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Combinatorial Mixture Synthesis and Biological Evaluation of Dihydrophenyl Triazine Antifolates

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Abstract—The traditional 'one-pot' three component synthesis was adapted successfully for combinatorial mixtures synthesis of dihydrophenyl triazines, which are nonclassical, dihydrofolate reductase (DHFR) inhibitors. Each library was designed to comprise eight reaction mixture pots and in every pot there were three dihydrophenyl triazines. A total of three libraries were synthesized and the final number of compounds harvested was 64. The products precipitated out of the reaction mixture and could be collected easily and cleansed by washing. Solid supports and further purification processes were not required. The reactions were monitored by TLC and a HPLC method was developed to determine the number of products in each pot. All 24 pots were screened for inhibitory activity against the rat liver DHFR. Two pots showed good inhibitory activity and the products in them were individually synthesized, characterized and biologically tested again. One lead compound was identified amongst all the compounds synthesized, and would be further optimized.

Introduction

The identification of a lead compound is normally the most tedious and time-consuming process in drug discovery. Traditional approaches to lead identification involve the random screening of natural products or synthesis and testing of hundreds of structurally-related compounds to identify the most active candidate. Recently, new methodologies involving the use of combinatorial chemistry and high throughput screening are developed for use in lead identification. Combinatorial chemistry allows the synthesis of large numbers of structurally distinct molecules in a fast and resource-effective manner. The key feature of combinatorial chemistry is that synthesis is designed to produce a range of analogues using similar reaction conditions, either in the same reaction vessel (mixture synthesis) or individually in parallel using semi-automated synthesis (parallel synthesis).[1–3] Mixture and parallel syntheses can be carried out in solution[4–6] or on solid-phases.[7–11] Michal Lebl et al.[12,13] carried out the 'one-bead-one-compound' method for solid-phase synthesis to ease the identification and screening process. Combinatorial chemistry was initially developed for peptide synthesis,[14] but its use was later extended to the synthesis of small organic molecules[15] such as heterocycles,[16–18] where the 1,4-benzodiazepine libraries are typical examples.[19,20]

By using combinatorial chemistry, larger and more diverse compound libraries can be formed and the probability of finding novel compounds of significant therapeutic value is higher. Combinatorial chemistry is faster, more efficient and cheaper than traditional synthesis for a small quantity of compounds is produced for biological screening and only the active compounds are produced in bulk.[1] Combinatorial synthesis is also superior to random screening of natural products because the entire synthetic route is known upon identification of the lead compound. Hence, optimization and further synthesis of analogues of the lead is tremendously simplified.

The 4,6-diamino-2,2-dialkyl-1,2-dihydro-1-phenyl-*s*-triazines are known to be inhibitors of DHFR. Inhibition of cellular DHFR reduces the source of tetrahydrofolates which eventually leads to cell death as a result of deficient DNA biosynthesis due to the lack of the availability of nucleic acids. Different dihydrophenyl triazines have been used therapeutically as antimalarial,[21] anticancer,[22,23] as well as antiparasitic agents.[24,25] This class of compounds was first synthesized by a 'one-pot' three component synthesis devised by E. J. Modest.[26] The main objective of this study is to investigate the feasibility of developing the 'one-pot' three component synthesis into a method for producing combinatorial libraries of the dihydrophenyl triazine antifolates. The libraries will then be screened for inhibitory activity

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against the rat liver DHFR and those compounds that show good activity will be identified and characterized.

Results and Discussion

Combinatorial mixture synthesis of dihydrophenyl triazines

In 1954, E. J. Modest reported that dihydrophenyl triazines having either *para*- or *meta*-halogen substitution in the phenyl ring and either a 2,2-dimethyl or a 2-*n*-hexyl substitution in the dihydro triazine ring showed promising inhibitory action against murine DHFR.[27] The physicochemical properties of the dihydrophenyl triazines are very much dependent on the nature of the substituents on both the phenyl ring and the dihydro triazine ring. These properties have a great influence on the way these molecules would bind to the enzyme and also their transport across the membranes.[28] In this pilot study, attempts were made to search for novel dihydrophenyl triazines by creating several libraries of dihydrophenyl triazines that have different physicochemical properties, through combinatorial synthesis. The 'one-pot' three component synthesis of dihydrophenyl triazines produces relatively clean hydrochloride salts of dihydrophenyl triazines which readily separate from the reaction mixture as a crystalline precipitate. This unique feature in the synthesis served as a basis for adapting the method for the combinatorial mixture synthesis of these compounds. The precipitation of the products would facilitate easy harvest by suction filtration and thereafter, easy washing with an appropriate solvent. The advantage of this step would be the elimination of the use of solid supports such as beads or resins for the isolation and purification of the synthesized products.

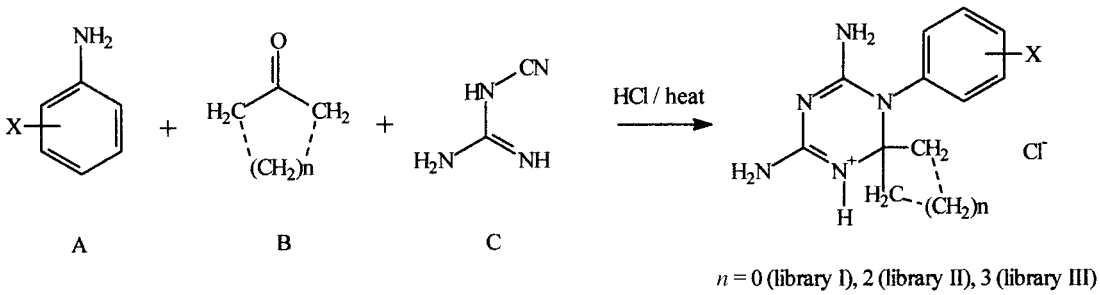
It was decided that to create the libraries of the dihydrophenyl triazines, each library would be synthesized by keeping two components unchanged while the third component would be varied (Fig. 1). The two unchanged components would be the ketone and cyanoguanidine and an array of differently substituted anilines would be used as the third component. To simplify the mixture synthesis further, it was decided that each library would be made up of eight pots of reaction mixtures. Each reaction mixture pot would contain a ketone, cyanoguanidine and three differently substituted anilines. Therefore, if the reactions were to proceed satisfactorily, a total of 24 dihydrophenyl triazines would be synthesized in each library.

Library I was synthesized using acetone, cyanoguanidine and anilines. The products formed were 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(substituted phenyl)-*s*-triazine hydrochlorides. Cyclopentanone and cyclohexanone were used separately in libraries II and III respectively and the products formed were the 2,2-cyclopentyl and 2,2-cyclohexyl analogues of the dihydrophenyl triazines. All the reaction mixtures were heated to reflux or just below reflux temperature and the progress of the reaction was closely monitored. The length of reaction time was determined by the use of TLC and a chemical reaction that involved the use of freshly prepared ammoniacal copper sulphate solution. The reaction was stopped when either all the reactants were consumed or when there was no further consumption of the reactants as shown by the TLC. The ammoniacal copper sulphate reagent was used to determine whether arylbiguanide (an intermediate) was present, and therefore, was also useful in the indication of the appropriate time to terminate the reaction. The results have shown that the rate of synthesis varied over a wide window of reaction time, ranging from half an hour to thirty hours (Tables 1–3). It was deemed useless to carry on with the reaction after 30h for no appreciable amount of the product was formed beyond that time. In all the reaction mixture pots, the products precipitated out of solution during the synthesis or after the reaction vessels were cooled down to room temperature (pots III-1 to pots III-4). The only exception was pot II-2 where additional acetone was used to precipitate the products in the cold. All the products were collected by suction and washed with the same solvent that was used in the reaction to get rid of reagents that had not reacted and soluble impurities. The products thus obtained were sufficiently pure to be used directly for the subsequent analytical steps.

Analysis of the combinatorial mixtures

The crystalline products from each pot were dissolved in water and the solutions were scanned in the UV region to determine the maximal absorbance wavelength. It was reported that the arylbiguanide intermediate absorbed maximally at around 254 nm while most dihydrophenyl triazines without strong electron withdrawing substituents would have λ_{max} values lower than 254 nm. The λ^{max} values of all the pots were recorded to be less than 254 nm, hence these ultraviolet scans confirmed the absence of the arylbiguanide intermediate in the products collected.

The use of TLC in the monitoring of the progression of the synthesis was not efficient enough to determine the number of products formed in each pot. Hence a HPLC analytical method was devised to ascertain the number of products present in every pot. The mixture of products collected from each pot was introduced on a reverse-phase C18 column and eluted with a mobile phase that contained a mixture of ammonium acetate buffer (0.05 M, pH 4) and acetonitrile as the modifier. The composition of the ammonium acetate buffer was changed linearly from 80 to 20% while the percentage of the acetonitrile used was altered accordingly. This linear gradient elution method was able to separate most of the components in each pot while pots which did not show good separation, the modifier was replaced by methanol, and better separation was subsequently obtained. All the chromatograms showed a relatively straight baseline and this was an indication that the products obtained were clean and free from contaminants. The chromatogram of every pot showed three major peaks (Fig. 2), corresponding to the formation of three dihydrophenyl triazines, except for pots



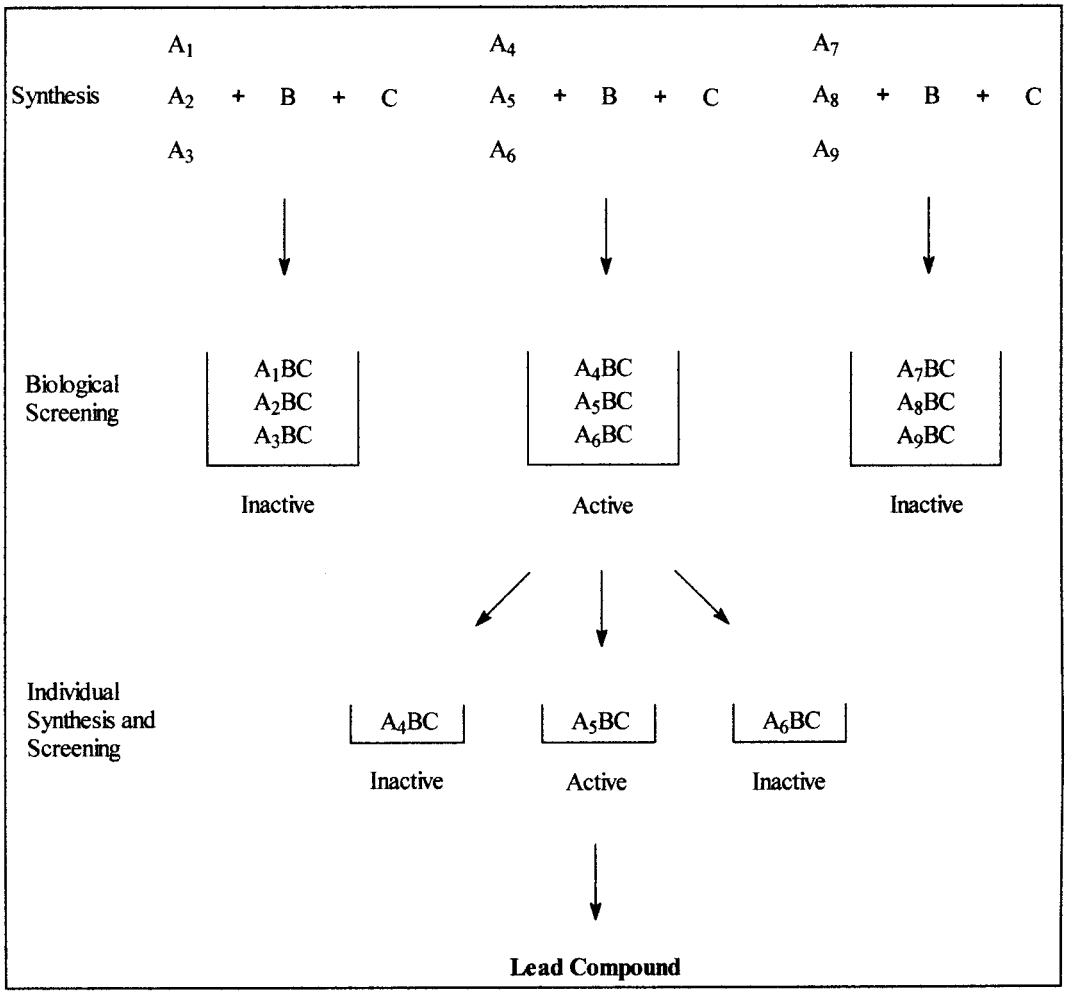


Figure 1. 'Three component' synthesis of dihydrophenyl triazines and the strategy for the combinatorial mixture synthesis.

I-5, I-7, II-3, II-4, II-5, II-7, III-4 and III-7, where only two peaks were observed. On the basis of the TLC results, anilines which did not participate in the synthesis would not have formed the corresponding dihydrophenyl triazine, hence those pots would contain only two products. For instance, in pots I-5 and II-5, the 2-nitroaniline did not react in the synthesis, therefore the 2′-nitrophenyl triazine could not be present as one of the products in these two mixtures. In the cases of pots I-7, II-7 and III-7, the 2,4-dinitroaniline was not consumed in the reaction, indicating that the 2,4-dinitrophenyl triazine was not among the dihydrophenyl triazines that were synthesized. The failure of the participation of the nitroanilines in the reactions is attributed to the electron-withdrawing effect of the nitro groups, which deactivates the anilines, making them less nucleophilic. In the case of pot II-3, TLC showed that 2-methylaniline did not react in the synthesis while for pots II-4 and III-4, 2-methoxyaniline was present in a large amount at the time the reaction was terminated.

Table 1. Reaction times, capacity factors of products, and IC_{50} values of pots in library I

Pot	Compound	X	Reaction time (h)	Capacity factors[a]	IC_{50} (μM)
I-1	1	2-Cl			
	2	3-Cl	8	1.47, 2.05, 2.27[A]	0.250
	3	4-Cl			
I-2	1	2-Br			
	2	3-Br	8	1.59, 2.30, 2.56[A]	0.270
	3	4-Br			
I-3	1	2-CH_3			
	2	3-CH_3	22	3.86, 4.62, 4.86[A]	0.380
	3	4-CH_3			
I-4	1	2-OCH_3			
	2	3-OCH_3	22	1.39, 1.56 3.23[A]	7.600
	3	4-OCH_3			
I-5	1	2-NO_2			
	2	3-NO_2	30	2.09, 2.32[B]	3.90
	3	4-NO_2			
I-6	1	3-Cl, 4-CH_3			
	2	3-Cl, 4-OCH_3	30	3.89, 5.34, 6.43[B]	0.022
	3	4-Cl, 3-NO_2			
I-7	1	2-NO_2, 4-NO_2			
	2	3-NO_2, 4-CH_3	30	1.49, 2.03[A]	0.200
	3	3-NO_2, 4-F			
I-8	1	3-CN			
	2	4-CN	30	0.69, 0.86, 3.08[A]	0.18
	3	3,4-diCH_3			

[a] The components of each plot were separated by linear gradient elution using a mobile phase of ammonium acetate buffer (0.05 M, pH 4) modified by A: acetonitrile and B: methanol.

Table 2. Reaction times, capacity factors of products and IC_{50} values of pots in library II

Pot	Compound	X	Reaction time (h)	Capacity factors[a]	IC_{50} (μM)
II-1	1	2-Cl			
	2	3-Cl	3	2.58, 3.33, 3.49[A]	0.220
	3	4-Cl			
II-2	1	2-Br			
	2	3-Br	3	2.74, 3.60, 3.86[A]	0.078
	3	4-Br			
II-3	1	2-CH_3			
	2	3-CH_3	6	2.66, 3.40[A]	6.000
	3	4-CH_3			
II-4	1	2-OCH_3			
	2	3-OCH_3	22	2.57, 2.74[A]	0.280
	3	4-OCH_3			
II-5	1	2-NO_2			
	2	3-NO_2	8	1.18, 2.22[A]	> 100
	3	4-NO_2			
II-6	1	3-Cl, 4-CH_3			
	2	3-Cl, 4-OCH_3	3	5.93, 7.12, 8.14[B]	0.220
	3	4-Cl, 3-NO_2			
II-7	1	2-NO_2, 4-NO_2			
	2	3-NO_2, 4-CH_3	½	2.58, 3.23[A]	0.140
	3	3-NO_2, 4-F			
II-8	1	3-CN			
	2	4-CN	3	1.33, 3.31, 7.77[B]	1.000
	3	3,4-diCH_3			

[a] The components of each pot were separated by linear gradient using a mobile phase of ammonium acetate buffer (0.05 M, pH 4) modified by A: acetonitrile and B: methanol.

Table 3. Reaction times, capacity factors of products and IC_{50} values of pots in library III

Pot	Compound	X	Reaction time (h)	Capacity factors[a]	IC_{50} (μM)
III-1	1	2-Cl			
	2	3-Cl	24	3.41, 4.30, 4.54[A]	12.000
	3	4-Cl			
III-2	1	2-Br			
	2	3-Br	24	3.57, 4.55, 4.95[A]	3.700
	3	4-Br			
III-3	1	2-CH_3			
	2	3-CH_3	3	3.57, 4.28, 4.40[A]	68.000
	3	4-CH_3			
III-4	1	2-OCH_3			
	2	3-OCH_3	8	6.38, 6.93[B]	> 100
	3	4-OCH_3			
III-5	1	2-NO_2			
	2	3-NO_2	30	1.24, 3.07, 4.00[A]	78.000
	3	4-NO_2			
III-6	1	3-Cl, 4-CH_3			
	2	3-Cl, 4-OCH_3	30	6.99, 7.90, 8.99[B]	17.000
	3	4-Cl, 3-NO_2			
III-7	1	2-NO_2, 4-NO_2			
	2	3-NO_2, 4-CH_3	30	3.40, 4.16[A]	18.000
	3	3-NO_2, 4-F			
III-8	1	3-CN			
	2	4-CN	8	1.28, 4.30, 6.34[B]	48.000
	3	3,4-diCH_3			

[a] The components of each pot were separated by linear gradient elution using a mobile phase of ammonium acetate buffer (0.05M, pH4) modified by A: acetonitrile and B: methanol.

For these cases, steric interaction between the *ortho*-substituted anilines and the bulky C2 substitution at the dihydro triazine ring might have prevented the formation of the product. Hence a combination of both the HPLC and TLC data could be used to identify the products that were formed in the mixture synthesis. This new method of combinatorial mixture synthesis which is adapted from the three component synthesis has generated a total of sixty-four differently substituted dihydrophenyl triazines.

Screening and identification of lead

All the 24 pots of dihydrophenyl triazine mixtures were screened separately for inhibitory activity against partially purified rat liver DHFR. Since the precipitated products were found to be relatively clean, they were simply dissolved in water and used directly for the biological assays. The results showed that all dihydrophenyl triazines from all the 24 pots exhibited a different extent of inhibition against the rat liver enzyme and some of them had shown inhibitory action that was so weak that they could be considered inactive. The compounds in library III showed the least inhibitory activity among the three libraries. Hence, a cyclohexyl substituent at position C2 of the dihydro triazine ring appeared to provide too much steric bulk to enable a favorable accommodation of the dihydrophenyl triazine at the active site of the enzyme. The cyclopentyl substituent, on the other hand, seemed able to achieve a suitable conformation to allow the inhibitor molecule to

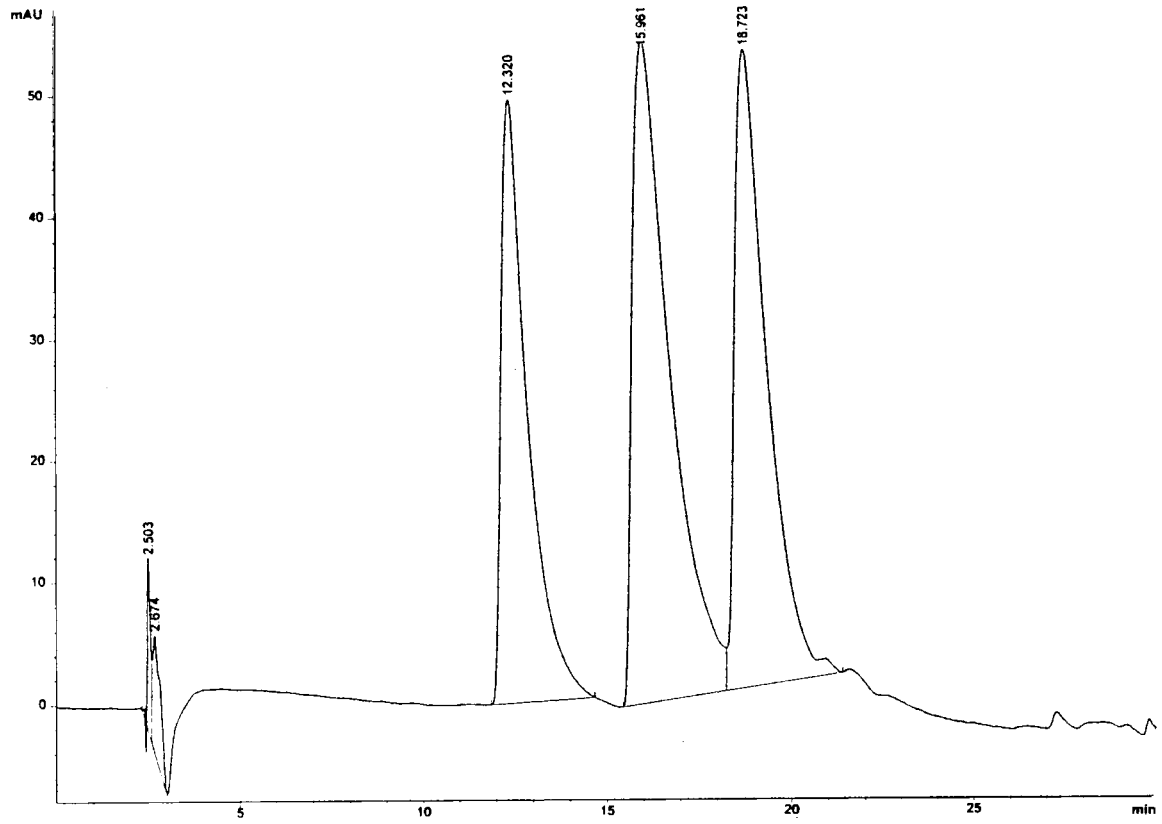


Figure 2. HPLC chromatogram showing the separation of the products in pot I-6 using a linear gradient elution with solvent system.

bind in the active site, thus showing relatively lower IC_{50} and K_i values compared to compounds in library III. Pot I-6 and pot II-2 each contained dihydrophenyl triazines that inhibited 50% of the enzyme at the concentrations of 0.022 μM and 0.078 μM respectively. From the results of the preliminary screening, it was decided that pots that exhibited IC_{50} values that were lower than 0.1 μM would warrant further evaluation of their components individually. Thus, the six dihydrophenyl triazines of pots I-6 and II-2 were synthesized and characterized separately.

When the six dihydrophenyl triazines were evaluated individually for inhibitory activity against the rat liver DHFR, the most active inhibitor turned out to be compound I-6.3 which exhibited an IC_{50} value of 0.006 μM and a K_i value of 2×10^{-4} μM (Table 4). On the basis of the K_i values, which indicate the binding affinity of an inhibitor to the enzyme, the binding affinities of compounds I-6.1, I-6.2 and II-2.2 were about 14 times lower than compound I-6.3. Whilst compounds II-2.1 and II-6.3 have far less affinity for the enzyme. Henceforth, compound I-6.3 was selected as the lead from the total of 64 compounds synthesized.

Conclusion

In conclusion, this pilot study has effectively demonstrated that the traditional 'one pot' three component synthesis can be adapted successfully for combinatorial mixture synthesis. One of the advantages of this method is the ease with which the products can be harvested without the need for the use of resin support for derivatization. It is the precipitation of the product in the form of crystals during the course of the reaction that facilitates this step in the synthesis. The products harvested are relatively clean after having passed through a few cycles of washing with an appropriate solvent and this has been demonstrated in the chromatograms. As such the crystalline products can be used directly in the biological assay without further purification. This method opens up the scope to further investigation on the combinatorial synthesis of novel dihydrophenyl triazines, with the only limitation of the availability of the various substituted or derivatized anilines.

Table 4. IC_{50} and K_i values of individually synthesized products

Compound	IC_{50} (μM)	K_i (μM)[a]
I-6.1	0.084	2.80×10^{-3}
I-6.2	0.088	2.93×10^{-3}
I-6.3	0.006	2.00×10^{-4}
II-2.1	82.00	2.73
II-2.2	0.096	3.20×10^{-3}
II-2.3	4.700	1.57×10^{-1}
II-6.3	0.074	2.46×10^{-3}
III-6.3	3.200	1.07×10^{-1}

[a] K_i values were determined using the formula:- $K_i = \frac{K_m \times IC_{50}}{S}$ where $K_m = 1.099\times10^{-3}$ mg/mL and S(substrate concentration) = 0.033 mg/mL.

Out of the three libraries of dihydrophenyl triazines, which comprised a total of 24 reaction pots, the theoretical number of compounds expected from the combinatorial synthesis was 72, however, only 64 compounds were detected by means of HPLC. The other eight compounds did not form under the reaction conditions stipulated. The compounds in every pot were screened for inhibitory activity against the rat liver DHFR and only two pots indicated activity that warranted individual synthesis of the components for further biological evaluation. Of the six dihydrophenyl triazines that were tested, the compound that showed the best inhibitory activity had an IC_{50} value of 0.006 μM and a K_i value of 2×10^{-4} μM. The 2,2-cyclopentyl analogue (II-6.3) and the 2,2-cyclohexyl analogue (III-6.3) were also synthesized to determine whether their inhibitory activities were as good as I-6.3. Unfortunately they both turned out to be less active, having K_i values of only 2.46×10^{-3} μM and 1.07×10^{-1} μM respectively.

Experimental

All the chemicals used in the syntheses were purchased from Aldrich Chemical Co. (USA) or Tokyo Kasei Organic Chemicals (TCI, Japan). They were used without further purification. Melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. Infrared spectra (using potassium bromide discs) were recorded with a Jasco FT/IR-430 Fourier transform infrared spectrometer and reported in wavenumbers (cm^{-1}). The ^1H NMR spectra were recorded on a Bruker ACF 300 MHz NMR Spectrometer. The chemical shift (δ) values are expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, dd = double doublet, m = multiplet. Maximum UV absorption wavelengths of the compounds were determined on the UV-160A UV-visible recording spectrophotometer. TLC was performed on silica gel coated aluminum plates with fluorescent indicator and visualized with UV light at 254 nm. Elemental analyses were performed on the Perkin–Elmer 2400 Elemental Analyzer Series II and all the values are within ±0.4% of calculated values.

Chemical syntheses

In the three component combinatorial mixture synthesis, a mixture of 3 substituted anilines (0.01 mol each), cyanoguanidine (2.772 g, 0.033 mol), a ketone (30 mL) and concentrated hydrochloric acid (2.49 mL, 0.03 mol) was refluxed for half an hour to 30 h. Gentle heating instead of reflux was applied when synthesizing mixtures in the second and third libraries. An inert solvent (ethanol) was used when synthesizing mixtures in the third library. The progress of the reaction was followed by TLC, using a mixture of methanol and chloroform as the mobile phase. A mixture of 10% methanol: 90% chloroform was used as the mobile phase for checking the extent of reaction in the first library; 5% methanol: 95% chloroform was used in the second library and 100% chloroform was used in the third library. The absence of arylbiguanide was noted by an absence of a coloured complex formation when an aqueous solution of the product was mixed with freshly prepared ammoniacal copper sulphate solution. The ammoniacal copper sulphate solution was prepared by dissolving 0.5 g of copper sulphate crystals in 100 mL of water and adding concentrated ammonia solution until a clear, deep blue solution was formed.

All the reactions were stopped when there was no arylbiguanide and the substituted anilines were completely consumed or when there was no further consumption of the anilines. All the mixtures precipitated out as a solid during the reaction or after the reaction vessel was cooled down to room temperature. The only exception was pot II-2, where 60 mL of cold acetone was poured into the cool mixture to induce precipitation. All the reaction vessels were kept at 4°C for a day before the solids were washed with the ketone or ethanol (for the third library only) and collected by suction filtration. The solid collected was used for further tests without purification.

For the synthesis of compounds I-6.1, I-6.2, I-6.3, II-2.1, II-2.2 and II-2.3, at least 0.02 mol of the aniline, 0.022 moles of the cyanoguanidine, 0.02 mol of hydrochloric acid together with either 20–30 mL of acetone or cyclopentanone were used. Characterization of the individual compounds was also carried out. Aqueous solutions at 5×10^{-7} M were used for UV scans. Compressed potassium bromide discs were used for FTIR scans and DMSO-d_6 was used to dissolve the compounds for ^1H NMR determinations.

4,6-Diamino-1-(3-chloro-4-methylphenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride, I-6.1. Yielded 3.14 g (51.99%) of colourless solid. Re-crystallization from absolute ethanol afforded 1.86 g of colourless crystals: mp 193–196°C; λ_{max} (water) = 240.4 nM; IR(KBr disc) 3303, 3259, 3140, 2973, 1644 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (s, 6H, 2-CH_3), 2.38 (s, 3H, 4′-CH_3), 7.24–7.27 (dd, 1H, J_{ortho} = 8 Hz, J_{meta} = 2 Hz, Ar−H), 7.47–7.48 (d, 1H, J_{meta} = 2 Hz, Ar−H), 7.49–7.51 (d, 1H, J_{ortho} = 8 Hz, Ar−H), 9.27 (s, 1H, NH); Anal. ($C_{12}H_{16}N_5Cl.HCl$) C, H, N.

4,6-Diamino-1-(3-chloro-4-methoxyphenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride, I-6.2. Yielded 4.10 g (64.47%) of colourless solid. Re-crystallization from a mixture of absolute ethanol and water afforded 1.99 g of colourless flaky crystals: mp 224–226 °C; λ_{max} (water) = 235.8 nm; IR(KBr disc) 3416, 3303, 3141, 2932, 1633, 1275, 1058 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.35 (s, 6H, 2-CH_3), 3.91 (s, 3H, 4′-OCH_3), 7.24–7.27 (d, 1H, J_{ortho} = 9 Hz, Ar−H), 7.31–7.34 (dd, 1H, J_{ortho} = 9 Hz, J_{meta} = 2 Hz, Ar−H), 7.48–7.49 (d, 1H, J_{meta} = 2 Hz, Ar−H), 9.19 (s, 1H, NH); Anal. ($C_{12}H_{16}N_5ClO.HCl$) C, H, N.

4,6-Diamino-1-(4-chloro-3-nitrophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride, I-6.3. Yielded 3.27 g (49.10%) of pale yellow solid. Re-crystallization from a

mixture of absolute ethanol and water afforded 1.78 g of pale yellow crystals: mp 218–222 °C; λ_{max} (water) = 241.0 nm; IR (KBr disc) 3303, 3222, 3098, 2979, 1644 cm^{-1}; ^1H NMR(DMSO-d_6) δ 1.38 (s, 6H, 2-CH_3), 7.73–7.77 (dd, 1H, J_{ortho} = 8 Hz, J_{meta} = 2 Hz, Ar−H), 7.88–7.91 (d, 1H, J_{ortho} = 8 Hz, Ar−H), 8.17–8.18 (d, 1H, J_{meta} = 2 Hz, Ar−H), 9.44 (s, 1H, NH); Anal. ($C_{11}H_{13}N_6O_2Cl.HCl$) C, H, N.

4,6-Diamino-1-(2-bromophenyl)-2-cyclopentyl-1,2-dihydro-*s*-triazine hydrochloride, II-2.1. Yielded 4.84 g (45.01%) of colourless solid. Re-crystallization from a mixture of absolute ethanol and water afforded 3.11 g of clear, colourless, needle-shaped crystals: mp 233–236 °C; λ_{max} (water) = 238.6 nm; IR (KBr disc) 3296, 3196, 3140, 2973, 1647 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.38–2.05 (m, 8H, 2-C_4H_8), 7.44–7.87 (m, 4H, Ar−H), 9.14 (s, 1H, NH); Anal. ($C_{13}H_{16}N_5Br.HCl$) C, H, N.

4,6-Diamino-1-(3-bromophenyl)-2-cyclopentyl-1,2-dihydro-*s*-triazine hydrochloride, II-2.2.. Yielded 6.31 g (58.69%) of colourless solid. Re-crystallization from a mixture of absolute ethanol and water afforded 3.74 g of fine, powdery, needle-shaped crystals: mp 214–218 °C; λ_{max} (water) = 240.6 nm; IR (KBr disc) 3296, 3209, 3137, 2973, 1645 cm^{-1}; ^1H NMR(DMSO-d_6) δ 1.50–1.97 (m, 8H, 2-C_4H_8), 7.39–7.75 (m, 4H, Ar−H), 9.34 (s, 1H, NH); Anal. ($C_{13}H_{16}N_5Br.HCl$) C, H, N.

4,6-Diamino-1-(4-bromophenyl)-2-cyclopentyl-1,2-dihydro-*s*-triazine hydrochloride, II-2.3. Yielded 6.69 g (62.22%) of colourless solid. Re-crystallization from a mixture of absolute ethanol and water afforded 3.99 g of large, clear, needle-shaped crystals: mp 221–224 °C; λ_{max} (water) = 239.0 nm; IR (KBr disc) 3303, 3203, 3142, 2973, 1643 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.49–1.98 (m, 8H, 2-C_4H_8), 7.32–7.35 (d, 2H, J_{ortho} = 8 Hz, Ar−H), 7.71–7.73 (d, 2H, J_{ortho} = 8 Hz, Ar−H), 9.15 (s, 1H, NH); Anal. ($C_{13}H_{16}N_5Br.HCl$) C,H,N.

HPLC analysis method

All the HPLC separations of mixtures were carried out on the Hewlett–Packard series 1050 HPLC system with diode array detection using a LiChrosorb reversed phase C18 (5 μm) column (4.6×250 mm). All the mixtures were individually dissolved in methanol to form a 0.01% w/v solution. Each sample (20 μL) was injected and separation of the products was achieved using a linear gradient elution. A mobile phase consisted of ammonium acetate buffer (0.05 M, pH 4) was used, where the percentage of the ammonium acetate buffer was changed from 80 to 20% over 30 min. The flow-rate was set at 1 mL/min and detection wavelength was made at 250 nm. The ammonium acetate buffer was prepared by dissolving 3.85 g of ammonium acetate in 1000 mL of milli-Q water and adjusting the pH to 4 with glacial acetic acid. If the products in the mixture did not separate, the experiment would be repeated using methanol to replace acetonitrile. All the separations were performed in duplicates.

Preparation of partially purified rat liver DHFR

Two male Wistar rats (250 g each) were killed and the livers were removed and washed with deionised water to remove blood. All further manipulations were performed at 4 °C. The livers (27.85 g) were blended with approximately 8 volumes of milli-Q water (final volume of 300 mL) for 2 min. The mixture was then centrifuged at 10,000 rpm using the Beckman Avanti J-25 centrifuge for 10 min. The pellet was discarded and the supernatant was adjusted to pH 5.1 with dilute acetic acid. The mixture was further centrifuged at 18,000 rpm for 20 min and the clear, wine-red supernatant was transferred to a Spectra/Por 6 Dialysis Membrane and dialyzed for 15 h against a phosphate buffer solution (0.01 M, pH 6.5). The clear preparation was then transferred to sterile Eppendorf tubes (1.5 mL) and stored at −20 °C prior to use.

DHFR inhibition assay

Phosphate buffer (0.15 M, pH 7) was used for all the DHFR assays. An aqueous solution of NADPH (2 mg/mL, 2 mM) was prepared immediately prior to use and maintained at 0 °C. A solution of dihydrofolic acid (DHF) (1 mg/mL, 2 mM) was prepared immediately before use by suspending DHF in the 2-mercaptoethanol solution (0.25 M) and adding sodium hydroxide (2 M) solution dropwise, with vigorous agitation, until dissolution had occurred. The solution was protected from light and maintained at 0 °C.

The assay was performed in a Hewlett–Packard 8453 Diode Array UV-visible spectrophotometer fitted with a HP 89090A Peltier temperature controller. The absorbance of NADPH was detected at the wavelength of 340 nm. The assay was carried out over 15 min with absorbance readings taken every 30 s. A graph of absorbance readings versus time was plotted and the gradient over the linear range from 400 to 900 s was taken to be the rate of the reaction. The percentage activity of each inhibitor was calculated by the following formulae:

(i) $$\text{Activity} = \frac{\text{Slope of inhibited enzyme}}{\text{Slope of uninhibited enzyme}} \times 100\%$$

(ii) $$\text{Inhibition} = 100\% - \text{Activity}$$

A graph of percentage inhibition against the logarithmic concentration (μM) was plotted for each pot and the IC_{50} value was taken to be the concentration (μM) at which 50% inhibition was observed.

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