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Synthesis of Solution-Phase Combinatorial Library of 4,6-Diamino-1,2-dihydro-1,3,5-triazine and Identification of New Leads Against A16V + S108T Mutant Dihydrofolate Reductase of *Plasmodium falciparum*

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Abstract—An efficient method to synthesize solution-phase combinatorial library of 1-aryl-4,6-diamino-1,2-dihydro-1,3,5-triazine was developed. The strategy involved an acid-catalyzed cyclocondensation between arylbiguanide hydrochlorides and carbonyl compounds in the presence of triethyl orthoacetate as water scavenger. A 96-membered combinatorial library was constructed from 6 aryl biguanides and 16 carbonyl compounds. Screening of the library by iterative deconvolution method revealed two candidate leads which are equally active against wild-type *Plasmodium falciparum* dihydrofolate reductase, but are about 100-fold more effective against the A16V + S108T mutant enzyme as compared to cycloguanil.

Introduction

The dihydrofolate reductase (DHFR) domain of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) enzyme of *Plasmodium falciparum* is the target of pyrimethamine (Pyr) and cycloguanil (Cyc) (Scheme 1), two historically important therapeutic drugs commonly used for the treatment of malaria infection.[1,2] However, in recent years, resistance to antifolate antimalarials in *P. falciparum* has become widespread, and hence reducing the effectiveness and the clinical utilities of the drugs. With the limited spectrum of the currently available antimalarials, there is an immediate need to search for new drugs and/or identify new targets in order to combat the resistant malaria. Resistance to Pyr and Cyc in *P. falciparum* has been shown to be associated with point mutations of the DHFR domain.While mutations at amino acids 51, 59, 108 and 164 are associated with Pyr- and Cyc-cross resistance, mutation at residue 16 has been shown to be responsible for Cyc resistance.[3–8] The mutations were found to affect both the binding affinity to inhibitors and kinetic properties of the enzymes.[9] The lack of three dimensional structure of *P. falciparum* DHFR has precluded rational inhibitor design and development of more effective inhibitors, therefore molecular modeling approach has been exploited as an alternative strategy.[10,11] Our previous works have led to the findings that steric clash imposed by A16V mutation effects binding of Cyc, and not Pyr, to the active site of the A16V + S108T mutant enzyme.[11] The hypothesis was verified by synthesizing and measuring the inhibition constants (K_i) of various Cyc derivatives (4,6-diamino-1,2-dihydro-1,3,5-triazine compounds) devoid of one or both of C-2 substituents, from which promising leads were identified.[12]

Recent advancement in the field of combinatorial chemistry has attracted much interest in drug discovery research due to the possibility of rapid generation of

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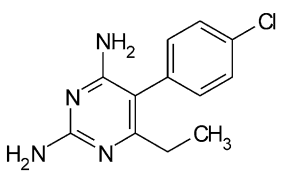
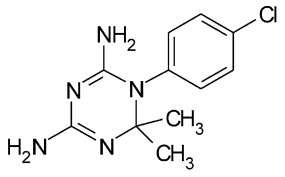
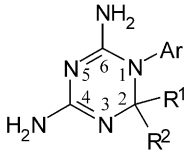


pyrimethamine



cycloguanil



I

4,6-diamino-1,2-dihydro-1,3,5-triazine derivatives

Scheme 1.

many structurally related compounds from which, in conjunction with a suitable screening technique, potential drug candidates can be rapidly identified.[13] In this paper, we wish to demonstrate the use of combinatorial technique to discover lead compounds with significantly more potent than the parent cycloguanil against cycloguanil-resistant DHFR from *P. falciparum* by synthesizing a small and well-defined combinatorial library of dihydrotriazines in solution phase. We also present the combinatorial library screening results and the identification of two potential drug candidates which are effective against both wild-type and A16V + S108T mutant DHFR of *P. falciparum*.

Results and Discussion

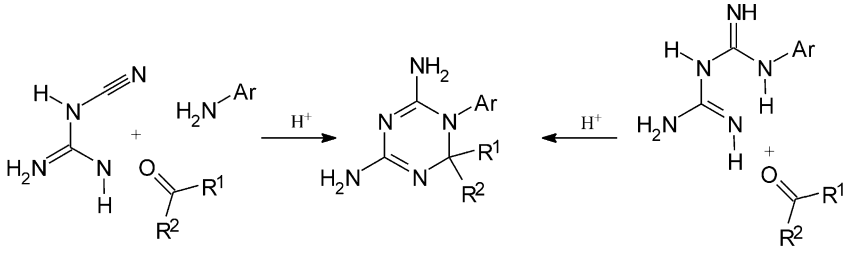
Chemistry

Synthesis of 1-aryl-4,6-diamino-1,2-dihydro-1,3,5-triazine (I) by the acid catalyzed three-component cyclocondensation between an aromatic amine, a carbonyl compound and dicyandiamide is well-documented (Scheme 2).[14] A synthesis of 72-membered combinatorial mixture of such library employing this method has been recently reported.[15] It was reported that only 64 out of 72 compounds were detected in the library. Furthermore, it is doubtful whether all the compounds successfully formed are present in equimolar quantities due to the difference in reactivities and/or the different crystallization behavior of each compound.

Our objective was to develop a technique by which a library of compounds comprising of pools of statistical distribution of the desired member, irrespective of the reactivities of starting materials and solubilities of the products, could be produced. The original Modest's three component[14] as well as two-component[16] dihydrotriazine syntheses (Scheme 2) gave satisfactory results with only limited range of substrates therefore a more efficient method is required in order to achieve our goal.

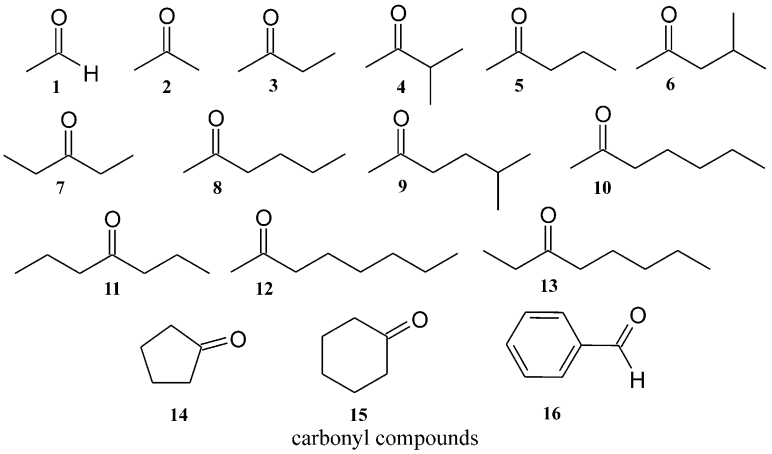
We have recently noted that addition of a water scavenger such as triethyl orthoacetate have dramatic effect to the formation of dihydrotriazine by 2-component synthesis reaction.[21] Reactions between a number of phenylbiguanide hydrochloride and excess of carbonyl compounds (5 equiv) proceeded smoothly at room temperature (30 °C) in the presence of triethyl orthoacetate and catalytic amounts of concentrated HCl. Evaporation of the solvents and trituration of the residue with diethyl ether afforded the dihydrotriazines (I) in good yield and purity (>90%). The insolubility of the dihydrotriazines in ether provides the advantage of solid phase synthesis in such a way that excess reagents and ether-soluble by-products may be removed by exhaustive washing without the need for expensive solid supports and attachment-cleavage steps. To investigate the range of suitable substrates to be included in the library, the rates of reactions of a representative set of carbonyl compounds (4-heptanone, 3-octanone, benzaldehyde, acetone, 4-methyl-3-pentanone, 3-methyl-2-butanone) and aryl biguanides (phenyl, 3,4-dichlorophenyl, 4-methylphenyl, 2,4-dichlorophenyl, 4-chlorophenylbiguanides) were examined by HPLC analyses. It was revealed that most substrates reacted completely within 12–72 h under the conditions described above. Only the reactions involving 2,4-dichlorophenylbiguanide was slow and incomplete even on prolonged stirring. Incidentally, according to the structure–activity relationship data available,[17] compounds (**I**) bearing *ortho*-substituent on the *N*-1 aryl group are unlikely to exhibit significant inhibitory activity against DHFRs, therefore 2,4-dichlorophenylbiguanide and other *ortho*-substituted biguanides were not included in the library.

Having established the optimal synthetic conditions for individual compounds, the libraries were then synthesized as mixture libraries by the split-and-mix methodology[18] for the purpose of screening by iterative deconvolution approach.[19,20] Chemical structures of the constituents of the library involved are given in Scheme



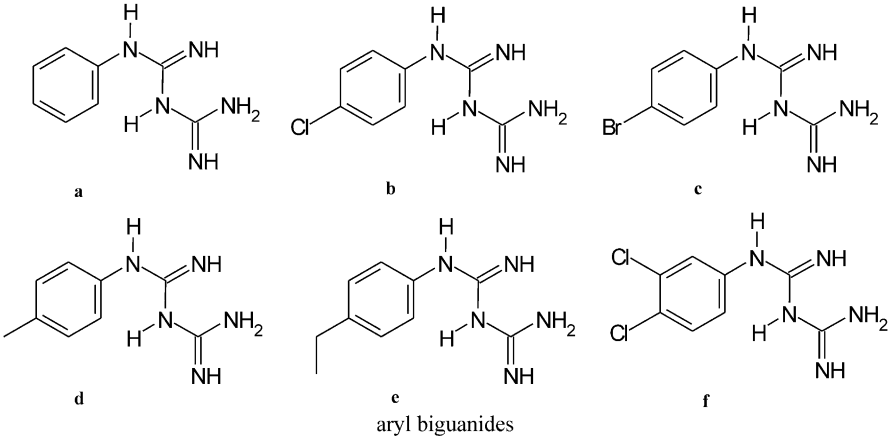
(I)

Scheme 2.



carbonyl compounds

1: acetaldehyde **2:** acetone **3:** 2-butanone **4:** 3-methylbutan-2-one
5: 2-pentanone **6:** 4-methylpentan-2-one **7:** 3-pentanone **8:** 2-hexanone
9: 5-methylhexan-2-one **10:** 2-heptanone **11:** 4-heptanone **12:** 2-octanone
13: 3-octanone **14:** cyclopentanone **15:** cyclohexanone **16:** benzaldehyde



aryl biguanides

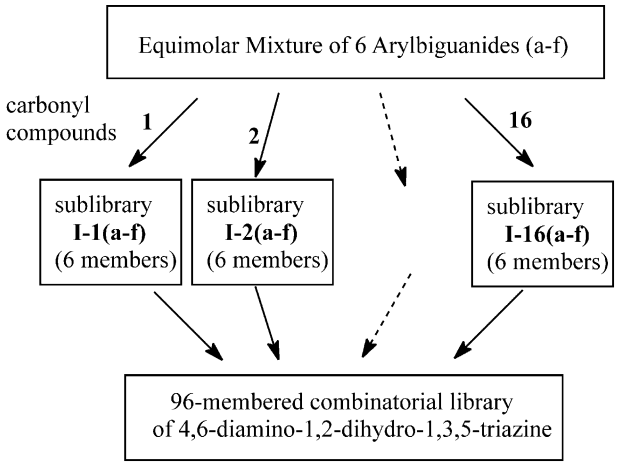
a: phenylbiguanide **b:** 4-chlorophenylbiguanide **c:** 4-bromophenylbiguanide
d: 4-methylphenylbiguanide **e:** 4-ethylphenylbiguanide **f:** 3,4-dichlorophenylbiguanide

Scheme 3.

3, and the strategy for creating a 96-membered combinatorial library from these compounds is illustrated in Scheme 4. According to this strategy, sixteen portions of an equimolar mixture of six aryl biguanides in the form of hydrochloride salts are separately treated with excess of 16 carbonyl compounds to give 16 sub-libraries, **I-1(a–f)**– **I-16(a–f)**, each of which had the C-2 substituents (derived from the carbonyl compounds **1**–**16**) fixed, and contained 6 compounds bearing 6 different N-1-phenyl substituents (derived from the biguanides **a**–**f**). As a result, a total number of 96 compounds, excluding those enantiomeric pairs whose C-2 of the dihydrotriazine is asymmetrically substituted, are synthesized.

A representative sublibrary **I-11(a–f)** was synthesized by treating the arylbiguanide mixture with 5 equivalents of 4-heptanone in the presence of triethyl orthoacetate and concentrated HCl in methanol at room temperature. After the reaction was completed, as judged by HPLC analysis, the solvent was removed and the product was triturated with diethyl ether to obtain the sublibrary as an off-white solid. Reverse phase HPLC analysis of the sublibrary **I-11(a–f)** revealed the presence of six major peaks with λ_{max} at 249 nm, characteristic for dihydrotriazines. The areas under each peak from the HPLC analysis profile (Fig. 1) were comparable and were in good agreement with the expected equimolar mixture of the six compounds (assuming the molar extinction coefficient of the triazine chromophore is independent of substituents). In addition, mass spectrometric analysis (ESI) revealed (M-Cl^+) peaks at $m/z = 273$, 287, 302, 307, 341 and 351, corresponding to the six desired products as anticipated. The remaining 15 sublibraries were synthesized and analyzed in the same way. All sublibraries **I-1(a–f)**–**I-16(a–f)** yielded six major peaks in approximately equimolar proportion upon HPLC analysis, and gave mass spectrometric data which corresponded to the desired products.

We have also explored the possibilities of creating combinatorial libraries in a complementary manner by reacting a biguanide with a mixture of carbonyl com-



Scheme 4.

pounds. Since the easily removable carbonyl compounds must be used in excess in order to drive the reaction to completion, complication arose due to the difference of reactivities of each carbonyl compound. Thus treatment of 4-chlorophenylbiguanide with excess equimolar mixture of 5-methylhexan-2-one, 2-pentanone, 2-octanone, 3-octanone, 2-hexanone and 2-heptanone gave variable amounts of products. Such difficulties in preparing isokinetic mixture of the carbonyl compounds made this approach less attractive and was not further investigated.

Enzyme assays

Primary screening of the 16 sublibraries was carried out with wild-type and A16V + S108T mutant pfDHFR of *P. falciparum* for the inhibitory activities (Table 1). Three sublibraries were found to exhibit reasonably good inhibition against both enzymes. These included sublibraries of acetaldehyde **(I-1(a–f)**: K_i wt 2.5 ± 0.2 nM; K_i mut 35.5 ± 7.3 nM), benzaldehyde **(I-16(a–f)**: K_i wt 6.4 ± 1.0 nM; K_i mut 146.3 ± 44.5 nM) and 2-octanone **(I-12(a–f)**: K_i wt 1.6 ± 0.2 nM; K_i mut 315.9 ± 60.4 nm). While the sublibraries **I-1(a–f)**, **I-12(a–f)** and **I-16(a–f)** were about as effective as Cyc against the wild-type *P. falciparum* DHFR, the libraries were significantly more active than Cyc against the A16V + S108T mutant enzyme. To assess the effectiveness of the compounds in the sublibraries, the ratios of the K_i values of the inhibitors for the A16V + S108T mutant enzyme and the wild-type enzyme (K_i-mut/K_i-wt) were calculated. As shown in Table 1, the **I-1(a–f)**, **I-12(a–f)** and **I-16(a–f)** sublibraries were about 38-, 4- and 84-fold more effective than Cyc, respectively, against the A16V + S108T mutant enzyme.

To further investigate and identify the active compounds from the pool of each sublibrary, all 18 members from the three sublibraries were individually synthesized, and the K_i values against both wild-type and A16V + S108T mutant enzymes were determined. As shown in Table 2, two most potent lead inhibitors **I-1f** and **I-16f** were identified from the sublibraries **I-1(a–f)** and **I-16(a–f)** respectively (Scheme 5). While the activities of the two compounds against the wild-type DHFR are comparable to that of Cyc (**I-1f**, K_i wt = 1.4 ± 0.2 nM; **I-16f** K_i wt = 1.6 ± 0.2 nM; Cyc K_i wt = 1.5 ± 0.3 nM), they are much more effective against the A16V + S108T mutant (**I-1f**, K_i mut = 17.8 ± 0.8 nM; **I-16f** K_i mut = 11.0 ± 1.8 nM; Cyc K_i mut = 1,313.7 ± 164.5 nM).

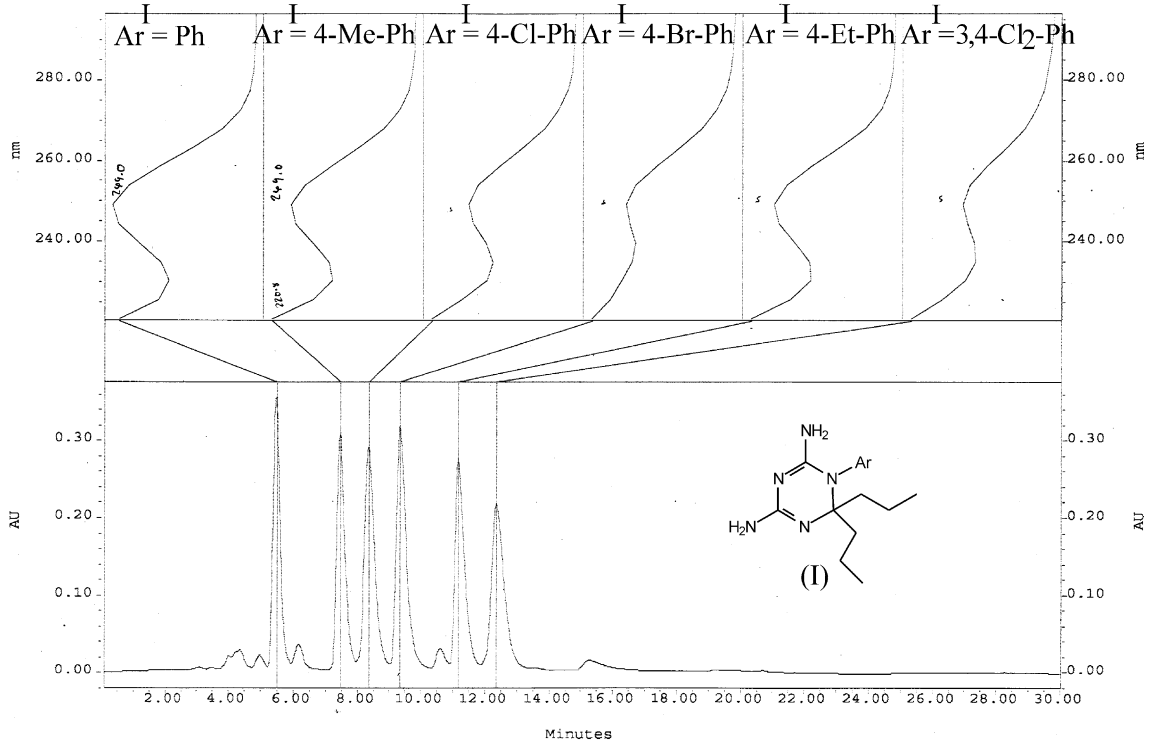


Figure 1. HPLC data of **I-11(a–f)**. Column: Hypersil™ 5 μm BDS C_{18} (4.6×250 mm); Mobile phase: 0.1 M triethylammonium acetate (pH 7.0):acetonitrile gradient from 15–60% over a period of 15 min with an equilibration period of 5 min; UV-detector at 254 nm.

Table 1. K_i values of 4,6-diamino-1,2-dihydro-1,3,5-triazine sublibraries for the wild-type and A16V + S108T mutant DHFRs of *P. falciparum*

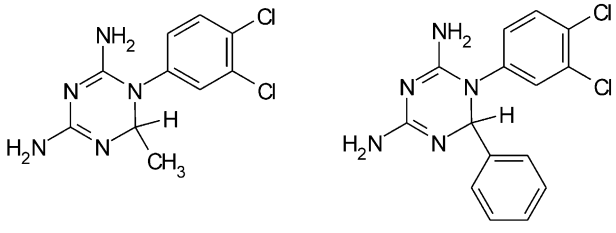
Sublibrary ID	Ar	R^1	R^2	K_i (wt)[a] (nM)	K_i (mut)[b] (nM)	K_i (mut)/K_i (wt)
I-1(a–f)		-H	-Me	2.5±0.2	35.5±7.3	14
I-2(a–f)		-Me	-Me	2.4±0.0	1428±286	595
I-3(a–f)		-Me	-Et	4.0±0.5	1939±225	485
I-4(a–f)		-Me	-$^{i}C_3H_7$	36.4±1.4	27,658±2096	760
I-5(a–f)		-Me	-$^{n}C_3H_7$	6.1±0.6	3499±601	574
I-6(a–f)	mixture of	-Me	-$CH_2CH(Me)_2$	5.0±0.3	4301±246	860
I-7(a–f)	4-H-C_6H_4-	-Et	-Et	11.4±1.7	5664±352	497
I-8(a–f)	4-ClC_6H_4-	-Me	-$^{n}C_4H_9$	5.5±0.3	2016±120	367
I-9(a–f)	4-BrC_6H_4-	-Me	-$(CH_2)_2CHMe_2$	3.9±0.3	1104±0.3	283
I-10(a–f)	4-MeC_6H_4-	-Me	-$^{n}C_5H_{11}$	4.9±0.4	1528±181	312
I-11(a–f)	4-EtC_6H_4-	-$^{n}C_3H_7$	-$^{n}C_3H_7$	470±32	39,546±10,153	83
I-12(a–f)	3,4-Cl_2	-Me	-$^{n}C_6H_{13}$	1.6±0.2	316±60.4	198
I-13(a–f)	C_6H_4-	-Et	-$^{n}C_5H_{11}$	43.0±2.6	12,233±438	284
I-14(a–f)		-$(CH_2)_4$-		19.4±3.2	668±99	34
I-15(a–f)		-$(CH_2)_5$-		414±58	5594±867	14
I-16(a–f)		-H	-Ph	6.4±1.0	146±45	23
Cyc	4-ClC_6H_4-	-Me	-Me	1.5±0.3	1314±165	876

[a]Wild-type pfDHFR.
[b]A16V + S108T mutant pfDHFR.

Table 2. K_i values of each member of the sublibraries **I-1(a–f), I-12(a–f)** and **I-16(a–f)** for the wild-type and A16V + S108T mutant DHFRs of *P. falciparum*

Compound ID	R^1	R^2	Ar	K_i (wt)[a] (nM)	K_i (mut)[b] (nM)	K_i (mut)/K_i (wt)
I-1a	-H	-Me	C_6H_5-	113±15	456±0.3	4.0
I-1b	-H	-Me	4-ClC_6H_4-	4.1±0.0[c]	127±13.6[c]	31[c]
I-1c	-H	-Me	4-BrC_6H_4-	5.7±0.5[c]	202±17[c]	35[c]
I-1d	-H	-Me	4-MeC_6H_4-	23.4±1.9[c]	186±22.4[c]	7.9[c]
I-1e	-H	-Me	4-EtC_6H_4-	11.0±3.0	322±56.4	29
I-1f	-H	-Me	3,4-$Cl_2C_6H_3$-	1.4±0.2[c]	17.8±0.8[c]	13[c]
I-12a	-Me	-$^{n}C_6H_{13}$	C_6H_5-	1.5±0.8	180±12.6	120
I-12b	-Me	-$^{n}C_6H_{13}$	4-ClC_6H_4-	0.7±0.5	564±151	806
I-12c	-Me	-$^{n}C_6H_{13}$	4-BrC_6H_4-	3.0±1.8	2186±338	729
I-12d	-Me	-$^{n}C_6H_{13}$	4-MeC_6H_4-	0.8±0.6	823±155	1029
I-12e	-Me	-$^{n}C_6H_{13}$	4-EtC_6H_4-	1.1±0.4	552±39.4	502
I-12f	-Me	-$^{n}C_6H_{13}$	3,4-$Cl_2C_6H_3$-	1.4±0.2	107±32.2	76
I-16a	-H	-Ph	C_6H_5-	49.1±3.5	61.8±15.6	1.3
I-16b	-H	-Ph	4-ClC_6H_4-	4.5±0.2[c]	49.3±3.3[c]	11[c]
I-16c	-H	-Ph	4-BrC_6H_4-	2.9±1.2[c]	90.3±11.4[c]	31[c]
I-16d	-H	-Ph	4-MeC_6H_4-	7.7±2.2[c]	170±13.8[c]	22[c]
I-16e	-H	-Ph	4-EtC_6H_4-	12.1±2.3	86.6±10.8	7.2
I-16f	-H	-Ph	3,4-$Cl_2C_6H_3$-	1.6±0.2[c]	11.0±1.8[c]	6.9[c]

[a]Wild-type pfDHFR.
[b]A16V + S108T mutant pfDHFR.
[c]Data from Yuthavong et al. (ref 12).



I-1f **I-16f**

I-1f: 4,6-Diamino-3',4'-dichlorophenyl-1,2-dihydro-2-methyl-1,3,5-triazine

I-16f: 4,6-Diamino-3',4'-dichlorophenyl-1,2-dihydro-2-phenyl-1,3,5-triazine

Scheme 5.

It is interesting to note that both compounds share two common features: (i) they possess only one substituent at C-2 position and (ii) they possess an extra chloro substituent at the 3′ position of the N-1 aromatic ring. The result confirmed that the presence of one hydrogen atom, or rather the absence of one substituent at the C-2 position of dihydrotriazine ring is an essential element of good binding with the A16V + S108T mutant enzyme as evident by the K_i-mut/K_i-wt ratio. This is in agreement with our previous model[11] which suggested that the steric effect resulted from interaction between one of the methyl group in cycloguanil at C-2 position with Val-16 in A16V + S108T pfDHFR is responsible for its poor activity. It also further emphasizes the contribution of the additional substituent at the 3′ position on the 1-aryl groups to good binding to the A16V + S108T pfDHFR, the fact that has been previously observed.[12] Although the two compounds had been previously identified as highly potent A16V + S108T pfDHFR inhibitors by conventional screening method,[12] the present study has demonstrated that the principle of combinatorial chemistry can be applied to the same problem to give the same outcome in a short time.

Conclusion

We have developed an efficient method to synthesize a 96-membered solution-phase combinatorial mixture library of 4,6-diamino-1,2-dihydro-1,3,5-triazines. Two highly potent lead inhibitors, one is over 100-folds more active against A16V + S108T mutant DHFR than cycloguanil, were identified from the library by iterative deconvolution technique.

Experimental

Materials and methods

Elemental analyses were performed by Miss A. Ungpakornkaew on a Perkin–Elmer CHN analyzer model PE2400 series II (Chulalongkorn Research Equipment Centre, Bangkok). ^{1}H NMR spectra were recorded on a Bruker ACF 200 (Chulalongkorn University, Bangkok) operating at 200 MHz. Chemical shifts are quoted in ppm downfield relative to tetramethylsilane. MALDI-TOF mass spectrum was recorded by Miss N. Panchan on a Bruker Biflex mass spectrometer (The Institute of Genetic Engineering and Biotechnology, Chulalongkorn University, Bangkok) using α-cyanocinnamic acid (CCA) matrix. ESI mass spectra were recorded on a Fisons Instruments Mass Spectrometer model Trio 2000 in ESI mode. Masses are quoted as m/z unless otherwise stated, only molecular ions and major fragments being quoted. Absolute methanol and other chemicals from standard suppliers were used without further purification. All samples were dried in vacuo before analyses and biological evaluation.

General procedure for synthesis of individual 4,6-diamino-1,2-dihydro-1,3,5-triazine sublibraries by a modified two-component synthesis. To a suspension of an equimolar mixture of the arylbiguanide hydrochloride (1 mmol) and the ketone or aldehyde (5 mmol) in absolute MeOH (5 mL) was added triethyl orthoacetate (0.75 mL) and concentrated HCl (0.025 mL). The reaction mixture was stirred at room temperature until the reaction is judged complete by HPLC analysis or by biguanide test (24–120 h). The residue from evaporation of the solvent was triturated and washed exhaustively with ether. The solid was collected by filtration and air dried.

Synthesis of dihydrotriazine libraries. Sixteen portions of a mixture of 6 biguanides were reacted with 16 different carbonyl compounds (Scheme 3) as described above (1 mmol scale reaction each) to give 16 sublibraries of dihydrotriazines: m/z (MALDI-TOF) **I-1(a–f)** 204.0, 218.0, 237.9, 281.9, 232.0, 271.9; **I-2(a–f)** 218.0, 232.0, 251.9, 296.0, 246.0, 287.0; **I-3(a–f)** 232.2, 246.2, 266.2, 310.2, 260.3, 301.1; **I-4(a–f)** 246.0, 260.0, 279.2, 324.0, 274.0, 314.0; **I-5(a–f)** 246.0, 260.4, 280.0, 324.0, 274.4, 314.0; **I-6(a–f)** 260.1, 274.1, 294.0, 338.1, 288.1, 328.0; **I-7(a–f)** 245.0, 259.0, 280.0, 324.0, 274.0, 314.0; **I-8(a–f)** 259.0, 273.0, 293.0, 338.0, 287.0, 327.0; **I-9(a–f)** 274.4, 288.4, 308.4, 352.0, 302.4, 342.0; **I-10(a–f)** 273.9, 287.9, 308.1, 352.1, 301.9, 342.1; **I-11(a–f)** 273.0, 287.0, 307.0, 351.0, 302.0, 341.0; **I-12(a–f)** 288.3, 302.3, 322.3, 366.2, 316.3, 356.3; **I-13(a–f)** 287.9, 301.8, 320.9, 365.9, 315.9, 355.9; **I-14(a–f)** 243.9, 257.9, 278.0, 322.0, 271.9, 312.0; **I-15(a–f)** 257.0, 271.0, 291.0, 335.0, 286.0, 325.0; **I-16(a–f)** 266.3, 280.4, 300.3, 344.3, 294.4, 334.3.

HPLC analysis of dihydrotriazine libraries. Samples for reverse phase HPLC analyses were dissolved in a suitable aqueous solvent and filtered through a Teflon filter (0.47 μ pore size). The HPLC analyses were performed on a Waters 600 system equipped with a Waters 996 photodiode array detector. HPLC conditions—Column: Hypersil™ 5 μm BDS C_{18} HPLC column (4.6×250 mm); Mobile phase: 0.1 M triethylammonium acetate (pH 7.0):acetonitrile gradient from 15–60% over a period of 15 min with an equilibration period of 5 min. Retention time (t_R): **I-1(a–f)** 3.7, 4.8, 5.3, 5.8, 7.0, 8.0; **I-2(a–f)** 4.4, 6.6, 7.4, 8.4, 10.2, 10.8; **I-3(a–f)** 3.7, 4.3, 4.5, 4.8, 5.4, 5.8; **I-4(a–f)** 4.3, 5.5, 6.0, 6.6, 8.0, 8.7; **I-5(a–f)** 4.4, 5.7, 6.3, 6.7, 7.7, 8.2; **I-6(a–f)** 4.7, 6.4, 7.1, 7.7, 9.3, 9.7; **I-7(a–f)** 3.7, 4.2, 4.5, 4.8, 5.5, 5.9; **I-8(a–f)** 5.1, 6.5, 7.1, 7.7, 9.2, 9.8; **I-9(a–f)** 6.0, 8.9, 10.0, 10.8, 12.5, 12.9; **I-10(a–f)** 6.4, 8.9, 9.6, 10.3, 11.9, 12.4; **I-11(a–f)** 5.5, 7.5, 8.4, 9.8, 11.2, 12.3; **I-12(a–f)** 11.1, 12.5, 12.8, 13.8, 14.7, 15.1; **I-13(a–f)** 10.7, 12.8, 13.4, 13.8, 14.8, 15.2; **I-14(a–f)** 4.5, 6.4, 7.1, 7.7, 9.1, 9.7; **I-15(a–f)** 4.7, 5.9, 6.3, 6.8, 7.6, 8.0; **I-16(a–f)** 4.0, 4.8, 5.2, 5.5, 6.0, 6.7 min.

The order of elution is determined by comparison with standards. This was found to be dependent on polarity of N-1 substituents: $C_6H_5 > 4\text{-}MeC_6H_4 > 4\text{-}ClC_6H_4 > 4\text{-}BrC_6H_4 > 4\text{-}EtC_6H_4 > 3,4\text{-}Cl_2C_6H_3$.

Synthesis of derivatives of 4,6-diamino-1,2-dihydro-1,3,5-triazines as individual compounds. The individual 4,6-diamino-1,2-dihydro-1,3,5-triazine derivatives were synthesized by the original Modest two-component synthesis (Method A)[14] or by the modified method employing

triethyl orthoacetate as water scavenger (Method B).[21] Samples for biological assays and microanalyses were recrystallized from methanol or ethanol-ether. All cycloguanil derivatives were characterized by ^{1}H NMR and mass spectra (ESI or MALDI-TOF) and also by elemental analyses (CHN) for new compounds. Spectroscopic and microanalytical data of **I-1b**, **I-1c**, **I-1d**, **I-1f**, **I-16b**, **I-16c**, **I-16d**, **I-16f** have been previously reported.[12]

4,6-Diamino-1,2-dihydro-2-methyl-1-phenyl-1,3,5-triazine hydrochloride (I-1a). Method A; 51% yield; colorless needles (MeOH–Et_2O); found: C, 47.0; H, 6.2; N, 27.7%. ($C_{10}H_{14}ClN_5 + H_2O$ requires C, 46.6; H, 6.2; N, 27.2%); δ_H (D_2O) 1.16 (3H, d, $J = 6.4$ Hz, C$\underline{H}_3$-2), 4.96 (1H, q, $J = 6.0$ Hz, $\underline{H}$-2), 7.20 and 7.36 (5H, 2×m, aromatic C-H); *m*/*z* (MALDI-TOF) 204 (M–C1)$^+$.

4,6-Diamino-(4′-ethylphenyl)-1,2-dihydro--2-methyl-1,3,5-triazine hydrochloride (I–1e). Method A; 43% yield; colorless needles (EtOH–Et_2O); found: C, 53.8; H, 6.6, N, 26.1% ($C_{12}H_{18}ClN_5$ requires C, 53.8; H, 6.7; N, 26.1%); δ_H (D_2O) 1.01 (3H, t, $J = 7.0$ Hz, C$\underline{H}_3$CH$_2$-4′), 1.16 (3H, d, $J = 5.9$ Hz, C$\underline{H}_3$-2), 2.05 (2H, q, $J = 7.4$ Hz, CH$_3$C$\underline{H}_2$-4′), 4.93 (1H, q, $J = 6.4$ Hz, $\underline{H}$-2), 7.13 and 7.24 (2×2H, AB doublet, $J = 8.2$ Hz, aromatic C-H); *m*/*z* (MALDI-TOF) 232 (M.H$^+$).

4,6-Diamino-2-hexyl-1,2-dihydro-2-methyl-1-phenyl-1,3,5-triazine hydrochloride (I-12a). Method B; 89% yield; light brown crystalline solid (MeOH–Et_2O); found C, 57.40; H, 8.39; N, 21.64% ($C_{16}H_{26}ClN_5 + 0.5H_2O$ requires C, 57.7; H, 8.8; N, 21.0%); δ_H (D_2O, 200 MHz); 0.63 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.05–1.73 (5×2H, m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.15 (3H, s, CH$_3$-2), 7.18 and 7.38 (5H, 2×m, aromatic C-H); *m*/*z* (MALDI-TOF) 288.5 (M–Cl)$^+$.

4,6-Diamino-1-(4′-chlorophenyl)-2-hexyl-1,2-dihydro-2-methyl-1,3,5-triazine hydrochloride (I-12b). Method B; 85% yield; colorless prisms (MeOH–Et_2O); found C, 53.6; H, 7.0; N, 19.6% ($C_{16}H_{25}Cl_2N_5$ requires C, 53.6 ;H, 7.0 ;N, 19.6%); δ_H (D_2O, 200 MHz); 0.65 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.08–1.70 (5×2H, 5×m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.18 (3H, s, CH$_3$-2), 7.20 (2H, m, aromatic C-H), 7.40 (2H, d, $J = 8.0$ Hz, aromatic C-H); *m*/*z* (MALDI-TOF) 322.5 (M–Cl)$^+$.

4,6-Diamino-1-(4′-bromophenyl)-2-hexyl-1,2-dihydro-2-methyl-1,3,5-triazine hydrochloride (I-12c). Method B; 86% yield; colorless needles (MeOH–Et_2O); Found C, 47.6; H, 6.3; N, 17.3% ($C_{16}H_{25}BrC1N_5$ requires C, 47.7 ; H, 6.3 ;N, 17.4%); δ_H (D_2O, 200 MHz); 0.67 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.07–1.72 (5×2H, m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.17 (3H, s, CH$_3$-2), 7.15 (2H, m, aromatic C-H), 7.55 (2H, d, $J = 8.0$ Hz, aromatic C-H); *m*/*z* (MALDI-TOF) 366.1 and 368.1 (M–Cl)$^+$.

4,6-Diamino-2-hexyl-1,2-dihydro-2-methyl-1-(4′-methylphenyl)-1,3,5-triazine hydrochloride (I-12d). Method B; 90% yield; pale yellow crystalline solid (MeOH–Et_2O); Found C, 60.2; H, 8.7; N, 20.9% ($C_{17}H_{24}ClN_5$ requires C, 60.4; H, 8.4; N, 20.7%); δ_H (D_2O, 200 MHz); 0.64 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.05–1.70 (5×2H, m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.17 (3H, s, CH$_3$-2), 2.20 (3H, s, CH$_3$-4′) 7.05 (2H, m, aromatic C-H), 7.20 (2H, d, $J = 8.0$ Hz, aromatic C-H); *m*/*z* (MALDI-TOF) 303.7 (M-Cl)$^+$.

4,6-Diamino-1-(4′-ethylphenyl)-2-hexyl-1,2-dihydro-2-methyl-1,3,5-triazine hydrochloride (I-12e). Method B, 84% yield; colorless prisms (MeOH–Et_2O); found C, 61.4; H, 8.6; N, 19.9% ($C_{18}H_{30}ClN_5$ requires C, 61.4; H, 8.6; N,19.9%); δ_H (D_2O, 200MHz); 0.67 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.05 (3H, t $J = 7.0$ Hz, C$\underline{H}_3$CH$_2$-4′), 1.10–1.70 (5×2H, m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.20 (3H, s, CH$_3$-2), 2.02 (2H, q, $J = 7.0$ Hz, C$\underline{H}_3$CH$_2$-4′), 7.12 (2H, m, aromatic C-H) 7.25 (2H, d, $J = 8.0$ Hz, aromatic C-H); *m*/*z* (MALDI-TOF) 361.5 (M–Cl)$^+$.

4,6-Diamino-1-(3′,4′-dichlorophenyl)-2-hexyl-1,2-dihydro-2-methyl-1,3,5-triazine hydrochloride (I-12f). Method B; 92% yield; colorless plates (MeOH–Et_2O); found C, 48.9; H, 6.1; N, 17.9% ($C_{16}H_{24}Cl_3N_5$ requires C, 48.9; H, 6.2; N, 17.8%); δ_H (D_2O, 200 MHz); 0.63 (3H, t, $J = 7.2$ Hz, C$\underline{H}_3$(CH$_2$)$_5$-2), 1.02–1.70 (5×2H, m, CH$_3$(C$\underline{H}_2$)$_5$-2), 1.68 (3H, s, CH$_3$-2), 7.13 and 7.47 (2×2H, 2×m, aromatic C-H); *m*/*z* (MALDI-TOF) 356.4 (M–C1)$^+$.

4,6-Diamino-1,2-dihydro-1,2-diphenyl-1,3,5-triazine hydrochloride (I–16a). Method A, 78% yield, white solid (MeOH–Et_2O); found: C, 50.7; H, 4.6; N, 19.2%. ($C_{15}H_{16}ClN_5 + 1.5HCl$ requires C, 50.5; H, 4.9; N, 19.6%); δ_H (D_2O) 5.98 (1H, s, $\underline{H}$-2), 7.02 (2H, dd, $J = 6.4$, 4.0 Hz, aromatic C–H) and 7.22 (8H, m, aromatic C–H); *m*/*z* (MALDI-TOF) 266 (M–Cl)$^+$.

4,6-Diamino-1-(4′-ethylphenyl)-1,2-dihydro-2-phenyl-1,3,5-triazine hydrochloride (I-16e). Method A, 78% yield; colorless needles (EtOH–Et_2O); found: C, 61.8; H, 6.2, N, 21.2% ($C_{17}H_{20}ClN_5$ requires C, 61.9; H, 6.1; N, 21.2%); δ_H (D_2O) 0.91 (3H, t, $J = 6.5$ Hz, C$\underline{H}_3$CH$_2$-4′), 2.36 (2H, q, $J = 7.3$ Hz, CH$_3$C$\underline{H}_2$-4′), 5.95 (1H, s, $\underline{H}$-2), 6.87 and 7.03 (2×2H, AB doublet, $J = 8.0$ Hz, aromatic C–H), 7.17 (5H, m, aromatic C–H); *m*/*z* (MALDI-TOF) 294 (M–Cl)$^+$.

Enzyme assays and inhibition by Cyc analogues. The wild-type and A16V + S108T mutant pfDHFRs were prepared and their activities were determined spectrophotometrically according to the method previously described.[22] The reaction (200 μL) contained 1x DHFR buffer (50 mM *N*-[tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.0, 75 mM β-mercaptoethanol, 1 mg/mL bovine serum albumin), 100 μM each of the substrate H_2folate and cofactor NADPH, and appropriate amount (0.001–0.005 units) of the affinity-purified enzymes. The inhibition of the enzymes by Cyc analogues and combinatorial libraries was determined in triplicate in a 96 well plate employing 200 μL samples from the above mixtures in the presence of antifolate. The reaction kinetic was followed on a microplate reader (Labsystems, Finland) and the K_i values of inhibitors were determined by the equation $IC_{50} = K_i\ (1 + ([S]/K_m))$,[23] where IC_{50} is the concentration of inhibitor which inhibits 50% of the enzyme activity under the

standard assay conditions and K_m is the Michaelis constant for the substrate dihydrofolate. The K_i values were expressed in nanomolar unit (nM). For K_i of mixture libraries, the average molecular weights of library members were used to calculate the molar concentration of the inhibitors.

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