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A Combinatorial Approach to 2,4,6-Trisubstituted Triazines with Potent Antimalarial Activity: Combining Conventional Synthesis and Microwave-Assistance

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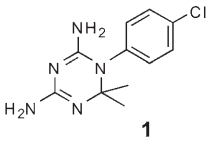
Malaria nowadays remains one of the world's greatest public health problems. It is responsible for two million deaths per year, mostly African children under five years old, particularly affecting peoples in developing countries.[1] Among the four malaria species that infect humans, the parasite *P. falciparum* is universally considered the most aggressive. Particularly impressive is its ability in mutating forms in response to administered antiplasmodial treatment, rapidly giving rise to adaptation and resistance.[2] Hence, it is extremely urgent to find an effective combination of antimalarial drugs, not only to improve the efficacy of the therapy, but also to prevent further development of resistance.[3] The discovery of the great potential of artemisinin has significantly encouraged the search in this area of medicinal chemistry. However, artemisinin and its active derivatives are ideal for rapid parasite clearance and clinical recovery, but they need to be combined with longer-acting drugs to prevent recrudescence.

Continuous efforts in the search for new drugs together with modeling and analytical investigations on the plasmodia mechanism of invasion have highlighted two possible important targets. The dihydrofolate reductase (DHFR) of *P. falciparum* is one of the few well-defined targets in antimalarial therapy. This enzyme catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. DHFR is considered the target of cycloguanil **1** and of other antifolates in use for anti-



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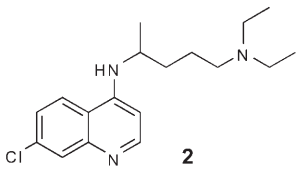

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malarial prophylaxis and therapy.[4] The inhibition of DHFR is expected to cause a significant decrease of pyrimidinic bases of DNA and, consequently, the inhibition of parasite growing. However, antifolate resistance in *P. falciparum* strains has rapidly developed against this promising generation of antimalarial agents.[5] The acute need of overcoming such problems has stimulated a great effort aimed at finding new effective antifolate antimalarials. In this context, the synthesis of novel chemical compounds affecting relative parasitic metabolism could lead to improved chemotherapeutic agents for malaria treatment. A second target is the acidic digestive vacuole. This food vacuole is involved in hemoglobin degradation and heme detoxification, which is considered the target of 4-aminoquinolines, such as chloroquine **2**.[6] Once again, as chloroquine (CQ) has been largely used in clinical therapy, rapid development of resistance often occurs in endemic disease. Hence, finding new effective 4-aminoquinoline-based antimalarials also represents a very urgent need.[7] Finally, a further challenge is to obtain antiplasmodial compounds, which are both highly effective and cheap, to make them accessible on a large scale for poor people.

With these objectives in mind, we reasoned that the use of trichlorotriazine as a core scaffold might give access to multifunctional architectures, potentially enabling us to address more than one of the above targets in a single molecule. Moreover, in light of the known sequential reactivity of the three chlorine atoms in the triazine backbone, such a scaffold is well-suited to get high variability, useful for combinatorial synthesis.[8] In principle, this concept gives access to a new generation of multivalent antimalarial agents for combination therapy based on a single active source. In the present paper, a library of 2,4,6-triamino-1,3,5-triazines was synthesized as both cycloguanil and chloroquine analogues, and assessed against *P. falciparum* strains cultured in human hematocyte.

The triazine derivatives were synthesized from cyanuric chloride by consecutive aromatic nucleophilic substitution (S_NAr) reactions under controlled conditions. We synthesized the monosubstituted triazine by adding the first nucleophilic amine to a solution of cyanuric chloride in acetone at 0 °C in the presence of diisopropylethylamine (DIPEA) or NaOH as activating bases. The second substitution was carried out in the same solvent at room temperature. Usually, deactivated disubstituted precursors, such as monochlorodiamino triazines, make it very difficult to perform the third nucleophilic attack.[9] The trisubstituted triazines were first approached by warming the monochlorotriazines with different nucleophiles in the presence of DIPEA in dimethylsulfoxide. In many cases, we found that reaction yields and kinetics with this procedure were unsatisfactory. As we have experience with microwave irradiation as an effective method for S_NAr reactions,[10] where possible, we chose to abort the chlorine replacement at the

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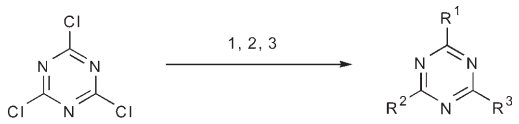
stage of disubstituted derivative, whereas the third substitution was accomplished by exploiting microwave heating. Reaction conditions for the microwave-assisted reaction were carefully optimized using a representative triazine target (data are summarized in the Supporting Information). The three-step procedure employed within this work is shown in Scheme 1.



3a–r

Scheme 1. Reaction conditions: 1) R^1H, base, acetone, 0 °C, 3-4 h; 2) R^2H, base, acetone, RT, 16 h; 3) R^3H, base, DMSO, MW, 6 bar, 180 °C, 18 min. R^{1-3} = nucleophilic amines.

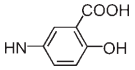
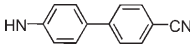
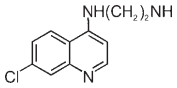
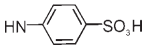
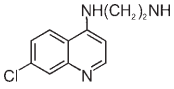
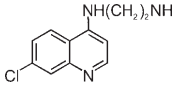
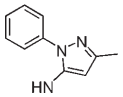
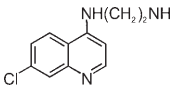
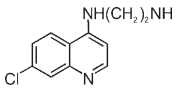
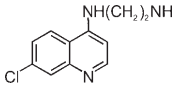
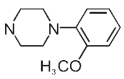
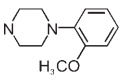
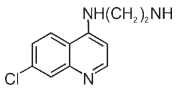
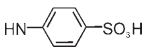
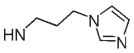
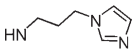
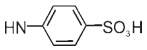
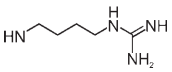
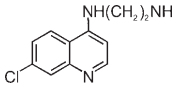
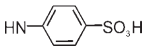
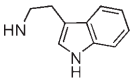
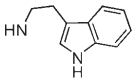
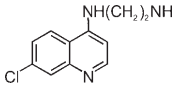
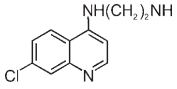
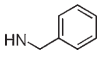
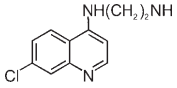
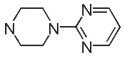
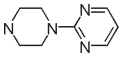
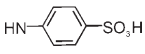
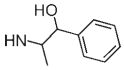
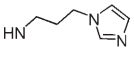
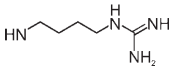
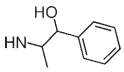
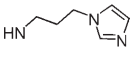
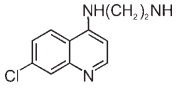
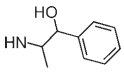
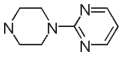
Using microwave irradiation in the first stage was not optimal, as control of the second and third substitution was very difficult, in most cases resulting in the formation of the homotrisubstituted derivative. On the contrary, our strategy combining conventional reactions and microwave-assisted heating not only improved chemical yields, but also allowed us to obtain triazine compounds loaded with two or three different targeting arms, thus increasing chemical diversity. We evaluated different primary and secondary amines, obtaining a library of eighteen triazine-based potential antimalarial agents. Results are summarized in Table 1.

All the compounds from this library were screened to assess their in vitro antimalarial activity, by using the pLDH assay, against D10 (CQ-sensitive) and W2 (CQ-resistant) strains of *P. falciparum*. *P. falciparum* cultures were carried out in vitro according to Trager and Jensen with slight modifications.[11] The D10 and W2 strains were maintained at 5% human hematocrit in RPMI 1640 medium with the addition of 10% heat-inactivated A-positive human plasma, 20 mM Hepes, and 2 mM glutamine. All the cultures were maintained at 37 °C. Compounds were dissolved and diluted with medium to achieve the required concentrations. Products were placed in 96 well flat-bottom microplates and serial dilutions were obtained. Asynchronous cultures with 1.0–1.5% parasitemia and 1.0% final hematocrit were aliquoted into the microplates and incubated at 37 °C for 72 h. Parasite growth was assessed spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (LDH), according to a modified version of Makler's method in control and treated cultures.[12] Antimalarial activity is expressed as the 50% inhibitory concentration (IC_{50}). The IC_{50} values of the synthesized compounds are reported in Table 1, whereas corresponding values for chloroquine (CQ) and cycloguanil (Cy) are provided in the table legend.

On the basis of our results, we found that new potent antimalarial compounds were obtained, exhibiting good growth inhibition and selective activity against the chloroquine-resistant W2 strain. A first look into our data suggests that lipophilicity of the amine substituents gives a significant positive contribution to antiparasitic activity. The most active compounds among our anti-W2 products, that is, **3c**, **3d**, **3l**, and **3m**, all contain hydrophobic groups. On the contrary, those molecules presenting polar groups, such as sulfonyl, carboxyl, or guanidinic head groups, generally exhibit poor activity, because low polarity is crucial to cross the parasite plasmatic membrane. However, only a more accurate consideration of the different lypophilic derivatives allows for a better correlation between the structure of NR_2 moieties on the triazine backbone and antimalarial activity. In fact, those compounds with two diethylamine substituents (**3a**, **3b**, **3k**) show a remarkable decrease in activity, confirming previous observations.[13] The NH_2 group as such is quite borderline, probably owing to a combination of a fine fitting within the DHFR active site and good inhibition of heme polymerization. Substituent steric hindrance seems also to play an important role. The presence of bulky groups, such as in **3e**, **3f**, **3g**, **3n**, and **3q**, results in a dramatic decrease of the antiplasmodial activity. Such loss of activity is probably attributable to an impediment in fitting the DHFR active site. Indeed, we may suppose that bulky side chains make it very difficult for the relevant molecules to approach to the DHFR active site and/or to assume the right conformation for the π–π interaction with the heme functionality. In apparent contrast, **3p**, one of the triazine derivatives with three different amines, shows very interesting IC_{50} values. This molecule presents large substituents, having a norephedrine, an aminoquinoline, and an aminoimidazole groups. Nevertheless, it shows an IC_{50} value in the same order as chloroquine against the CQ-sensitive strain, and a good activity against the CQ-resistant strain. We may speculate that these substituent groups promote the formation of strong complexes with iron-heme. Indeed, imidazole is known to induce axial coordination and the phenyl group of norephedrine functionality is able to enforce the π–π interaction with heme. Interestingly, **3i** has two polar functionalities: a sulfonic acid group and a guanidine-terminating arm. Despite its evident hydrophilic character, this product exhibits a weak antiplasmodial activity against the W2 strain, probably due to the ability to influence the action of the parasite in its first stages of invasion. Indeed, sulfonic acid and guanidinic group are known to be involved in cell to cell adhesion mechanisms, extremely crucial to parasite survival and reproduction. Amine basicity does not seem to appreciably affect the overall antiplasmodial character of the synthesized compounds. Indeed, compounds with low pK_a, such as **3d**, **3l**, and **3m**, exhibit a potent antiparasitic activity. Notably, the presence of at least one chloroquine-based side arm is required to achieve a good activity against W2 CQ-R strains, but the improvement obtained in the effectiveness of the monochloroquine agent once conjugated to *bis*-benzylamino triazine (**3m**) is quite impressive.

Cytotoxic activity of the synthesized compounds was estimated on human fibroblast (HDF) and on human microvascular endothelial cells (HMEC-1). In Table 2, we have summarized the results relative to compounds **3c**, **3d**, **3l**, **3m**, and **3p**, which proved very active against the W2 CQ-R strains. All the active compounds showed low toxicity against mammalian cells with a therapeutic index (IC_{50} value on human cells/IC_{50} value on *P. falciparum* strains) ranging between 73 and 569.

Table 1. Synthesized triaminosubstituted triazines and their evaluation as antimalarial agents.[a]

3	R^1	R^2	R^3	Yield [%]	D10 CQ-S strain[b] IC_{50} [nM][c]	W2 CQ-R strain[b] IC_{50} [nM][c]
3a		NEt_2	NEt_2	74	1450(±250)	1610(±300)
3b		NEt_2	NEt_2	79	NA	NA
3c	HN*i*Pr	HN*i*Pr		74	65(±9)	108(±24)
3d				74	106(±26)	63(±11)
3e				59	378(±4)	252(±18)
3f				67	268(±38)	171(±29)
3g				62	120(±36)	186(±31)
3h				84	NA	NA
3i				64	159(±24)	386(±70)
3j				83	NA	NA
3k	NEt_2	NEt_2		70	232(±36)	371(±41)
3l	NHEt	NHEt		66	125(±23)	94(±12)
3m				77	41(±2)	32(±3)
3n				83	NA	2970(±360)
3o				77	154(±57)	510(±125)
3p				66	65(±14)	124(±37)
3q				83	1615(±570)	1452(±360)
3r	NH_2	NH_2		79	243(±60)	337(±85)

[a] CQ shows a D10 IC_{50} value of 30±5 nM and a W2 IC_{50} value of 744±28 nM, whereas we found a W2 IC_{50} value of approximately 5000 nM for Cy (not active against D10 strands). [b] Values are means of three experiments in triplicate, standard deviation is given in parentheses (NA = not active).

Noticeably, all the compounds were over 50 times less toxic on human cells than on parasite.

In conclusion, a diversity-oriented library of eighteen 2,4,6-trisubstituted-1,3,5-triazines were synthesized as cycloguanil and chloroquine analogues, and they were assessed in terms of in vitro antimalarial activity and toxicity. The most active products, that is, **3d**, **3l**, and **3m**, showed higher activity against CQ-resistant parasite strains, whereas **3c**, **3m**, and **3p**

Table 2. Compound cytotoxicity assessed on HMEC-1 and HDF human cells.

Compd	HMEC-1[a] IC_{50} [μM]	HDF[a] IC_{50} [μM]
3c	12.1(±1.2)	7.9(±1.2)
3d	11(±5.2)	35.9(±7.7)
3l	20.4(±0.4)	16.9(±5.8)
3m	41.9(±0.5)	3.4(±1.1)
3p	17.5(±4.8)	14.4(±3.4)

[a] Values are means (± SD) of three experiments in triplicate.

exhibited CQ-sensitive activity in the same order as chloroquine itself. The other compounds showed variable activity, from good to very weak compared to chloroquine diphosphate and cycloguanil. In general, we noticed that the active compounds were well tolerated by human fibroblasts and endothelial cell lines, thus supporting the potential interest for this new class of antimalarial agents. All the synthesized compounds were obtained starting from commercial sources with a simple and effective approach. The combined use of conventional S_NAr reaction and microwave irradiation proved very valuable in producing derivatized triazines with tunable substituents. Remarkably, compound **3m** was identified as a cheap, triazine-based candidate new lead for antimalarial combination therapies. Work is in progress to evaluate the in vivo activity and toxicity of this product and its analogues.

Experimental Section

General synthetic procedure. Diisopropylethylamine (DIEA, 5 equiv) was added to a solution of cyanuric chloride (1 equiv) in acetone at 0 °C, followed by 1.2 equiv of the amine. After 3–4 h, a solid precipitate slowly formed, which was isolated by filtration. The second amine (2.5 equiv) and DIEA (1 mL) was added to this product (1 equiv) in acetone (10 mL). After 16 h, addition of water (8 mL) to the solution induced precipitation of the product, which was isolated by filtration. The third amine substitution was carried out under microwave irradiation. In a 5 mL capped vial, 1.06 equiv amine and 1 equiv disubstituted triazine were dissolved in 3 mL DMSO and 1 equiv *N*-methylmorpholine was added under stirring at room temperature. The vial was irradiated at 180 °C (measured by the internal infrared sensor of the microwave apparatus) for 18 min within the microwave cavity at 6 bar pressure. Then, the mixture was left to cool to RT and the product was isolated by addition of alkaline water. The purity and identity of all the products were checked by NMR spectroscopy, elemental analysis, and HPLC-MS.

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Keywords: aminoquinolines · antimalarial agents · combinatorial chemistry · microwave assistance · triazines

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