(s) of this compound class.

4. Experimental details

4.1. Chemistry

Reagents and solvents obtained from commercial suppliers were used without further purification. All reactions were monitored by TLC aluminum/silica gel plate with UV light visualization. Column chromatography was performed using silica gel 60 (40e63 mm). 1H NMR spectra were recorded on

both Varian instrument at 400 MHz and on a Bruker instrument at 500 MHz. Chemical shifts were reported in ppm (d) using the residual solvent line as the internal standard. Mass spectra (MS) were performed on Agilent 1100 HPLC instrument coupled to a LCjMSD Trap mass spectrometer, in ESI(þ) mode or APCI mode. Compounds showed a purity ?95% as determined from the corresponding UV absorbance HPLC chromatogram.

4.2. Preparation of anilines 3aec

4.2.1. 3-Methyl-4-(4-methylpiperazin-1-yl)aniline (3a)

A mixture of 1-fluoro-2-methyl-4-nitro-benzene (3.22 mmol), 1-methylpiperazine (3.63 mmol) and potassium carbonate (3.83 mmol) in dimethyl sulfoxide (4 ml) were stirred and heated to 100 ?C for 4 h. The resultant reaction solution was cooled down to room temperature, added water (50 ml) to the reaction mixture, filtered off the solid residue, washed with water (50 ml), and air-dried to give the desired product of 1-methyl-4-(2-methyl-4-nitrophenyl-piperazine (2a)) as a brown solid, 77% yield. 1H NMR (500 MHz, Chloroform-d) d 8.04 (d, J ¼ 8.3 Hz, 2H), 7.01 (d, J ¼ 8.4 Hz, 1H), 3.07 (t, J ¼ 4.8 Hz, 4H), 2.67e2.57 (m, 4H), 2.39 (s, 3H), 2.37 (s, 3H). [MþH]þ, ESI m/z 236.29. MW: 235.28 g/mol.

Afterwards, the obtained compound, 2a was hydrogenated. Pd/C (2.78 mmol) was added to a stirred solution of 1-methyl-4-(2-methyl-4-nitrophenyl-piperazine (5.53 mmol)) in methyl alcohol (15 ml) in N2 atmosphere. Following this it was hydrogenated overnight by maintaining the reaction flask in an atmosphere of H2 gas (balloon). The resultant was filtered thorough celite plate and concentrated under vacuum. The crude product was purified with silica-gel column chromatography (5%MeOH/DCM, 1%NH3) to yield the desired product, 3a as a brown solid, 87% yield. 1H NMR (500 MHz, DMSO-d6) d 6.74 (d, J ¼ 8.4 Hz, 1H), 6.38 (d, J ¼ 2.7 Hz, 1H), 6.34 (dd, J ¼ 8.4, 2.7 Hz, 1H), 4.61 (s, 2H), 2.68 (t, J ¼ 4.8 Hz, 4H), 2.41 (s, 4H), 2.20 (s, 3H), 2.10 (2, 3H). [MþH]þ, ESI m/z 206.23. MW: 205.30 g/mol.

4.2.2. 4-(4-Methylpiperazin-1-yl)aniline (3b)

A solution of 4-fluoronitrobenzene (7.1 mmol) and potassium carbonate (8.52 mol) in dimethyl sulfoxide (2 ml) was stirred at room temperature for 0.5 h 1-methylpiperazine (7.1 mmol) was added dropwise in the mixture solution. The resulting reaction mixture was stirred at room temperature for 6 h. The mixture was then poured into cold water. A yellow precipitate formed and was collected to give 1-methyl-4-(4-nitrophenyl) piperazine (2b), 93%yield. 1H NMR (400 MHz, CDCl3) d 8.16e8.09 (m, 2H), 6.86e6.80 (m, 2H), 3.53e3.46 (m, 4H), 2.70e2.60 (m, 2H), 2.42 (s, 3H). [MþH]þ, APCI m/z 222.1. MW: 221.26 g/mol.

Following this it was hydrogenated using the same procedure as compound 2a to yield the desired product, 3b as a brown solid, 100% yield. 1H NMR (400 MHz, DMSO) d 6.69e6.64 (m, 2H), 6.50e6.44 (m, 2H), 4.53 (s, 1H), 2.91e2.85 (m, 4H), 2.45e2.37 (m, 4H). methyl proton overlaps with the solvent peak. [MþH]þ, APCI m/z 192.2. MW: 191.27 g/mol.

4.2.3. 4-(Morpholin-4-yl)aniline (3c)

A mixture of 1-bromo-4-nitrobenzene (5.0 mmol), morpholine (5.0 mol) and potassium carbonate (6.0 mmol) in dimethyl sulf-oxide (2 ml) were stirred and heated to 100 ?C for 7 h. The resultant reaction solution was allowed to cool to room temperature. The mixture solution was extracted with EtOAc. The organic layer was collected, washed with brine, dried over Na2SO4, filtered, and dried under reduced pressure. The crude product was purified with silica-gel column chromatography (30% EtOAc/hexane) to give the desired product of 4-(4-nitrophenyl)morpholine, 2c as a yellow

solid, 47% yield. 1H NMR (400 MHz, CDCl3) d 8.18e8.09 (m, 2H), 6.86e6.79 (m, 2H), 3.88e3.82 (m, 4H), 3.39e3.31 (m, 4H).

Following this compound 2c was hydrogenated using the same procedure as compound 2a to yield the desired product, 3c as a purple solid, 97% yield. 1H NMR (400 MHz, DMSO) d 6.70e6.65 (m, 2H), 6.51e6.47 (m, 2H), 4.57 (s, 2H), 3.71e3.65 (m, 4H), 2.91e2.84 (m, 4H). [MþH]þ, APCI m/z 179.1. MW: 178.23 g/mol.

4.3. General procedure for the preparation of intermediate compounds 4aee

DIPEA (0.86 mmol) was added to the solution of 2,4-dichloropyrimidine-5-carnonitrile (0.57 mmol) in DMF (0.5 ml) and stirred at room temperature for 10 min. A solution of aminobenzene reagent corresponding to desired product (0.57 mmol) in DMF (0.5 ml) was added dropwise to the reaction mixture and stirred for 1 h. The resultant was evaporated under vacuum and extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pres-sure. 4c was obtained from the reaction of 4b, whereby substitution at the 2-position occurred.

4.3.1. 2-[(2-Chloro-5-cyano-pyrimidin-4-yl)amino]benzamide (4a)

2-aminobenzamide is used as an aminobenzene reagent, fol-lowed the general procedure and purified by silica-gel column chromatography (25%EtOAc/hexane) to yield 4a as a yellow solid, yield 41%, 1H NMR (500 MHz, DMSO-d6) d 12.23 (s, 1H), 8.95 (s, 1H), 8.40 (d, J ¼ 8.4 Hz, 1H), 8.31 (s, 1H), 7.84 (dd, J ¼ 7.9, 1.5 Hz, 1H), 7.80 (s, 1H), 7.58 (t, J ¼ 7.6 Hz, 1H), 7.21 (t, J ¼ 7.6 Hz, 1H), MS-ESI: m/z 273.67 [MþH]þ. MW: 273.68 g/mol.

4.3.2. 3-[(2-Chloro-5-cyano-pyrimidin-4-yl)amino] benzenesulfonamide (4b)

2-Aminobenzensulfonamide is used as an aminobenzene re-agent, followed the general procedure and purified by silica-gel column chromatography (10%EtOAc/hexane) to yield 4b as a beige solid, yield 32%, 1H NMR (500 MHz, DMSO-d6) d 11.07 (s, 1H), 8.95 (s, 1H), 8.20 (s, 1H), 7.89e7.83 (m, 1H), 7.61e7.55 (m, 2H), 7.39 (s, 2H). MS-ESI: m/z 309.89 [MþH]þ. MW: 309.73 g/mol.

4.3.3. 3-[(4-Chloro-5-cyano-pyrimidin-4-yl)amino] benzenesulonamide (4c)

4c was obtained as a side product from the reaction of 4b, whereby substitution at the 2-position occurred. Purified by silica-gel column chromatography (10%EtOAc/hexane), a beige solid, yield 35%, 1H NMR (500 MHz, DMSO-d6) d 10.56 (s, 1H), 8.79 (s, 1H), 7.99 (t, J ¼ 1.8 Hz, 1H), 7.80 (ddd, J ¼ 8.1, 2.2, 1.1 Hz, 1H), 7.67 (dt, J ¼ 7.9, 1.4 Hz, 1H), 7.60 (t, J ¼ 7.9 Hz, 1H), 7.40 (s, 2H). MS-ESI: m/z 310.06 [MþH]þ. MW: 309.73 g/mol.

4.3.4. 2-Chloro-4-(3-methylsulfonylanilino) pyrimidine-5-carbonitrile (4d)

3-(Methylsulfonyl)aniline hydrochloride is used as an amino-benzene reagent, followed the general procedure and purified by silica-gel column chromatography (5%MeOH/DCM) to yield 4d as a yellow solid, yield 13%, 1H NMR (500 MHz, DMSO-d6) d 11.14 (s, 1H), 8.99 (s, 1H), 8.31 (s, 1H), 7.97 (dt, J ¼ 6.4, 2.4 Hz, 1H), 7.69e7.62 (m, 2H), 3.21 (d, J ¼ 1.4 Hz, 3H). MS-ESI: m/z 309.0 [MþH]þ.MW: 308.74 g/mol.

4.3.5. N-tert-butyl-3-[(2-chloro-5-cyano-pyrimidin-4-yl] benzenesulfonamide (4e)

N-t-butyl-3-aminobenesulfonamide is used as an amino-benzene reagent, followed the general procedure and purified by silica-gel column chromatography (0e5%MeOH/DCM) to yield 4e as a beige solid, yield 30%, 1H NMR (500 MHz, DMSO-d6) d 11.06 (s, 1H), 8.94 (s, 1H), 8.21 (s, 1H),

7.85e7.81 (m, 1H), 7.56 (dd, J ¼ 2.6, 1.4 Hz, 1H), 7.55 (d, J ¼ 4.6 Hz, 2H), 1.12 (s, 9H). MS-ESI: m/z 366.08 [MþH]þ. MW: 365.84 g/mol.

4.4. General procedure for the preparation of intermediate compounds 4f

4.4.1. 3-[(2-Chloro-5-methoxypyrimidin-4-yl)amino]benzene-1-sulfonamide (4f)

3-aminobenzenesulfonamide (3.07 mmol) and DIPEA (8.38 mmol) was added to the mixture of 2,4-dichloro-5-methoxypyrimidine (2.79 mmol) in 1,4-dioxane (15 ml). The reac-tion mixture was stirred at 100 C for 3 days. The solvent was evaporated under reduce pressure and extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pressure. The crude product was purified with column chromatography (5% MeOH/DCM) to give the desired product, 4f as a light yellow solid with a 22% yield, 1H NMR (500 MHz, DMSO-d6) d 9.58 (s, 1H), 8.29e8.24 (m, 1H), 8.00 (s, 1H), 7.99e7.95 (m, 1H), 7.56e7.52 (m, 2H), 7.34 (s, 2H), 3.95 (s, 3H). MS-ESI: m/z 315.28 [MþH]þ. MW: 314.75 g/mol.

4.5. General procedure for the preparation of compounds 4ge4i

4.5.1. 2-[(2-Chloropyrimidin-4-yl)amino]benzamide (4g)

A mixture of 2,4-dichloropyrimidine and 2-aminobenzamide in 0.1 M HCl was stirred at 100 C for 3 h. The precipitated obtained was filtered and the product was washed with water and recrys-tallized in methanol. The crystal product was collected and air-dried to give the desired intermediate compound, 4g as a light green solid, with a 39% yield. 1H NMR (400 MHz, DMSO-d6) d 11.24 (s, 1H), 8.18 (dd, J ¼ 11.3, 7.1 Hz, 3H), 7.76 (dd, J ¼ 7.9, 1.5 Hz, 1H), 7.70 (s, 1H), 7.56e7.50 (m, 1H), 7.17 (td, J ¼ 7.7, 1.1 Hz, 1H), 6.87 (d, J=5.9Hz, 1H). MS-APCI:m/z,249.1 [M+H]+.MW:248.67 g/mol.

4.5.3. 2-Chloro-N-(3-methylphenyl)pyrimidin-4-amine (4i)

4i was prepared using the same procedure as 4h, with a 25 h of reaction time. A desired intermediate product 4i was obtained as beige solid, with a 65% yield, 1H NMR (400 MHz, DMSO-d6) d 9.94 (s, 1H), 8.13 (dd, J ¼ 5.8, 3.2 Hz, 1H), 7.40 (d, J ¼ 7.8 Hz, 1H), 7.34 (s, 1H), 7.25 (t, J ¼ 7.8 Hz, 1H), 6.92 (d, J ¼ 7.3 Hz, 1H), 6.75e6.72 (m, 1H), 2.30 (s, 3H), MS-APCI: m/z 220.1 [MþH]þ. MW: 219.67 g/mol.

4.6. General procedure for the synthesis of compound 1a, 5aed

A chloro-intermediate compound (4aee) (0.22 mmol) and DIPEA (0.33 mmol) were dissolved in DMF (0.5 ml). A solution of amino-intermediate compound (3aec) (0.2 mmol) in DMF (0.5 ml) was then added dropwise into the solution. The reaction mixture was stirred at room temperature for 4 h. The resultant solution was concentrated under vacuum, and extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pressure.

4.6.1. 2-[[5-Cyano-2-[3-methyl-4-(4-methylpiperazin-1-yl)aniline] pyrimidin-4-yl]amino]benzamide (1a)

Compound 3a as the amino-intermediate and 4a as the chlorointermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3) to give the desired product, 1a with a 25% yield as a beige solid. 1H NMR (500 MHz, DMSO-d6) d 11.67 (s, 1H), 9.51 (s, 1H), 8.49 (s, 1H), 8.39 (d, J ¼ 8.5 Hz, 1H), 8.20 (s, 1H), 7.74 (dd, J ¼ 7.9, 1.5 Hz, 1H), 7.69 (s, 1H), 7.33 (d, J ¼ 2.5 Hz, 1H), 7.29e7.24 (m, 1H), 7.20 (t, J ¼ 8.0 Hz, 1H), 7.03 (dd, J ¼ 16.6, 8.2 Hz, 2H), 2.87 (t, J ¼ 4.7 Hz, 4H), 2.25 (s, 3H),2.24 (s, 3H). MS-ESI: m/z 443.13 [MþH]þ. MW: 442.53 g/mol.

4.6.2. 3-[[5-Cyano-2-[3-methyl-4-(4-methylpiperazin-1-yl)aniline] pyrimidin-4-yl]amino]benzenesulfonamide (5a)

Compound 3a as the amino-intermediate and 4b as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a beige solid, yield 23%, 1H NMR (500 MHz, DMSO-d6) d 10.07 (s, 1H), 9.38 (s, 1H), 8.50 (d, J ¼ 1.3 Hz, 1H), 7.99 (d, J ¼ 8.1 Hz, 1H), 7.90 (s, 1H), 7.45e7.40 (m, 1H), 7.31 (s, 3H), 7.27 (s, 2H), 7.02 (d, J ¼ 8.2 Hz, 1H), 2.85 (t, J ¼ 4.7 Hz, 4H), 2.25 (s, 3H), 2.23 (s, 3H). MS-ESI: m/z 479.20 [MþH]þ. MW: 478.58 g/mol.

4.6.3. 3-[[5-Cyano-4-[3-methyl-4-(4-methylpiperazin-1-yl)aniline] pyrimidin-2-yl]amino]benzenesulfonamide (5b)

Compound 3a as the amino-intermediate and 4c as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a beige solid, yield 15%, 1H NMR (500 MHz, DMSO-d6) d 9.67 (s, 1H), 8.48 (s, 1H), 8.35 (d, J ¼ 2.5 Hz, 1H), 7.71 (dt, J ¼ 6.6, 2.3 Hz, 1H), 7.51 (dd, J ¼ 5.0, 2.8 Hz, 2H), 7.30 (d, J ¼ 7.0 Hz, 2H), 6.74 (d, J ¼ 8.4 Hz, 1H), 6.41 (d, J ¼ 2.4 Hz, 1H), 6.34 (dd, J ¼ 8.5, 2.6 Hz, 1H), 4.66 (s, 2H), 2.72 (d, J ¼ 5.4 Hz, 4H), 2.16 (s, 3H). MS-ESI: m/z 479.12 [MþH]þ.MW: 478.58 g/mol.

4.6.4. 2-[3-Methyl-4-(4-methylpriperazin-1-yl)aniline]-4-(3-methylsulfonylanilino)pyrimidine-5-carbonitrile (5c)

Compound 3a as the amino-intermediate and 4d as the chloro-column chromatography (0e10%MeOH/DCM, 1%NH3), a yellow solid, yield 26%, 1H NMR (500 MHz, DMSO-d6) d 10.12 (s, 1H), 9.42 (s, 1H), 8.52 (s, 1H), 8.10 (dd, J ¼ 8.0, 2.1 Hz, 1H), 7.98 (s, 1H), 7.50 (dt, J ¼ 7.8, 1.3 Hz, 1H), 7.37 (s, 1H), 7.30 (d, J ¼ 11.7 Hz, 2H), 7.02 (d, J ¼ 8.4 Hz, 1H), 3.11 (s, 3H), 2.85 (t, J ¼ 4.6 Hz, 4H), 2.24 (s, 4H), 2.22 (s, 3H), 1.98 (s, 1H). MS-ESI: m/z 478.25 [MþH]þ. MW: 477.59 g/mol.

4.6.5. N-tert-butyl-3-(5-cyano-2-(3-methyl-4-(4-methylpiperazin-1 -yl)phenylamino)pyrimidin-4-ylamino)benzene-sulfonamide (5d)

Compound 3a as the amino-intermediate and 4e as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a beige solid, yield 39%, 1H NMR (500 MHz, DMSO-d6) d 10.06 (s, 1H), 9.38 (s, 1H), 8.49 (s, 1H), 8.00 (d, J ¼ 8.2 Hz, 1H), 7.90 (s, 1H), 7.44e7.40 (m, 2H), 7.29 (d, J ¼ 16.0 Hz, 3H), 7.02 (d, J ¼ 8.5 Hz, 1H), 2.85 (t, J ¼ 4.7 Hz, 4H), 2.25 (s, 3H), 2.22 (s, 3H), 1.09 (s, 9H). 13C NMR (101 MHz, DMSO) d 164.71, 162.44, 160.56, 159.89, 158.68, 147.38, 144.62, 139.53, 133.06, 132.33, 122.64, 119.88, 119.15, 118.21, 117.79, 116.86, 116.39, 53.00, 50.92, 46.34, 35.66, 29.88, 21.80, 17.43, 13.93. MS-ESI: m/z 535.24 [MþH]þ. MW: 534.68 g/mol.

4.7. General procedure for the synthesis of compound 5eeg

4.7.1. 2,4-bis(3-methylanilino)pyrimidine-5-carbonitrile (5e)

3-Methylaniline (0.29 mmol) and DIPEA were added to a solu-tion of 2,4-dichloropyrimidine-5-carbonitrile (0.29 mmol) in DMF (5 ml). The reaction mixture was stirred at room temperature for 4.5 h. The reaction mixture was then extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pressure to yield the desired product, 5e which disubstituted reaction at 2- and 4-position occurred, with a 50% yield as a white solid. 1H NMR (500 MHz, DMSO-d6) d 9.80 (s, 1H), 9.40 (s, 1H), 8.48 (s, 1H), 7.40 (s, 1H), 7.36 (q, J ¼ 18.1, 10.4 Hz, 4H), 7.30 (t, J ¼ 8.1 Hz, 1H), 7.24 (t, J ¼ 8.0 Hz, 1H), 7.09 (d, J ¼ 7.6 Hz, 1H), 7.03 (d, J ¼

4.9 Hz, 3H), 6.99 (d, J ¼ 7.7 Hz, 1H), 6.78 (d, J ¼ 7.4 Hz, 1H), 2.28 (s, 3H), 2.14 (s, 3H). MS-ESI: m/z 316.31 [MþH]þ. MW: 315.38 g/mol.

316.31 [MþH]þ. MW: 315.38 g/mol.

4.7.2. 2,4-bis(3-methylanilino)pyrimidine-5-carboxamide (5f)

5e (0.09 mmol), hydrogen peroxide solution (0.86 mmol) and 1 M NaOH (0.17 mmol) in methyl alcohol (1 ml) were stirred to 40 C for 24 h. The reaction mixture was concentrated under vac-uum and extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pressure. The crude product was purified by column chromatography (0e10%MeOH/DCM, 1%NH3) to give 5f, with a 48%yield as a beige solid. 1H NMR (500 MHz, DMSO-d6) d 11.52 (s, 1H), 9.55 (s, 1H), 8.69 (s, 1H), 7.99 (s, 1H), 7.51 (s, 2H), 7.47 (dd, J ¼ 8.4, 2.1 Hz, 1H), 7.40 (s, 1H), 7.21 (t, J ¼ 7.8 Hz, 1H), 7.13 (t, J ¼ 7.8 Hz, 1H), 6.90 (dd, J ¼ 7.4, 1.7 Hz, 1H), 6.83e6.79 (m, 1H), 2.27 (s, 3H), 2.22 (s, 3H). MS-ESI: m/z 334.13 [MþH]þ. 333.40 g/mol.

4.7.3. 3-[2-Methoxy-5-[3-methyl-4-(4-methylpiperazin-1-yl)1-aniline]aniline]benzenesul-fonamide (5g)

Compound 3a was dissolved in tetrahydrofuran (0.4 ml) and treated with a solution of 1 M HCl (0.33 mmol) in ethyl ether. The mixture was stirred for 15 min and then concentrated under vac-uum. The residue was then treated with 4f (0.16 mmol) and 2-propanol (0.8 ml). The suspension was stirred at 100 C for 5 days. The reaction mixture was added to a NaHCO3 solution (10 ml) and extracted with EtOAc. The organic layer was collected, washed with brine, dried over MgSO4, filtered, and dried under reduced pressure to give the desired product, 5g with a 91% yield as a beige solid. 1H NMR (500 MHz, Methanol-d4) d 8.32 (t, J ¼ 2.0 Hz, 1H), 8.04 (ddd, J ¼ 8.2, 2.2, 1.0 Hz, 1H), 7.71 (s, 1H), 7.58 (dt, J ¼ 7.9, 1.3 Hz, 1H), 7.46 (t, J ¼ 8.0 Hz, 1H), 7.33e7.35 (m, 2H), 7.01 (d, J ¼ 8.2 Hz, 1H), 3.92 (s, 3H), 2.96 (t, J ¼ 4.9 Hz, 4H), 2.76 (s, 4H), 2.46 (s, 3H), 2.26 (s, 3H). MS-ESI: m/z 484.23 [MþH]þ. MW: 483.59 g/mol.

4.8. General procedure for the synthesis of compounds 5he5r

A chloro-intermediate (4gei) (0.32 mmol) and amino-intermediate (3aec) (0.32 mmol) were combined in a round bot-tom flask with 5 ml of isopropanol and catalytic amounts of TFA. The reaction mixture was stirred at 80 C overnight. The reaction was then cooled down to room temperature, neutralized with 1 M NaOH, and evaporated under vacuum to give a crude product. The crude product was purified by silica-gel column chromatography to yield a desired product.

4.8.1. 2-[(2-{[3-Methyl-4-(4-methylpiperazin-1-yl)phenyl]amino} pyrimidin-4-yl)amino]benzamide (5h)

Compound 3a as the amino-intermediate and 4g as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5e10%MeOH/DCM, 1%NH3), a beige solid, yield 67%, 1H NMR (400 MHz, DMSO-d6) d 11.10 (s, 1H), 9.05 (s, 1H), 8.57 (d, J ¼ 8.3 Hz, 1H), 8.21 (s, 1H), 8.04 (d, J ¼ 5.7 Hz, 1H), 7.77e7.73 (m, 1H), 7.66 (s, 1H), 7.52 (d, J ¼ 2.3 Hz, 1H), 7.46e7.37 (m, 2H), 7.04 (t, J ¼ 7.0 Hz, 1H), 6.93 (s, 1H), 6.20 (d, J ¼ 5.7 Hz, 1H), 2.80 (t, J ¼ 4.6 Hz, 4H), 2.23 (s, 3H), 2.20 (s, 3H). Another set of 4 protons is overlapped under water peak. MS-APCI: m/z, 418.3 [MþH]þ.MW: 417.52 g/mol.

4.8.2. 2-[(2-{[4-(4-Methylpiperazin-1-yl)phenyl]amino}pyrimidin-4-yl)amino]benzamide (5i)

Compound 3b as the amino-intermediate and 4g as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a brown solid,

yield 56%, 1H NMR (400 MHz, DMSO-d6) d 11.07 (s, 1H), 8.99 (s, 1H), 8.57 (d, J ¼ 8.5 Hz, 1H), 8.20 (s, 1H), 8.01 (t, J ¼ 5.2 Hz, 1H), 7.76 (dd, J ¼ 7.9, 1.5 Hz, 1H), 7.65 (s, 1H), 7.52 (d, J ¼ 9.0 Hz, 2H), 7.44 (dd, J ¼ 11.4, 4.3 Hz, 1H), 7.03 (dd, J ¼ 11.1, 4.1 Hz, 1H), 6.87 (d, J ¼ 9.1 Hz, 2H), 6.17 (d, J ¼ 5.7 Hz, 1H), 3.09e3.03 (m, 4H), 2.48e2.44 (m, 4H), 2.22 (s, 3H). MS-APCI: m/z, 404.3 [MþH]þ. MW: 403.49 g/mol.

4.8.3. 2-[(2-{[4-(Morpholin-4-yl)phenyl]amino}pyrimidin-4-yl) amino]benzamide (5j)

Compound 3c as the amino-intermediate and 4g as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a white solid, yield 11%, 1H NMR (400 MHz, DMSO-d6) d 11.08 (s, 1H), 9.02 (s, 1H), 8.57 (d, J ¼ 8.4 Hz, 1H), 8.21 (s, 1H), 8.03 (d, J ¼ 5.7 Hz, 1H), 7.76 (d, J ¼ 7.8 Hz, 1H), 7.66 (s, 1H), 7.54 (d, J ¼ 9.0 Hz, 2H), 7.45 (t, J ¼ 7.1 Hz, 1H), 7.04 (t, J ¼ 7.6 Hz, 1H), 6.88 (d, J ¼ 9.0 Hz, 2H), 6.18 (d, J ¼ 5.7 Hz, 1H), 3.78e3.72 (m, 4H), 3.07e3.01 (m, 4H), MS-APCI: m/z,391.3 [MþH]þ. MW: 390.45 g/mol.

4.8.4. 3-[(2-{[3-Methyl-4-(4-methylpiperazin-1-yl)phenyl]amino} pyrimidin-4-yl)amino]benzene-1-sulfonamide (5k)

Compound 3a as the amino-intermediate and 4h as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a brown solid, yield 70%, 1H NMR (400 MHz, DMSO-d6) d 9.63 (s, 1H), 8.94 (s, 1H), 8.25 (d, J ¼ 8.3 Hz, 1H), 8.03 (d, J ¼ 5.7 Hz, 1H), 7.89 (s, 1H), 7.45 (ddd, J ¼ 7.7, 7.2, 1.5 Hz, 4H), 7.37 (s, 2H), 6.95 (d, J ¼ 8.7 Hz, 1H), 6.21 (d, J ¼ 5.7 Hz, 1H), 2.80 (t, J ¼ 4.5 Hz, 4H), 2.24 (s, 3H), 2.20 (s, 3H). 13C NMR (101 MHz, DMSO-d6) d 160.27, 159.58, 156.38, 145.46, 144.50, 140.64, 135.80, 131.82, 129.41, 122.46, 122.02, 118.84, 118.58, 117.85, 116.19, 98.65, 55.25, 51.56, 45.81, 40.19, 40.15, 39.98, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89, 17.65. MS-APCI: m/z 454.2 [MþH]þ.MW: 453.57 g/mol.

4.8.5. 3-[(2-{[4-(4-methylprperazin-1-yl)phenyl]amino)pyrimidin-4-yl)amino]benzene-1-sulfonamide (5l)

Compound 3b as the amino-intermediate and 4h as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (10%MeOH/DCM, 1%NH3), a beige solid, yield 62%, 1H NMR (400 MHz, DMSO-d6) d 9.60 (s, 1H), 8.89 (s, 1H), 8.19 (d, J ¼ 8.3 Hz, 1H), 8.01 (d, J ¼ 5.7 Hz, 1H), 7.93 (s, 1H), 7.52 (d, J ¼ 9.0 Hz, 2H), 7.44 (ddd, J ¼ 11.1, 7.9, 4.7 Hz, 2H), 7.37 (s, 1H), 6.88 (d, J ¼ 9.1 Hz, 1H), 6.18 (d, J ¼ 5.7 Hz, 1H), 3.07 (t, J ¼ 3.2 Hz 3H), 2.26 (s, 3H). MS-APCI: m/z 440.2 [MþH]þ. MW: 439.54 g/mol.

4.8.6. 3-[(2-{[4-(Morpholin-4-yl)phenyl]amino}pyrimidin-4-yl) amino]benzene-1-sulfonamide (5m)

Compound 3c as the amino-intermediate and 4h as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5e10%MeOH/DCM, 1%NH3), a purple solid, yield 75%, 1H NMR (400 MHz, DMSO-d6) d 9.62 (s, 1H), 8.92 (s, 1H), 8.18 (d, J ¼ 8.5 Hz, 1H), 8.01 (d, J ¼ 5.7 Hz, 1H), 7.93 (s, 1H), 7.53 (d, J ¼ 9.0 Hz, 2H), 7.47 (t, J ¼ 7.8 Hz, 1H), 7.42 (dt, J ¼ 7.8, 1.4 Hz, 1H), 7.37 (s, 2H), 6.88 (d, J ¼ 9.1 Hz, 2H), 6.19 (d, J ¼ 5.7 Hz, 1H), 3.77e3.68 (m, 4H), 3.06e2.99 (m, 4H). 13C NMR (101 MHz, DMSO-d6) d 160.29, 159.69, 156.38, 146.10, 144.48, 140.64, 133.05, 129.37, 122.57, 120.76, 118.59, 116.18, 115.62, 98.46, 66.20, 49.35, 40.19, 40.15, 39.98, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89. MS-APCI: m/z 427.2 [MþH]þ. MW: 426.50 g/mol.

4.8.7. 2-N-[4-(3,4-Dimethylpiperazin-1-yl)phenyl]-4 -N-(3-methylphenyl)pyrimidine-2,4-diamine (5n)

Compound 3a as the amino-intermediate and 4i as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a brown solid,

yield 80%, 1H NMR (400 MHz, DMSO-d6) d 9.20 (s, 1H), 8.90 (s, 1H), 7.96 (d, J ¼ 5.7 Hz, 1H), 7.48 (dd, J ¼ 15.8, 8.0 Hz, 4H), 7.16 (t, J ¼ 7.8 Hz, 1H), 6.92 (d, J ¼ 8.5 Hz, 1H), 6.80 (d, J ¼ 7.5 Hz, 1H), 6.15 (d, J ¼ 5.7 Hz, 1H), 2.78 (d, J ¼ 4.3 Hz, 4H), 2.27 (s, 3H), 2.23 (s, 3H), 2.18 (s, 3H). 13C NMR (101 MHz, DMSO-d6) d 160.44, 159.71, 155.99, 145.29, 140.07, 137.77, 136.01, 131.78, 128.48, 122.65, 122.01, 120.26, 118.72, 117.79, 116.96, 98.28, 55.28, 51.63, 45.87, 40.20, 40.15, 39.99, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89, 21.23, 17.58. MS-APCI: m/z 389.4 [MþH]þ. MW: 388.52 g/mol.

4.8.8. 4-N-(3-Methylphenyl)-2-N-[4-(4-methylpiperazin-1-yl) phenyl]pyrimidine-2,4-diamine (5o)

Compound 3b as the amino-intermediate and 4i as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a yellow solid, yield 82%, 1H NMR (400 MHz, DMSO-d6) d 9.18 (s, 1H), 8.85 (s, 1H), 7.94 (d, J ¼ 5.7 Hz, 1H), 7.59e7.47 (m, 3H), 7.43 (d, J ¼ 6.6 Hz, 1H), 7.16 (t, J ¼ 7.7 Hz, 1H), 6.85 (d, J ¼ 8.9 Hz, 2H), 6.79 (d, J ¼ 7.1 Hz, 1H), 6.13 (d, J ¼ 5.7 Hz, 1H), 3.04 (t, J ¼ 2.8 Hz 4H), 2.46 (t, J ¼ 4.4 Hz, 4H), 2.27 (s, 3H), 2.22 (s, 3H). 13C NMR (101 MHz, DMSO-d6) d 160.45, 159.85, 155.99, 145.97, 140.11, 137.77, 132.96, 128.43, 122.57, 120.79, 120.19, 116.89, 115.84, 98.03, 54.73, 48.98, 45.79, 40.19, 40.15, 39.98, 39.94, 39.77, 39.73, 39.52, 39.31, 39.10, 38.89, 21.31. MS-APCI: m/z 375.3 [MþH]þ. MW: 374.49 g/mol.

4.8.9. 4-N-(3-Methylphenyl)-2-N-[4-(morpholin-4-yl)phenyl] pyrimidine-2,4-diamine (5p)

Compound 3c as the amino-intermediate and 4i as the chloro-intermediate, following the general procedure. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a brown solid, yield 78%, 1H NMR (400 MHz, DMSO-d6) d 9.18 (s, 1H), 8.88 (s, 1H), 7.95 (d, J ¼ 5.7 Hz, 1H), 7.58e7.51 (m, 3H), 7.44 (d, J ¼ 8.1 Hz, 1H), 7.16 (dd, J ¼ 10.1, 5.5 Hz, 1H), 6.88e6.83 (m, 2H), 6.80 (d, J ¼ 7.5 Hz, 1H), 6.14 (d, J ¼ 5.7 Hz, 1H), 3.78e3.69 (m, 4H), 3.05e2.97 (m, 4H). MS-APCI: m/z 362.3 [MþH]þ. MW: 361.45 g/mol.

4.8.10. N,N0-bis(3-methylphenyl)pyrimidine-2,4-diamine (5q)

5q was a by-product obtained from the double substitution re-action from the aniline used to produce 4i. Purified by silica-gel column chromatography (10% MeOH/DCM), a beige solid, yield 2%, 1H NMR (400 MHz, DMSO-d6) d 9.25 (s, 1H), 9.05 (s, 1H), 7.99 (d, J ¼ 5.7 Hz, 1H), 7.53 (d, J ¼ 7.1 Hz, 3H), 7.46 (s, 1H), 7.18 (t, J ¼ 7.8 Hz, 1H), 7.14e7.07 (m, 1H), 6.82 (d, J ¼ 7.5 Hz, 1H), 6.73 (d, J ¼ 7.4 Hz, 1H), 6.20 (d, J ¼ 5.7 Hz, 1H), 2.28 (s, 3H), 2.24 (s, 3H), MS-APCI: m/z 291.2 [MþH]þ. MW: 290.37 g/mol.

4.8.11. 2-N,4-N-bis[4-(morpholin-4-yl)phenyl]pyrimidine-2,4-diamine (5r)

5r was a by-product from the elimination of 2-aminobenzamide on the 2-position of compound 5j. Purified by silica-gel column chromatography (5%MeOH/DCM, 1%NH3), a yellow solid, yield 20%, 1H NMR (400 MHz, DMSO-d6) d 10.13 (s, 1H), 9.02 (s, 1H), 8.51 (d, J ¼ 8.9 Hz, 1H), 8.06 (d, J ¼ 5.6 Hz, 1H), 7.94 (dd, J ¼ 8.0, 1.6 Hz, 1H), 7.60e7.49 (m, 3H), 7.15e7.08 (m, 1H), 6.86 (d, J ¼ 9.1 Hz, 2H), 6.26 (d, J ¼ 5.7 Hz, 1H), 3.76e3.71 (m, 4H), 3.06e2.99 (m, 4H), MS-APCI: m/z, 434.3 [MþH]þ. MW: 432.53 g/mol.

4.9. Biological assays

4.9.1. Anti-malarial activity

Multidrug resistant Plasmodium falciparum (K1 strain) were cultivated as previously described [43,52]. Briefly, P. falciparum were cultivated in RPMI-1640 medium supplement with 25 mM HEPES, 25 mM NaHCO3, 10% heat-activated human serum and 3%erythrocytes. The culture is incubated at

37 C in a humidified incubator with 3% CO2 and daily passaged to fresh medium containing erythrocyte to maintain parasite growth. The in vitro anti-malarial activity of the test peptides were tested against multidrug resistant Plasmodium falciparum (K1strain) using a semi-automated microdilution technique [42].200ml of early ring state parasite mixture (1% parasitemia, 1.5% Hct) were added into each well of a 96 well plate. Then, 25 ml tested peptide were added in duplicate. 96 well plate. Then, 25 ml tested peptide were added in duplicate. Plates were incubated in CO2 incubator for 24 h, then 25 mlof medium containing 0.5 mCi [3H] hypoxanthine (Perkin Elmer, USA) were added and incubated for 18e20 h. The levels of incorporated radioactive labeled hypoxanthine, indicating parasite growth, were determined by using the TopCount microplate scintillation counter (Packard, USA). Dihydroartemisinine chloroquine and mefloquine were used as positive control and 0.5% DMSO was used as negative controls. The antimalarial activity was expressed as the inhibitory concentration (IC50), representing the concentration of drug that reduced 50% of parasite growth. Data fitting was performed using SOFTMax Pro (Molecular Devices USA).

4.9.2. Cytotoxicity

The percent cytotoxicity against the Vero cell line (ATCC CCL-81) at 10 mM employed the method described by Hunt and co-workers [44]. The GFP-expressing Vero cell line was generated by stably transfecting the Vero cells with pEGFP-N1 plasmid Clontech and maintained in minimal essential medium supplemented with 10%heat inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM so-dium pyruvate, 1.5 g/L sodium bicarbonate, and 0.8 mg/ml genet-icin at 37 C in an incubator with 5% CO2. Vero cell suspension (45 ml, 3.3 104 cells/ml) was added into each well of 384-well plates containing 5 mL of test compounds. Plates were incubated in a CO2 incubator at 37 C for 4 days. Fluorescence signals were measured using SpectraMaxM5 microplate reader (Molecular Devices, U.S.A.). Fluorescence signals were measured using Spec-traMax M5 microplate reader (Molecular devices, U.S.A.) in the bottom-reading mode at the excitation and emission wavelengths of 530 and 590 nm. Ellipticine was used as the positive control and 0.5% DMSO as the negative control.

Dose response curves for a subset of compounds were deter-mined in Vero (ATCC CCL-81) and A549 (ATCC CCL-185) cells. The MTT based method described by Mosman el al was employed [45]. Cells were cultured in DMEM medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37 C in a humidified atmosphere with 5% CO2. The cells were cultured in complete DMEM for 16e18 h, then cultured with compound for 72 h. Each well was charged with DMEM containing 3-[4,5-dimehyl-2-thiazolyl]-2,5- diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml in normal sodium), and then cells were incubated for 3 h. The medium was carefully decanted and 50 ml of dimethyl sulfoxide was added. The plate was shaken mechanically for 5 min, and then absorbance readings at a wavelength of 570 nm were performed on a Sunrise microplate reader (Tecan). Gefitinib was used as the positive control and 0.5% DMSO as the negative control.

4.9.3. Protein kinase activity

All kinase assays were constituted and performed in a similar manner except for the following differences in reagents: For JAK2 kinase assays, the reaction contained 10 ng JAK2 enzyme, 0.1 mg/ml poly (Glu:Tyr) substrate and 0.1 mg/ml ATP. For JAK3 kinase assays, the reaction contained 3 ng JAK3 enzyme, 0.1 mg/ml poly (Glu:Tyr) substrate and 0.1 mg/ml ATP.

Inhibition was determined by using Antibody Beacon™ Tyrosine Kinase Assay Kit (A-35725; Invitrogen, USA). For the EGFR kinase assay, the detection complex contained the following components in 1X kinase assay buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM EGTA, 0.01% Brig and 2 mM DTT), 0.0267 mg/ml poly (Glu:Tyr), 6.7 nM anti-phosphotyrosine antibody, 0.33 nM

Oregon Green 488 ligand and 0.5 mM ATP. To the reaction was added with 12.5 ml of test compounds and 12.5 ml of EGFR enzyme (3 ng enzyme/reaction). After that, 25 ml of the detection complex was added into each well. The mixture solution was analyzed for 1½ hat 30 Cinafluorescence microplate reader using excitation at 485 nm and emission at 535 nm using an Infinite F200 fluorescence microplate reader (Tecan). The IC50 data was obtained by fitting using Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Erlotinib (EGFR) and compound-7c [53] (JAK2/3) were used as the positive control and 0.5% DMSO as the negative control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2016.08. 055. These data include MOL files and InChiKeys of the most important compounds described in this article.

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