Targeting the human malaria parasite Plasmodium falciparum: In vitro identification of a new antiplasmodial hit in 4-phenoxy-2-trichloromethylquinazoline series

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Original article

Targeting the human malaria parasite *Plasmodium falciparum*: *In vitro* identification of a new antiplasmodial hit in 4-phenoxy-2-trichloromethylquinazoline series

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ABSTRACT

From the promising results we previously obtained in quinazoline series and to complete the evaluation of the *in vitro* antiplasmodial activity of original 2-trichloromethylquinazolines, we synthesized new quinazolines possessing a variously substituted phenoxy group at position 4 through a simple and efficient two-step-synthesis approach. The studies of their activity toward the multi-resistant W2 *Plasmodium falciparum* strain and of their cytotoxicity on the human hepatocyte HepG2 cell line highlighted a hit compound (molecule **7**) displaying a W2 IC_{50} value of 1.1 μM and a HepG2 CC_{50} value of 50 μM, comparable to chloroquine and doxycycline. Structure-activity- and toxicity relationships indicate that the trichloromethyl group plays a key role in the antiplasmodial activity of such chemical scaffold and also that the phenoxy group substitution as a direct influence on the molecules selectivity. Moreover, molecule **7** displays significant specific activity against the *Plasmodium* genus in comparison with *Toxoplasma* and does not show any mutagenic property at the Ames test.

1. Introduction

Plasmodium falciparum is a protozoan parasite belonging to the *Apicomplexa* phylum and is the causative agent of the most severe type of malaria: cerebral malaria. According to the W.H.O., 225 million people were suffering from malaria in 2009 among which about 800.000 died, especially African children aged less than 5 years [1]. Because of the emergence of multi-drug-resistant *P. falciparum* strains, new antimalarial drug-compounds presenting original mechanisms of action are needed in order to contribute to solving such alarming public health problem [2].

Since more than 40 years, it is well known that the quinazoline ring can display antiplasmodial properties, as many 2,4-diaminoquinazolines were known to inhibit strongly *P. falciparum* dihydrofolate reductase (*Pf*D.H.F.R.), an essential enzyme to the parasite DNA synthesis [3]. Quite recently, new molecules belonging to the quinazoline series, substituted at position 2, 4 or 6, were identified as exerting promising antiplasmodial activities [4–8]. Among them, three different series were issued from our research team (Fig. 1) specialized on the design and the synthesis of original molecules with anti-infectious properties [9–11].

In order to carry on with the investigation of the antiplasmodial potential of new quinazoline derivatives, we focused on the evaluation of the influence of the substitution of position 4 of the quinazoline ring toward this activity by preparing a new series of 2-trichloromethylquinazolines bearing a phenoxy group at position 4. Then, their *in vitro* antiplasmodial properties on the W2 multi-resistant *P. falciparum* strain were evaluated. In parallel, a cytotoxicity study was conducted on the human HepG2 cell line, in order to

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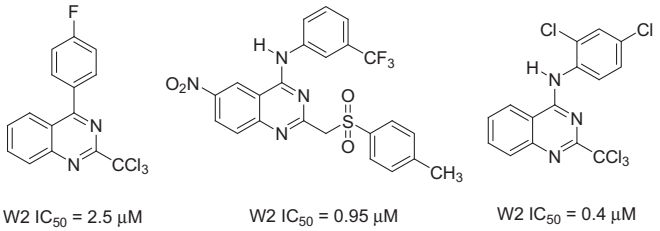
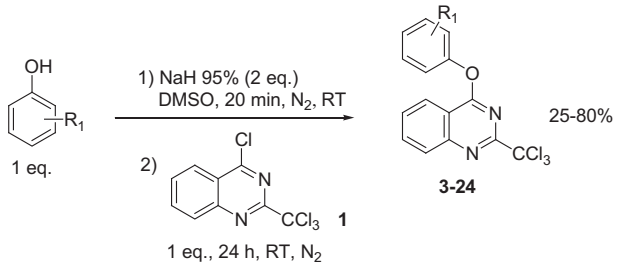


Fig. 1. Quinazolines displaying W2 *P. falciparum* antiplasmodial properties.



Scheme 2. Preparation of phenoxyquinazolines **3**–**24**.

assess their selectivity. These two studies permitted us to identify a new antiplasmodial hit (molecule **7**) which biological profile was finally refined with an antitoxoplasmic evaluation and an Ames mutagenic test.

2. Results and discussion

2.1. Chemistry

2.1.1. Preparation of substrate *1* and compound *2*

Key starting material 4-chloro-2-trichloromethylquinazoline **1** was prepared from commercial 2-methyl-4-(3*H*)quinazolinone by a microwave-assisted chlorination protocol we previously described [12]. This substrate reacts easily with sodium hydroxide in DMSO, at RT, to afford 4-hydroxyquinazoline **2** which exhibits in solution a double lactam-lactim prototropic tautomerism (Scheme 1).

2.1.2. Preparation of molecules *3*–*24*

4-Chloroquinazolines and especially molecules such as compound **1**, bearing a strong electro-attracting group at position 2, display a good reactivity at position 4 toward most of nucleophiles. Thus, substrate **1** was involved in S_NAr reactions with a series of aryl alcoholates, extemporaneously generated by the action of an excess of 95% sodium hydride on the corresponding phenols in anhydrous DMSO, at RT under nitrogen atmosphere, to afford phenoxyquinazolines **3**–**24** (Scheme 2). Sodium hydride was chosen as a base displaying weak nucleophilic properties in order to avoid the formation of undesirable substitution products. Depending on the phenol reagent used, reaction yields varied from 25 to 80% (mean yield = 63%). In order to define a single general operating procedure, the reaction time was fixed to 24 h even if most reactions were much faster.

2.1.3. Preparation of compound *25*

Reduction of the trichloromethyl hit molecule **7** was operated in refluxing acetic acid with a large excess of iron and permitted to isolate the methylquinazoline **25** in a fast and efficient way (Scheme 3).

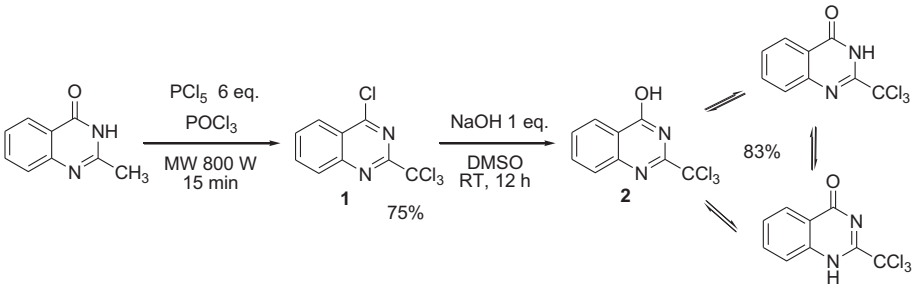
2.1.4. Preparation of compound *26*

The trifluoromethylquinazoline **26**, a close structural analog of hit compound **7** was synthesized by applying the same protocol as for molecules **3**–**24**. The reaction between 4-chloro-2-trifluoromethylquinazoline and 4-chlorophenate, formed as above, afforded **26** in 78% yield (Scheme 4).

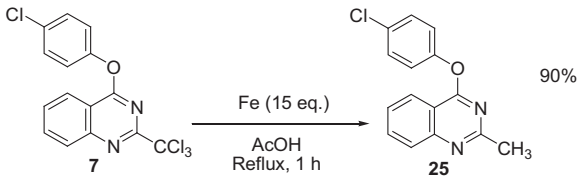
2.2. Biology

2.2.1. Antiplasmodial activity

Molecules were tested *in vitro* on the W2 multi-resistant strain of *P. falciparum* by using the SYBR green I assay with an incubation time of 72 h. This method appears to be one of the most relevant methods for the *in vitro* screening of antiplasmodial candidates nowadays [13]. Chloroquine and doxycycline were used as positive controls (Table 1). Despite the presence of a trichloromethyl group on molecules **1** and **2**, none of them displayed any antiplasmodial activity which indicates that such substituent does not confer antiplasmodial properties alone. By considering the activities of molecules **3**–**5**, it appears that the substitution of position 4 of the quinazoline ring by aryl alcoholate derivatives improves very significantly the IC_{50} values. Among such derivatives, α- and β-naphtols were not retained for further investigations as regards of their pronounced toxic profile. Then, a series of 19 compounds (**6**–**24**) including a substituted phenol ring were prepared and evaluated. Their antiplasmodial activities were rather good, ranging from 1.1 to 6 μM. Among them, molecule **7** appears to be a hit compound, displaying an IC_{50} value of 1.1 μM, intermediate to the ones of the reference-drugs chloroquine (0.4 μM) and doxycycline (4.8 μM). The most active molecules were the ones including electro-attracting groups on the phenoxy moiety, apart with *ortho*-substituted ones which were always less active. The evaluation of nitro-substituted molecules **16** and **18** revealed that such functional group is responsible for a poor solubility of the corresponding molecules. Compounds bearing electrodonating substituents (**19**–**24**) were either globally less active or toxic. In order to set up the importance of the trichloromethyl group to the antiplasmodial activity of this quinazoline scaffold, a methyl and a trifluoromethyl



Scheme 1. Preparation of compounds **1** and **2** from 2-methyl-4-(3*H*)-quinazolinone.



Scheme 3. Preparation of methylquinazoline **25**.

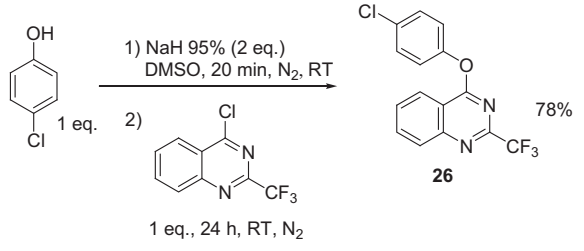
analog (**25** and **26**) were prepared and their activities where compared to the one of hit compound **7**. In both cases, antiplasmodial properties were not maintained which demonstrates that the trichloromethyl group, even if not bringing antiplasmodial activity alone (see molecules **1** and **2**), plays a key role in the anti-infectious activity of such a series.

2.2.2. Cytotoxicity and selectivity index

In parallel, all compounds were assessed regarding their *in vitro* cytotoxicity so as to be able to determine their selectivity indexes and validate their real potential as selective antiplasmodial agents (Table 1). Because of its metabolic activation potential, the HepG2 human cell line is one of the most frequently used cell line for conducting *in vitro* toxicological screenings [14]. By using these cells, we wanted to investigate both the toxic potential of each molecule and the ones of some of their eventual metabolites. Cytotoxicity concentrations 50% (CC_{50}) were measured and compared to the one of doxorubicin, used as positive control. Among the 26 tested molecules, only 5 appeared as significantly toxic. As already mentioned above, naphtol-substituted quinazolines **4** and **5** were quite toxic. Among other molecules, 4-phenoxy-2-trichloromethylquinazolines including a methyl group at position *meta* (**20**) or a methoxy group at position *ortho* or *meta* (**23** and **24**) were also cytotoxic. As already noted in oxazolidine series [15], although being nitated, molecules **16** and **17** did not exhibit *in vitro* cytotoxicity. Most of compounds bearing a trichloromethyl group at position 2 of the quinazoline ring did not display cytotoxic properties, in comparison with both reference drug-compounds and methyl (**25**) or trifluoromethyl (**26**) analogs of molecule **7**. Thus, selectivity indexes (CC_{50}/IC_{50} ratio) calculated in the whole 4-phenoxy series are ranging from 0.3 to 45, indicating that the type of substitution of the phenoxy moiety has a major influence on the biological profile of the series and can be responsible for its cytotoxicity.

2.2.3. Complementary investigations

Then, in order to get more informations about the biological profile of molecule **7**, complementary investigations were carried out (Table 2). *P. falciparum* belonging to the *Apicomplexa* phylum, we decided to evaluate the *in vitro* biological activity of hit compound **7** on an other closely related *Apicomplexa* protozoan, *Toxoplasma gondii*. In parallel, the cytotoxicity of the tested molecules was assessed on the HFF fibroblast cell line which is the one



Scheme 4. Preparation of trifluoromethylquinazoline **26**.

required for performing the *in vitro* antitoxoplasmic evaluation which we applied. In comparison with pyrimethamine used as a reference-drug, molecule **7** displays a weak antitoxoplasmic activity which appears in favor of its selective antiplasmodial profile. Moreover, in addition to the HepG2 cell line, molecule **7** remains non toxic on the HFF cell line. Finally, to insure the *in vitro* toxicological evaluation of hit compound **7**, its mutagenic potential was evaluated by using the Ames Test [16]. No significant number of revertants was noted for molecule **7**, in comparison with negative controls, indicating that this trichloromethylated compound is not mutagenic *in vitro*.

3. Conclusion

A new series of 4-phenoxy-2-trichloromethylquinazolines was prepared in two steps through a simple and efficient synthesis pathway. Among these molecules, compound **7** was identified as an *in vitro* antiplasmodial hit, displaying an IC_{50} value of 1.1 μM on the W2 multi-resistant strain of *P. falciparum* and being safe as regards both of its 50 μM CC_{50} value on the human HepG2 cell line and its >50 μM value on the HFF cell line, in comparison with chloroquine and doxycycline chosen as reference-drugs. Moreover, molecule **7** appears rather selective toward the *Plasmodium* genus, in comparison with the *Toxoplasma* one and does not exert *in vitro* mutagenic properties. Structure–activity and –toxicity relationships show that the trichloromethyl group plays a key role in the antiplasmodial activity which can be significantly improved by modifying the substitution of the phenoxy moiety, especially with electro-attracting groups in *para* or *meta* positions (except nitro group because of poor solubility of corresponding nitrated derivatives). The ortho substitution of the phenoxy moiety appears as unfavorable to the activity while the presence of electrodonating substituents in position *ortho* and essentially *meta* are responsible for cytotoxicity increase. These medicinal chemistry data support the idea that some 4-substituted-2-trichloromethylquinazolines constitute attractive chemical scaffolds for the conception of new molecular entities with antimalarial potential.

4. Experimental section

4.1. Chemistry

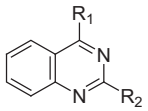
4.1.1. General

Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were determined on a Bruker Avance 200 MHz instrument, at the Faculté de Pharmacie de Marseille. Chemical shifts are given in δ values referenced to the solvent. Elemental analyses were carried out with a Thermo Finnigan EA 1112 apparatus at the Spectropôle department of the Faculté des Sciences et Techniques de S^t Jérôme. Silica Gel 60 (Merck 70-230) was used for column chromatography. The progress of the reactions was monitored by thin layer chromatography using Kieselgel 60 F254 (Merck) plates.

*4.1.2. Preparation of 4-chloro-2-trichloromethylquinazoline **1** [12]*

Compound **1** was prepared as described previously [12] and was obtained after purification by silica gel column chromatography, eluting with dichloromethane/petroleum ether (1:1), as a white solid in 75% yield; mp 127 °C ^1H NMR (200 MHz, $CDCl_3$) δ: 7.82–7.90 (m, 1H), 8.03–8.12 (m, 1H), 8.20–8.24 (m, 1H), 8.33–8.38 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 95.9 (C), 122.9 (C), 126.0 (CH), 129.7 (CH), 130.6 (CH), 135.9 (CH), 150.2 (C), 159.9 (C), 164.0 (C). Anal. Calcd for $C_9H_4Cl_4N_2$: C, 38.34; H, 1.43; N, 9.94. Found: C, 38.25; H, 1.14; N, 9.60.

Table 1
Antiplasmodial activity and human cell toxicity of the studied series.



Entry	R_1–	–R_2	*W2 P. falciparum* antiplasmodial[a] activity IC_{50} (μM)	HepG2 human cell toxicity[a] CC50 (μM)	Selectivity index[b]
1[12]	Cl–	$-CCl_3$	54.5	>125[e]	>2.3
2	HO–	$-CCl_3$	>50[e]	21	<0.42
3	Ph–O–	$-CCl_3$	3.1	50	16
4	1-Naphthyl–O–	$-CCl_3$	3.9	2.4	0.6
5	2-Naphthyl–O–	$-CCl_3$	2.3	3.6	1.6
6	3,4-Di–CH_3–Ph–O–	$-CCl_3$	3.3	29	8.8
7	**4-Cl–Ph–O–**	**$-CCl_3$**	**1.1**	**50**	**45**
8	3-Cl–Ph–O–	$-CCl_3$	2.4	26.1	10.9
9	2-Cl–Ph–O–	$-CCl_3$	4.4	33.6	7.6
10	4-F–Ph–O–	$-CCl_3$	2.4	29.5	12.3
11	3-F–Ph–O–	$-CCl_3$	2.3	41.7	18.1
12	2-F–Ph–O–	$-CCl_3$	5.0	38.3	7.7
13	4-CF_3–Ph–O–	$-CCl_3$	1.8	29.6	16.4
14	3-CF_3–Ph–O–	$-CCl_3$	2.3	25.1	10.9
15	2–CF_3– Ph–O–	$-CCl_3$	3.3	34.9	10.6
16	4-NO_2–Ph–O–	$-CCl_3$	3.5	>16[f]	>4.6
17	3-NO_2–Ph–O–	$-CCl_3$	3.0	36.3	12.1
18	2-NO_2–Ph–O–	$-CCl_3$	4.9	>8[f]	>1.6
19	4-CH_3–Ph–O–	$-CCl_3$	3.2	42.8	13.4
20	3-CH_3–Ph–O–	$-CCl_3$	2.9	7.6	2.6
21	2-CH_3–Ph–O–	$-CCl_3$	5.5	25.1	4.6
22	4-OCH_3–Ph–O–	$-CCl_3$	2.9	34.1	11.8
23	3-OCH_3–Ph–O–	$-CCl_3$	1.9	0.6	0.3
24	2-OCH_3–Ph–O–	$-CCl_3$	6.0	6.7	1.1
25	4-Cl–Ph–O–	$-CH_3$	28	26	0.9
26	4-Cl–Ph–O–	$-CF_3$	68	72	1.1
Ref.	Chloroquine[c]		0.4	30	75
Ref.	Doxycycline[c]		4.8	20	4.2
Ref.	Doxorubicin[d]		–	0.2	–

[a] Mean of three independent experiments.
[b] Selectivity index was calculated according to the following formula: $SI_{W2 Plasmodium}$ = HepG2 CC_{50}/W2 *P. falciparum* IC_{50}.
[c] Chloroquine and doxycycline were used as antiplasmodial drug–compounds of reference.
[d] Doxorubicin was used as a drug–compound of reference for human cell toxicity.
[e] No significant antiplasmodial activity or toxicity noted at highest concentration tested.
[f] Molecule could not be tested at higher concentration because of poor solubility.

*4.1.3. Preparation of 4-hydroxy-2-trichloromethylquinazoline **2** [17]*

Compound **1** (1 eq.) was dissolved in DMSO. 1 eq. of sodium hydroxide was then added and the reaction mixture was stirred at RT for 12 h. Water was then added to the reaction mixture which was extracted three times with dichloromethane. The organic layer was washed several times with water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Compound **2** was finally isolated, after purification by silica gel column chromatography, eluting with dichloromethane, as a white solid in 83% yield; mp 215 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.58–7.66 (m, 1H), 7.80–7.90 (m, 2H), 8.33–8.37 (m, 1H), 11.01 (br s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 92.2 (C), 121.2 (C), 126.8 (CH), 128.9 (CH), 129.0 (CH), 135.4 (CH), 146.6 (C), 149.3 (C), 162.1 (C). Anal. Calcd for $C_9H_5Cl_3N_2O$: C, 41,02; H, 1.91; N, 10.63. Found: C, 41.12; H, 1.89; N, 10.49.

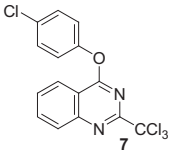
*4.1.4. General procedure for the preparation of compounds **3**–**24***

1 equivalent of the appropriate aryl alcohol was dissolved in dimethylsulfoxyde (DMSO), then added onto 2 eq. of NaH 95%, and stirred under N_2 for 20 min. 1 eq. of 4-chloro-2-trichloromethylquinazoline was dissolved in DMSO, added to the mixture and stirred at RT for 24 h under nitrogen atmosphere. The reaction was stopped by addition of water and the reaction mixture was then extracted three times with dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography on silica gel with an appropriate solvent to afford the corresponding nucleophilic aromatic substitution product.

*4.1.4.1. 4-Phenoxy-2-trichloromethylquinazoline (**3**) [18].* Compound **3** was obtained, after purification by column chromatography (eluent: cyclohexane-ethyl acetate 8:2) as a white solid in 76% yield; mp 106 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.27–7.51 (m, 5H), 7.72–7.80 (m, 1H), 7.94–8.03 (m, 1H), 8.12–8.17 (m, 1H), 8.41–8.45 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.7 (C), 115.3 (C), 121.4 (CH*2), 123.6 (CH), 125.8 (CH), 128.9 (CH), 129.0 (CH), 129.4 (CH*2), 134.8 (CH), 151.0 (C), 152.2 (C), 160.1 (C), 167.2 (C). Anal. Calcd for $C_{15}H_9Cl_3N_2O$: C, 53.05; H, 2.67; N, 8.25. Found: C, 53.13; H, 2.69; N, 8.16.

*4.1.4.2. 4-(Naphthalen-1-yloxy)-2-trichloromethylquinazoline (**4**).* Compound **4** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 9:1) as a pale beige solid in 60% yield; mp 126 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.43–7.63 (m, 4H), 7.79–8.08 (m, 5H), 8.18–8.22 (m, 1H), 8.62 (dd, J = 0.7 Hz and 8.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.6 (C), 115.3 (C), 118.0 (CH), 121.3 (CH), 123.6 (CH), 125.4 (CH), 126.0 (CH), 126.3 (CH), 126.4 (CH), 126.8 (C), 128.1 (CH), 129.1 (CH), 129.2 (CH), 134.8 (C), 134.9 (CH), 148.0 (C), 151.2 (C), 160.3

Table 2
Comparison of anti-apicomplexa activities and mutagenicity evaluation of hit compound **7**.

Entry	*W2 P. falc.* antiplasmodial activity[a] IC_{50} (μM)	*T. gondii* antitoxoplasmic activity[a] IC_{50} (μM)	HFF fibroblast toxicity[a] CC_{50} (μM)	HepG2 human cell toxicity[a] CC_{50} (μM)	Ames test result[b]
 **7**	1.1	8.8	>50	50	Negative
Chloroquine[c]	0.4	–	–	30	–
Doxycycline[c]	4.8	–	–	20	–
Pyrimethamine[d]	–	1.1	>50	7.1	–
Doxorubicin[e]	–	–	0.03	0.2	Positive

[a] Mean of three independent experiments.
[b] A product is considered mutagenic when it induces, at least, a two-fold increase of the number of revertants in comparison with negative control.
[c] Chloroquine and doxycycline were used as antiplasmodial drug–compounds of reference.
[d] Pyrimethamine was used as an antitoxoplasmic drug–compound of reference.
[e] Doxorubicin was used as a drug–compound of reference for cytotoxicity.

(C), 167.7 (C). Anal. Calcd for $C_{19}H_{11}Cl_3N_2O$: C, 58.56; H, 2.85; N, 7.19. Found: C, 59.03; H, 3.02; N, 7.06.

*4.1.4.3. 4-(Naphthalen-2-yloxy)-2-trichloromethylquinazoline (**5**).* Compound **5** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 9:1) as a pale beige solid in 80% yield; mp 155 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.48–7.57 (m, 3H), 7.74–8.05 (m, 6H), 8.16–8.20 (m, 1H), 8.46–8.50 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.7 (C), 115.4 (C), 118.2 (CH), 121.1 (CH), 123.7 (CH), 125.8 (CH), 126.6 (CH), 127.7 (CH), 127.8 (CH), 128.9 (CH), 129.1 (CH), 129.2 (CH), 131.4 (C), 133.8 (C), 134.9 (CH), 149.9 (C), 151.1 (C), 160.1 (C), 167.3 (C). Anal. Calcd for $C_{19}H_{11}Cl_3N_2O$: C, 58.56; H, 2.85; N, 7.19. Found: C, 58.43; H, 3.06; N, 7.11.

*4.1.4.4. 4-(3,4-Dimethylphenoxy)-2-trichloromethylquinazoline (**6**).* Compound **6** was obtained, after purification by column chromatography (eluent: cyclohexane-ethyl acetate 8:2) as a white solid in 64% yield; mp 104 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.30 (s, 6H), 7.15–7.17 (m, 3H), 7.70–7.78 (m, 1H), 7.93–8.01 (m, 1H), 8.11–8.15 (m, 1H), 8.40 (dd, J = 0.9 Hz and 8.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 19.3 (C), 20.0 (C), 96.9 (C), 115.5 (C), 118.4 (CH), 122.2 (CH), 123.8 (CH), 128.9 (CH), 129.0 (CH), 130.2 (CH), 134.0 (C), 134.8 (CH), 137.8 (C), 150.2 (C), 151.0 (C), 160.3 (C), 167.4 (C). Anal. Calcd for $C_{17}H_{13}Cl_3N_2O$: C, 55.54; H, 3.56; N, 7.62. Found: C, 56.00; H, 3.72; N, 7.66.

*4.1.4.5. 4-(4-Chlorophenoxy)-2-trichloromethylquinazoline (**7**).* Compound **7** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a white solid in 74% yield; mp 167 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.32–7.46 (m, 4H), 7.73–7.81 (m, 1H), 7.95–8.04 (m, 1H), 8.13–8.17 (m, 1H), 8.38–8.42 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.6 (C), 115.2 (C), 122.9 (CH*2), 123.6 (CH), 129.0 (CH), 129.2 (CH), 129.5 (CH*2), 131.2 (C), 135.0 (CH), 150.6 (C), 151.1 (C), 160.0 (C), 167.0 (C). Anal. Calcd for $C_{15}H_8Cl_4N_2O$: C, 48.16; H, 2.16; N, 7.49. Found: C, 48.46; H, 2.23; N, 7.49.

*4.1.4.6. 4-(3-Chlorophenoxy)-2-trichloromethylquinazoline (**8**) [18].* Compound **8** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 80% yield; mp 109 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.27–7.48 (m, 4H), 7.74–7.82 (m, 1H), 7.97–8.05 (m, 1H), 8.14–8.19 (m, 1H), 8.38–8.42 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.6 (C), 115.1 (C), 119.9 (CH), 122.2 (CH), 123.5 (CH), 126.1 (CH), 129.0 (CH), 129.3 (CH), 130.1 (CH), 134.7 (C), 135.1 (CH), 151.1 (C), 152.5 (C), 160.0 (C), 166.8 (C). Anal. Calcd for $C_{15}H_8Cl_4N_2O$: C, 48.16; H, 2.16; N, 7.49. Found: C, 48.04; H, 2.22; N, 7.89.

*4.1.4.7. 4-(2-Chlorophenoxy)-2-trichloromethylquinazoline (**9**).* Compound **9** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a white solid in 53% yield; mp 133 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.23–7.54 (m, 4H), 7.74–7.81 (m, 1H), 7.96–8.04 (m, 1H), 8.14–8.18 (m, 1H), 8.47 (d, J = 8.1 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.5 (C), 114.9 (C), 123.7 (CH), 123.8 (CH), 126.9 (C), 127.1 (CH), 127.8 (CH), 128.9 (CH), 129.2 (CH), 130.4 (CH), 135.0 (CH), 148.3 (C), 151.1 (C), 159.9 (C), 166.8 (C). Anal. Calcd for $C_{15}H_8Cl_4N_2O$: C, 48.16; H, 2.16; N, 7.49. Found: C, 47.83; H, 2.38; N, 8.06.

*4.1.4.8. 4-(4-Fluorophenoxy)-2-trichloromethylquinazoline (**10**).* Compound **10** was obtained, after purification by column chromatography (eluent: petroleum ether-dichloromethane 1:1) as a white solid in 45% yield; mp 128 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.54–7.59 (m, 1H), 7.74–7.85 (m, 3H), 7.99–8.08 (m, 1H), 8.17–8.22 (m, 1H), 8.41–8.46 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.7 (C), 115.2 (C), 115.8 (CH), 116.3 (CH), 123.0 (d, J = 8.4 Hz, CH*2), 123.6 (CH), 129.0 (d, J = 8.8 Hz, CH*2), 134.9 (CH), 147.9 (d, J = 3 Hz, C), 151.1 (C), 157.8 (C), 160.1 (C), 164.9 (d, J = 232 Hz, C). Anal. Calcd for $C_{15}H_8Cl_3FN_2O$: C, 50.38; H, 2.25; N, 7.83. Found: C, 50.74; H, 2.22; N, 8.08.

*4.1.4.9. 4-(3-Fluorophenoxy)-2-trichloromethylquinazoline (**11**).* Compound **11** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 63% yield; mp 107 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 6.99–7.09 (m, 1H), 7.18–7.23 (m, 2H), 7.38–7.49 (m, 1H), 7.74–7.81 (m, 1H), 7.97–8.05 (m, 1H), 8.14–8.19 (m, 1H), 8.41 (dd, J = 0.6 and 8.2 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.6 (C), 109.7 (d, J = 25 Hz, CH), 112.9 (d, J = 21 Hz, CH), 115.2 (C), 117.3 (d, J = 3 Hz, CH), 123.5 (CH), 129.0 (CH), 129.2 (CH), 130.2 (d, J = 10 Hz, CH), 135.0 (CH), 151.1 (C), 152.9 (d, J = 11 Hz, C), 160.0 (C), 162.9 (d, J = 247 Hz, C), 166.8 (C). Anal. Calcd for $C_{15}H_8Cl_3FN_2O$: C, 50.38; H, 2.25; N, 7.83. Found: C, 50.73; H, 2.33; N, 7.74.

*4.1.4.10. 4-(2-Fluorophenoxy)-2-trichloromethylquinazoline (**12**).* Compound **12** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a white solid in 53% yield; mp 116 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.19–7.46 (m, 4H), 7.74–7.82 (m, 1H), 7.97–8.05 (m, 1H), 8.15–8.19 (m, 1H), 8.43–8.47 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.5 (C), 114.8 (C), 116.8 (d, J = 18 Hz, CH), 123.6 (d, J = 4 Hz, CH),

123.7 (CH), 124.5 (d, $J = 4$ Hz, CH), 127.2 (d, $J = 7$ Hz, CH), 128.9 (CH), 129.2 (CH), 135.0 (CH), 139.5 (d, $J = 12$ Hz, C), 151.1 (C), 154.3 (d, $J = 250$ Hz, C), 160.0 (C), 166.7 (C). Anal. Calcd for $C_{15}H_8Cl_3FN_2O$: C, 50.38; H, 2.25; N, 7.83. Found: C, 50.88; H, 2.45; N, 7.77.

*4.1.4.11. 2-Trichloromethyl-4-[4-(trifluoromethyl)phenoxy]quinazoline (**13**).* Compound **13** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 31% yield; mp 120 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.55–7.61 (m, 2H), 7.74–7.85 (m, 3H), 8.00–8.08 (m, 1H), 8.17–8.21 (m, 1H), 8.41–8.46 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.5 (C), 115.1 (C), 121.9 (CH*2), 123.5 (CH), 124.0 (q, $J = 272$ Hz, C), 126.8 (q, $J = 4$ Hz, CH*2), 128.1 (q, $J = 33$ Hz, C), 129.1 (CH), 129.4 (CH), 135.2 (CH), 151.2 (C), 154.6 (C), 159.9 (C), 166.7 (C). Anal. Calcd for $C_{16}H_8Cl_3F_3N_2O$: C, 47.15; H, 1.98; N, 6.87. Found: C, 47.70; H, 2.20; N, 6.55.

*4.1.4.12. 2-Trichloromethyl-4-[3-(trifluoromethyl)phenoxy]quinazoline (**14**).* Compound **14** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 9:1) as a white solid in 55% yield; mp 84 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.58–7.66 (m, 3H), 7.76–7.85 (m, 2H), 7.99–8.08 (m, 1H), 8.16–8.21 (m, 1H), 8.41–8.46 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.5 (C), 115.1 (C), 119.1 (q, $J = 4$ Hz, CH), 122.6 (q, $J = 4$ Hz, CH), 123.5 (CH), 124.9 (CH), 123.6 (q, $J = 272$ Hz, C), 129.1 (CH), 129.4 (CH), 130.0 (CH), 131.9 (q, $J = 33$ Hz, C), 135.2 (CH), 151.2 (C), 152.1 (C), 159.9 (C), 166.7 (C). Anal. Calcd for $C_{16}H_8Cl_3F_3N_2O$: C, 47.15; H, 1.98; N, 6.87. Found: C, 47.40; H, 2.01; N, 7.10.

*4.1.4.13. 2-Trichloromethyl-4-[2-(trifluoromethyl)phenoxy]quinazoline (**15**).* Compound **15** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 7:3) as a beige solid in 68% yield; mp 116 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.42–7.46 (m, 1H), 7.67–7.84 (m, 4H), 7.98–8.06 (m, 1H), 8.15–8.20 (m, 1H), 8.42–8.46 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.5 (C), 115.0 (C), 122.8 (q, $J = 31$ Hz, C), 123.1 (q, $J = 273$ Hz, C), 123.7 (CH), 124.3 (CH), 125.9 (CH), 127.0 (q, $J = 5$ Hz, CH), 128.9 (CH), 129.5 (CH), 132.9 (CH), 135.1 (CH), 149.5 (C), 149.6 (C), 151.2 (C), 159.8 (C), 166.9 (C). Anal. Calcd for $C_{16}H_8Cl_3F_3N_2O$: C, 47.15; H, 1.98; N, 6.87. Found: C, 47.64; H, 1.94; N, 6.98.

*4.1.4.14. 4-(4-Nitrophenoxy)-2-trichloromethylquinazoline (**16**).* Compound **16** was obtained, after purification by column chromatography (eluent: petroleum ether-dichloromethane 6:4) as a white solid in 75% yield; mp 158 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.59–7.66 (m, 2H), 7.78–7.86 (m, 1H), 8.01–8.09 (m, 1H), 8.18–8.22 (m, 1H), 8.36–8.45 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 97.9 (C), 115.0 (C), 122.4 (CH*2), 123.4 (CH), 125.3 (CH*2), 129.2 (CH), 129.6 (CH), 135.4 (CH), 145.4 (C), 151.3 (C), 156.8 (C), 159.7 (C), 166.4 (C). Anal. Calcd for $C_{15}H_8Cl_3N_3O_3$: C, 46.84; H, 2.10; N, 10.93. Found: C, 47.35; H, 2.11; N, 11.07.

*4.1.4.15. 4-(3-Nitrophenoxy)-2-trichloromethylquinazoline (**17**).* Compound **17** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 73% yield; mp 116 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.64–7.74 (m, 1H), 7.79–7.87 (m, 2H), 8.01–8.10 (m, 1H), 8.19–8.25 (m, 2H), 8.38–8.47 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.4 (C), 114.9 (C), 117.4 (CH), 120.9 (CH), 123.4 (CH), 128.0 (CH), 129.2 (CH), 129.5 (CH), 130.0 (CH), 135.4 (CH), 148.8 (C), 151.3 (C), 152.3 (C), 159.7 (C), 166.5 (C). Anal. Calcd for $C_{15}H_8Cl_3N_3O_3$: C, 46.84; H, 2.10; N, 10.93. Found: C, 47.23; H, 2.27; N, 10.59.

*4.1.4.16. 4-(2-Nitrophenoxy)-2-trichloromethylquinazoline (**18**).* Compound **18** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 70% yield; mp 174 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.47–7.56 (m, 2H), 7.75–7.84 (m, 2H), 7.99–8.06 (m, 1H), 8.15–8.25 (m, 2H), 8.43–8.47 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 96.2 (C), 114.7 (C), 123.7 (CH), 125.2 (CH), 126.0 (CH), 126.9 (CH), 129.0 (CH), 129.5 (CH), 134.9 (CH), 135.3 (CH), 141.9 (C), 145.3 (C), 151.2 (C), 159.5 (C), 166.8 (C). Anal. Calcd for $C_{15}H_8Cl_3N_3O_3$: C, 46.84; H, 2.10; N, 10.93. Found: C, 47.24; H, 2.30; N, 10.58.

*4.1.4.17. 4-(4-Methylphenoxy)-2-trichloromethylquinazoline (**19**).* Compound **19** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 79% yield; mp 138 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.42 (s, 3H), 7.22–7.32 (m, 4H), 7.72–7.80 (m, 1H), 7.95–8.03 (m, 1H), 8.12–8.17 (m, 1H), 8.40–8.45 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 21.0 (CH_3), 96.8 (C), 115.4 (C), 121.1 (CH*2), 123.7 (CH), 128.9 (CH), 129.0 (CH), 129.9 (CH*2), 134.7 (CH), 135.4 (C), 150.0 (C), 151.0 (C), 160.2 (C), 167.4 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O$: C, 54.34; H, 3.14; N, 7.92. Found: C, 54.87; H, 3.36; N, 7.88.

*4.1.4.18. 4-(3-Methylphenoxy)-2-trichloromethylquinazoline (**20**).* Compound **20** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 70% yield; mp 116 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.42 (s, 3H), 7.10–7.39 (m, 4H), 7.71–7.80 (m, 1H), 7.94–8.03 (m, 1H), 8.13–8.17 (m, 1H), 8.39–8.44 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 21.4 (CH_3), 96.8 (C), 115.4 (C), 118.3 (CH), 122.0 (CH), 123.7 (CH), 126.6 (CH), 128.9 (CH), 129.0 (CH), 129.1 (CH), 134.8 (CH), 139.5 (C), 151.0 (C), 152.1 (C), 160.2 (C), 167.3 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O$: C, 54.34; H, 3.14; N, 7.92. Found: C, 54.63; H, 3.28; N, 7.68.

*4.1.4.19. 4-(2-Methylphenoxy)-2-trichloromethylquinazoline (**21**).* Compound **21** was obtained, after purification by column chromatography (eluent: petroleum ether-ethyl acetate 8:2) as a white solid in 65% yield; mp 91 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.22 (s, 3H), 7.21–7.34 (m, 4H), 7.73–7.81 (m, 1H), 7.95–8.04 (m, 1H), 8.14–8.18 (m, 1H), 8.43–8.45 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 16.4 (CH_3), 96.7 (C), 115.1 (C), 121.7 (CH), 123.7 (CH), 126.1 (CH), 126.9 (CH), 128.9 (CH), 129.0 (CH), 130.4 (C), 131.2 (CH), 134.8 (CH), 150.8 (C), 151.0 (C), 160.3 (C), 167.2 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O$: C, 54.34; H, 3.14; N, 7.92. Found: C, 54.21; H, 3.05; N, 7.70.

*4.1.4.20. 4-(4-Methoxyphenoxy)-2-trichloromethylquinazoline (**22**).* Compound **22** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a white solid in 64% yield; mp 129 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.86 (s, 3H), 6.93–7.01 (m, 2H), 7.26–7.34 (m, 2H), 7.70–7.79 (m, 1H), 7.93–8.02 (m, 1H), 8.12–8.16 (m, 1H), 8.39–8.43 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.6 (CH_3), 96.8 (C), 114.3 (CH*2), 115.4 (C), 122.2 (CH*2), 123.7 (CH), 128.9 (CH), 129.0 (CH), 134.7 (CH), 145.7 (C), 151.0 (C), 157.2 (C), 160.2 (C), 167.5 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O_2$: C, 51.99; H, 3.00; N, 7.58. Found: C, 52.01; H, 3.09; N, 7.46.

*4.1.4.21. 4-(3-Methoxyphenoxy)-2-trichloromethylquinazoline (**23**).* Compound **23** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a yellow solid in 25% yield; mp 101 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.83 (s, 3H), 6.83–6.89 (m, 1H), 6.96–7.05 (m, 2H), 7.32–7.40 (m, 1H), 7.72–7.80 (m, 1H), 7.95–8.03 (m, 1H), 8.13–8.17 (m, 1H), 8.39–8.43 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.5 (CH_3), 96.8 (C), 107.2 (CH), 112.0 (CH), 113.5 (CH), 115.4 (C), 123.7 (CH), 128.9 (CH), 129.1 (C), 129.7 (CH), 134.8 (CH), 151.0 (C), 153.1 (C), 160.1 (C), 160.4 (C), 167.4 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O_2$: C, 51.99; H, 3.00; N, 7.58. Found: C, 52.16; H, 3.07; N, 7.67.

*4.1.4.22. 4-(2-Methoxyphenoxy)-2-trichloromethylquinazoline (**24**).* Compound **24** was obtained, after purification by column chromatography (eluent: dichloromethane-petroleum ether 1:1) as a white solid in 71% yield; mp 85 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.72 (s, 3H), 7.01–7.07 (m, 2H), 7.27–7.32 (m, 2H), 7.73–7.78 (m, 1H), 7.93–7.98 (m, 1H), 8.12–8.16 (m, 1H), 8.43–8.48 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.7 (CH_3), 96.8 (C), 112.4 (CH), 115.0 (C), 120.7 (CH), 122.4 (CH), 123.9 (CH), 127.0 (CH), 128.7 (CH), 128.8 (CH), 134.6 (CH), 141.1 (C), 150.9 (C), 151.3 (C), 160.2 (C), 167.3 (C). Anal. Calcd for $C_{16}H_{11}Cl_3N_2O_2$: C, 51.99; H, 3.00; N, 7.58. Found: C, 52.54; H, 3.26; N, 7.28.

*4.1.5. Preparation of 4-(4-chlorophenoxy)-2-methylquinazoline (**25**)*

To a refluxing mixture of acetic acid and iron (15 eq.), 1 eq. of 4-(4-chlorophenoxy)-2-trichloromethylquinazoline (**7**) was added. The reaction mixture was then stirred for 1 h and filtered over celite. Acetic acid was evaporated under reduced pressure, affording a yellow residue which was dissolved in dichloromethane. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Compound **25** was obtained, after purification by silica column chromatography, eluting with dichloromethane-ethyl acetate (8:2), as a white solid in 90% yield; mp 95 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.63 (s, 3H), 7.19–7.25 (m, 2H), 7.38–7.46 (m, 2H), 7.54–7.62 (m, 1H), 7.82–7.94 (m, 2H), 8.27–8.31 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 26.2 (CH_3), 114.2 (C), 123.3 (CH*2), 123.4 (CH), 126.7 (CH), 127.0 (CH), 129.5 (CH*2), 130.9 (C), 134.1 (CH), 150.9 (C), 151.9 (C), 163.7 (C), 166.2 (C). Anal. Calcd for $C_{15}H_{11}ClN_2O$: C, 66.55; H, 4.10; N, 10.35. Found: C, 66.93; H, 4.34; N, 10.13.

*4.1.6. Preparation of 4-(4-chlorophenoxy)-2-trifluoromethylquinazoline (**26**)*

One eq. of 4-chlorophenol was dissolved in dimethylsulfoxyde (DMSO), then added onto 2 eq. of NaH 95%, and stirred under N_2 for 20 min. 1 eq. of 4-chloro-2-trifluoromethylquinazoline was dissolved in DMSO, then added to the mixture, and stirred at RT for 24 h. The organic layer was then extracted three times by dichloromethane. The organic layer was washed with water five times, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Compound **26** was obtained after purification by silica column chromatography, eluting with dichloromethane/petroleum ether (1:1) as a white solid in 78% yield; mp 143 °C ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.27 (d, $J = 8.8$ Hz, 2H), 7.45 (d, $J = 8.8$ Hz, 2H), 7.77–7.85 (m, 1H), 7.99–8.07 (m, 1H), 8.14–8.18 (m, 1H), 8.41–8.45 (m, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 116.4 (C); 119.4 (q, $J = 275.5$ Hz, C); 122.9 (CH*2); 123.7 (CH); 128.8 (CH); 129.6 (CH); 129.7 (CH*2); 131.5 (C); 135.2 (CH); 150.4 (C); 151.1 (C); 151.6 (q, $J = 36.9$ Hz, C); 167.5 (C). Anal. Calcd for $C_{15}H_8ClF_3N_2O$: C, 55.49; H, 2.48; N, 8.63. Found: C, 55.71; H, 2.50; N, 8.62.

4.2. Biology

4.2.1. Antiplasmodial evaluation

In this study, a W2 culture-adapted *P. falciparum* strain resistant to chloroquine, pyrimethamine and proguanil was used in an *in vitro* culture. Maintenance in continuous culture was done as described previously by Trager and Jensen [19]. Cultures were maintained in fresh A+ human erythrocytes at 2.5% hematocrit in complete medium (RPMI 1640 with 25 mM HEPES, 25 mM $NaHCO_3$, 10% of A+ human serum) at 37 °C under reduced O_2 atmosphere (gas mixture 5% O_2, 5% CO_2, and 90% N_2). Parasitaemia was maintained daily between 1% and 6%. The *P. falciparum* drug susceptibility test was carried out by comparing quantities of DNA in treated and control cultures of parasite in human erythrocytes according to an SYBR Green I fluorescence-based method [20] using a 96-well fluorescence plate reader. Parasite culture was synchronized at ring stage with 5% sorbitol. Compounds were incubated in a total assay volume of 200 µl (RPMI, 2% hematocrit and 1% parasitaemia) for 72 h in a humidified atmosphere (5% O_2 and 5% CO_2) at 37 °C, in 96-well flat bottom plates. Triplicate assays were performed for each sample. After incubation, 170 µl supernatant was discarded and cells were washed with 150 µl 1× PBS. 15 µl re-suspended cells were transferred to 96-well flat bottom non-sterile black plates (Greiner Bio-one) already containing 15 µl of the SYBR Green lysis buffer (2XSYBR Green, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). Negative control, treated by solvents (DMSO or H_2O) and positive controls (chloroquine, doxycycline) were added to each set of experiments. Plates were incubated for 15 min at 37 °C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 497 and 520 nm, respectively. The concentrations of compounds required to induce a 50% decrease of parasite growth (IC_{50W2}) were calculated from three independent experiments.

4.2.2. Antitoxoplasmic evaluation

The effects of the tested compounds on the growth of *T. gondii* tachyzoites (PRU-β-Gal strain) [21] were assessed by a colorimetric microtiter assay adapted from Mc Fadden et al. [22]. Briefly, tachyzoites were maintained by serial passage in confluent monolayer of human foreskin fibroblasts HFF (ATCC, Manassas, USA). For assay, 96-well microtiter plates were seeded with 3×10^4 HFF cells per well and allowed to grow to confluence in RPMI 1640 (without phenol red) supplemented with 10% FCS and 1% L-glutamine/penicillin-streptomycin mix at 37 °C with 6% CO_2. Cell monolayers were infected with 1×10^4 parasites per well and incubated for 3 h at 37 °C with 6% CO_2. Then, various concentrations of compounds dissolved in DMSO (final concentration less than 0.5% v/v) were incorporated in triplicate. Appropriate controls treated by DMSO or the reference-drug pyrimethamine were added to each set of experiments. Negative control consisted in cell monolayers incubated without parasite and drug. After a 72 h incubation period at 37 °C with 6% CO_2, cell medium was removed and 100 µl of a 1 mM chlorophenol red-β-D-galactopyranoside (CPRG) solution were added to each well. The plates were incubated at 37 °C with 6% CO_2 for 6 h, at which time β-galactosidase activity was measured by reading plates at 570 and 630 nm on a Biotek microtiter plate reader. Blanking was made on the negative-control wells. The concentration of compounds required to induce a 50% decrease of parasite growth (IC_{50}) was calculated by non-linear regression analysis processed on dose–response curves, using the Table Curve software 2D v.5.0. IC_{50} values represent the mean value calculated from three independent experiments.

4.2.3. Cytotoxicity evaluation

The evaluation of the tested molecules cytotoxicity on the HepG2 (purchased from ATCC, ref HB-8065) and HFF cell lines was done according to the method of Mosmann [23] with slight modifications. Briefly, cells in 100 µL of complete medium, [RPMI supplemented with 10% foetal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL)/streptomycin (100 µg/mL)] were inoculated into each well of 96-well plates and incubated at 37 °C in a humidified 6% CO_2 with 95% air atmosphere. After a 24 h incubation, 100 µl of medium with various product concentrations was added and the plates were incubated for 72 h. At the end of the treatment and incubation, each plate-well was microscope-examined for detecting possible

precipitate formation before the medium was aspirated from the wells. 10 μL of MTT solution (5 mg MTT/mL in PBS) were then added to each well with 100 μl of medium without foetal calf serum. Cells were incubated for 2 h at 37 °C to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After this time, the MTT solution was removed and DMSO (100 μL) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with 630 nm as reference wavelength with a microplate spectrophotometer. DMSO was used as blank and doxorubicine as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration was determined from the dose–response curve.

4.2.4. Ames test

Compounds were assessed for mutagenicity by a modified version of the liquid incubation assay of the classical Ames test at five concentrations (25–125 nM) [24]. *Salmonella* tester strains (TA97a, TA98, TA100 and TA102) were grown overnight in a Nutrient Broth n°2 (Oxoid, France). After this period, products dissolved in DMSO (Sigma) were added to 0.1 mL of culture and incubated for 1 h at 37 °C with shaking. Each sample was assayed in duplicate. After incubation, 2 mL of molten top agar were mixed gently with the pre-incubated solution and poured onto Vogel-Bonner minimal agar plates. After 48 h at 37 °C, the number of spontaneous- and drug-induced revertants per plate was determined for each dose with a laser bacterial colony counter (laser bacterial colony counter 500A, Interscience). A product was considered mutagenic when it induced a two-fold increase of the number of revertants, compared with the spontaneous frequency (negative control). For each *Salmonella* strain, a specific positive- and solvent-control were performed.

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