Med Chem Res (2011) 20:116–120
DOI 10.1007/s00044-009-9283-8

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Combinatorial approach: identification of potential antifungals from triazole minilibraries

Manish Sudesh Bhatia · Bandu Eknath Zarekar · Prafulla Balkrishna Choudhari · Kundan Bhanudas Ingale · Neela Manish Bhatia

Received: 30 March 2009 / Accepted: 3 December 2009 / Published online: 9 January 2010

Abstract Employing basic principles of solution phase combinatorial chemistry, a solution phase combinatorial synthesis and screening of mini libraries of 1,2,4-triazole derivatives has been carried out. 6 × 6 indexed mini libraries were synthesized comprising of 36 compounds. The libraries were analyzed by liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) analysis. All the synthesized mini libraries were screened for antifungal activity and by deconvolution methodology leads for every fungi used for study were identified. The leads were synthesized individually and screened for activity. The antifungal activity of individually synthesized leads was improved as anticipated, in comparison with that of any of the mini libraries.

Keywords Solution phase · Combinatorial synthesis · 1,2,4-Triazole · Antifungal activity

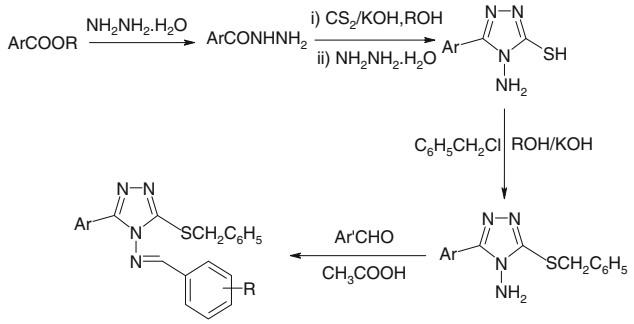
Introduction

Combinatorial chemistry is a technique by which large numbers of structurally distinct molecules can be synthesized in a short time and submitted for pharmacological assay. This process is thus rapid and interactive fine tuning similar to conventional lead optimization is much faster and yields much more analogues. The advantage of solution phase over solid phase combinatorial chemistry is that there is no need to develop strategy for either attachment of substrate to the solid support or its subsequent cleavage. Solution phase techniques are, however, limited to short reaction sequences that either use reagents in stoichiometric quantities, or use reagents that can be easily separated from the final products (Patrick, 2003; Terret, 1998). Various derivatives of 1,2,4-triazole are known to exhibit pharmacological properties like antibacterial, antifungal anticancer, anti-viral, anti-inflammatory, CNS depressant and anticonvulsant activity (Amir and Azam, 2004; Amir *et al.*, 1999; Banachiewicz *et al.*, 2004, Colanceska-Ragenovic *et al.*, 2001, Metwally *et al.*, 2007, Mishra *et al.*, 1991; Parmar *et al.*, 1972; Ravi and Rajkannan, 2004). In this work, focus is mainly on combinatorial synthesis and high throughput screening of 1,2,4-triazole derivatives (shown in Scheme 1 and Table 1) and identification of hits for antifungal activity by 2D deconvolution analysis. The synthesized mini libraries were confirmed by the separation of the components by TLC, and co-spotting of the individually synthesized components of library. The synthesized mini libraries were screened for antifungal activity and deconvolution studies identified hits which were synthesized individually and screened for their antifungal activity.

Result and discussion

Esters of acids like benzoic acid, anthranilic acid, salicylic acid, 4-aminobenzoic acid, phenyl acetic acid were reacted with hydrazine hydrate (99%) to obtain the respective hydrazides. These hydrazides were cyclized to form 1,2,4-triazole using carbon disulphide and potassium hydroxide in

M. S. Bhatia (✉) · B. E. Zarekar · P. B. Choudhari · K. B. Ingale · N. M. Bhatia
Department of Pharmaceutical Chemistry, Bharati Vidyapeeth College of Pharmacy, Near Chitranagri, Kolhapur 416013, Maharashtra, India
e-mail: manish_bhatia13@yahoo.co.in



Scheme 1 For synthesis of mini-libraries

the presence of alcohol via formation of potassium-3-dithiocarbazinate. These 3-substituted-4-amino-5-mercapto-4H-1,2,4-triazoles were further reacted with benzyl chloride in the presence of KOH to form substituted triazoles which were used as building blocks of the mini-combinatorial libraries. The minilibraries were prepared by reacting substituted triazole and aromatic aldehydes to form pool of imines with 1,2,4-triazole as basic core and vise a versa to form a pool of imines with a common aromatic structure and different 1,2,4-triazoles. The libraries thus formed were confirmed by TLC, IR, and liquid chromatography–mass spectrometry–mass spectrometry (LC–MS–MS) data and screened for antifungal activity. The hits were identified by 2D deconvolution methodology and then synthesized individually. Hits identified were synthesized by the condensation of 4-amino-5-benzylthio-3-subtituted-4H-1,2,4-triazole with selected aromatic aldehydes in acetic acid. The synthesized lead molecules were also screened for their antifungal activity and the screening results are shown in Table 2. The mini-libraries with best activity against each fungal strain are highlighted in bold and were employed for identifying the lead structures. The synthesized lead molecules were characterized by IR, 1H-NMR, and elemental analysis. In each of the synthesized mini-libraries, the absence of signals in the region 1,240–1,275 cm^{-1} in IR spectral data established the absence of C=S. The IR spectra of schiff bases showed characteristic absorption bands between 1,570 and 1,600 cm^{-1}. The 1H-NMR characteristic signals of compounds were observed at δ 8.58–8.78 ppm (s, 1H, N=CH). Moreover, the elemental analysis results were all in good agreement with the structures proposed for lead molecule. The hits identified were 5-benzylthio-*N*-[(4-methoxyphenyl)methylene]-3-pyridin-4-yl-4H-1,2,4-triazol-4-amine (L1) for A. niger, 3-(2-aminophenyl)-N-[(4-chlorophenyl)methylene]-5-(benzylthio)-4H-1,2,4-triazol-4-amine (L2) for *A. flavus*, 3-(2-aminophenyl)-*N*-[(4-methoxyphenyl)methylene]-5-benzylthio-4H-1,2,4-triazol-4-amine (L3) for *P. cryogenus* and 3-(2-aminophenyl)-*N*-[(4-dimethlyphenyl) methylene]-5-(benzylthio)-4H-1,2,4-triazol-4-amine (L4) for *F. oxysporium*. The hits were synthesized individually and complete characterization of the hits was performed to confirm the synthesis. The IR spectrum of the compounds showed peaks at 1710 C=O, 1105 C–O–C, 1670 C=N. The peak of NH stretching disappeared and emergence of C=N peak confirmed formation of imines. The

Table 1 Combinatorial Libraries

Sr.no.	BM1	BM2	BM3	BM4	BM5	BM6
Ar	C_6H_5–	2-$NH_2C_6H_5$–	2-OHC_6H_5–	4-NH_2–C_6H_5–	C_6H_5–CH_2–	4-C_5H_5N–
R						
1	H	H	H	H	H	H
2	4-Cl	4-Cl	4-Cl	4-Cl	4-Cl	4-Cl
3	2-OH	2-OH	2-OH	2-OH	2-OH	2-OH
4	4-$N(CH_3)_2$	4-$N(CH_3)_2$	4-$N(CH_3)_2$	4-$N(CH_3)_2$	4-$N(CH_3)_2$	4-$N(CH_3)_2$
5	4-OCH_3	4-OCH_3	4-OCH_3	4-OCH_3	4-OCH_3	4-OCH_3
6	3,4,5-OCH_3	3,4,5-OCH_3	3,4,5-OCH_3	3,4,5-OCH_3	3,4,5-OCH_3	3,4,5-OCH_3
Sr.no.	MB1	MB2	MB3	MB4	MB5	MB6
R	H	2-Cl	2-OH	4-$N(CH_3)_2$	4-OCH_3	3,4,5-OCH_3
Ar						
1	C_6H_5N–	C_6H_5N–	C_6H_5N–	C_6H_5N–	C_6H_5N–	C_6H_5N–
2	2-$NH_2C_6H_5$–	2-$NH_2C_6H_5$–	2-$NH_2C_6H_5$–	2-$NH_2C_6H_5$–	2-$NH_2C_6H_5$–	2-$NH_2C_6H_5$–
3	2-OHC_6H_5–	2-OHC_6H_5–	2-OHC_6H_5–	2-OHC_6H_5–	2-OHC_6H_5–	2-OHC_6H_5–
4	4-$NH_2C_6H_5$–	4-$NH_2C_6H_5$–	4-$NH_2C_6H_5$–	4-$NH_2C_6H_5$–	4-$NH_2C_6H_5$–	4-$NH_2C_6H_5$–
5	C_6H_5–CH_2–	C_6H_5–CH_2–	C_6H_5–CH_2–	C_6H_5–CH_2–	C_6H_5–CH_2–	C_6H_5–CH_2–
6	4-C_5H_5–	4-C_5H_5–	4-C_5H_5–	4-C_5H_5–	4-C_5H_5–	4-C_5H_5–

Table 2 Showing MIC of mini libraries

Library code	MIC (μg/ml)			
	Aspergillus niger	*Penicillium chrysogenum*	*Aspergillus flavus*	*Fusarium oxysporum*
BM1	250	125	250	**62**
BM2	250	**62**	**62**	125
BM3	250	1000	250	250
BM4	250	125	125	250
BM5	250	250	125	250
BM6	**62**	1000	500	125
MB1	125	125	250	500
MB2	**62**	250	250	125
MB3	500	250	500	125
MB4	500	**62**	250	**62**
MB5	125	500	**62**	125
MB6	250	1000	500	250
Fluconazole	–	–	–	–

Table 3 Table showing IR and NMR data of lead molecules

Lead	R	Ar	I.R (KBr) cm^{-1}	NMR (DMSO)δ ppm
L1	4-OCH_3	C_6H_5N–	2290 (CH), 1647.36 (C=N), 2830.15(O-CH_3).	9.19 (s, 1H, CH=N), 8.48 (s, 2H, pyridine), 7–8.3 (m, 9H Ar) 6.73 (s, 2H, pyridine), 3.28 (S, 3H OCH_3), 2.51 (s, 2H, SCH_2).
L2	4-Cl	2-$NH_2C_6H_5$–	3457 (N–H), 2290 (CH), 1647.36 (C=N), 742.46 (C–Cl).	9.19 (s, 1H, CH=N), 7–8.5 (13H), 2.52 (s, 2H, SCH_2), 2.1 (s, 2H, NH_2).
L3	4-OCH_3	2-$NH_2C_6H_5$–	3447 (N–H), 1647.36(C=N), 2830.15(O-CH_3).	9.12 (s, 1H, CH=N), 6.9–7.8 (m, 13H, Ar), 3.28 (S, 3H OCH_3), 2.51 (s, 2H, SCH_2), 1.44 (s, 2H, NH_2)
L4	4-OCH_3	$C_6H_5CH_2$	3138 (C–H), 2830.15 (O-CH_3), 1626 (C=N), 830 (C–N).	9.13 (s, 1H, CH=N), 7.1–8.2 (m, 14H, Ar), 3.30 (s, 3H, OCH_3), 2.66 (s, 2H, $C_6H_5CH_2$), 2.51 (s, 2H, SCH_2).

Table 4 Table showing antibacterial activity of the lead molecules

Library code	MIC (μg/ml)			
	Aspergillus niger	*Penicillium chrysogenum*	*Aspergillus flavus*	*Fusarium oxysporum*
L1	31	125	250	250
L2	125	31	125	62
L3	62	125	32	250
L4	62	250	125	32
Fluconazole	–	–	–	–

imine CH=N peak at 8.717 in NMR spectrum confirms the formation of predicted structures. The IR and NMR spectral data of lead compounds is given in Table 3. The leads were then screened for antifungal activity and the results of screening are given in Table 4. From the results of screening it was seen that the compounds synthesized as hits exhibited better activity, than that observed in case of any of the mini libraries. These results indicate the reliable utility of the 2D deconvolution studies in the inexhaustible drug discovery process.

Conclusion

Synthesis of mini-combinatorial libraries and subsequent screening carried out was based on extensive study of literature and text. The appealing content and correlation of reported and known facts with various hypotheses can be discussed to satisfactorily justify our attempt of combinatorially synthesizing and screening for antifungal activity of 1,2,4-triazole derivatives. All the synthesized hits were

found to be active against fungi. Activities of the hits for most of the biological activities attempted were better than those of the mini-combinatorial libraries for that activity. These results justify our approach of employing solution phase combinatorial synthesis and screening for this work, and support the utility of combinatorial methodologies in saving time and material for obtaining significant research outcome. Further optimization of the lead structures employing targeted or focused combinatorial libraries with similar approach could yield clinically useful antifungal agents.

Experimental

Melting points of synthesized compounds were determined by an open capillary method and are uncorrected. Analytical TLC was performed using silica gel-G as stationary phase. The IR (KBr) spectra were recorded on a Jasco-FTIR 4100 instrument. The 1H NMR spectra of the compounds were recorded on 400 MHz Varian NMR and DMSO-*d6* was used as solvent. Microwave synthesizer of Catalyst systems, cata-4RI, was used for microwave assisted synthesis. The LC–MS–MS spectrometer used for library analysis was of Varian Inc, USA, Model 500 MS IT with 410 Prostar Binary LC.

Step-I: general procedure for preparation of hydrazide

Placed a solution of acid ester 2 g (0.0232 mol) in ethanol (15 ml) in a flask, to this solution add hydrazine hydrate (7 ml, 99%). The reaction mixture was heated under reflux in microwave at 455 W for 12 min. The reaction mixture was poured on ice cold water; hydrazide which separated out was filtered, product was washed with two 10 ml portions of ethanol, dried and re-crystallized from ethanol.

Step II: General procedure for preparation of potassium 3-aroylthiocarbazates

To a mixture of 8.4 g (0.15 mol) of potassium hydroxide, 200 ml of absolute ethanol and 0.1 mol of the aroylhydrazide, 11.4 g (0.15 mol) of carbon disulfide was added and the resulting mixture was stirred for 24 h. It was then diluted with 200 ml of dry ether, and the precipitated solid was filtered, washed with 2 portions of 50 ml of ether and vacuum dried.

Step III: general procedure for preparation of 3-substituted-4-amino-5-mercapto-(4H)-1,2,4-triazoles

A mixture of potassium dithiocarbazate (15 g, 0.1 mol), hydrazine hydrate 99% (5 ml, 0.1 mol) and 2 ml water was refluxed for 10 min in microwave at 455 W, with continuous stirring. The color of the reaction mixture changed to green, hydrogen sulfide was evolved (lead acetate paper and odor), and a homogeneous solution resulted. Reaction mixture was diluted with 100 ml of cold water and acidified with 1:1 HCl to Congo red. The white precipitate obtained was filtered, dried and recrystallized using ethanol.

Step IV: general procedure for preparation of 3-substituted-4-amino-5-benzylthio-(4H)-1,2,4-triazoles

A mixture of suitable 3-substituted triazole-5-thiol (1.48×10^{-3} mol, 0.4 g) and benzyl chloride (1.48×10^{-3} mol, 0.4 g) in ethanolic alkali (0.08 g KOH in 20 ml aqueous ethanol) was refluxed in microwave. On cooling of the reaction mixture a crude precipitate was obtained, which was recrystallized from appropriate solvents.

Step V: procedure for preparation of combinatorial libraries

To a solution of 3-substituted-4-amino-5-benzylthio-(4H)-1,2,4-triazole 1 g (0.1 mol) in glacial acetic acid add different aldehydes (0.01 mol each). The mixture was refluxed for 15 min at 700 W in microwave. The mixture was poured into a beaker containing 100 ml ice-water, precipitate formed was filtered to obtain dry library. The pool of imines with aromatic region and different triazoles was prepared with same procedure.

Analysis of mini libraries by LC–MS–MS

LC–MS–MS was used to establish that a sizable fraction of the expected compounds are produced in the above synthesis of mini libraries. Experiments were designed to determine if the combination of a 3-substituted-4-amino-5-benzylthio-(4H)-1,2,4-triazoles core with a set of aldehydes results in the formation of a mixture in which all of the expected imino compounds are present above a certain concentration threshold. Observation of a library of six compounds was done on HPLC and was found to be satisfactory. Figure 1 shows the resolved components on the binary LC showing six different components. To investigate the mass-spectrometric behavior of these molecules, several pure imino derivatives of 3-substituted-4-amino-5-benzylthio-(4H)-1,2,4-triazoles prepared by condensing the core molecule with one aldehyde at a time was analyzed. Taken together, the molecular ion peaks obtained from these measurements are a set of data directly correlated to the diversity of a given molecular library. Since fragmentation was not a factor, we were able to compare the

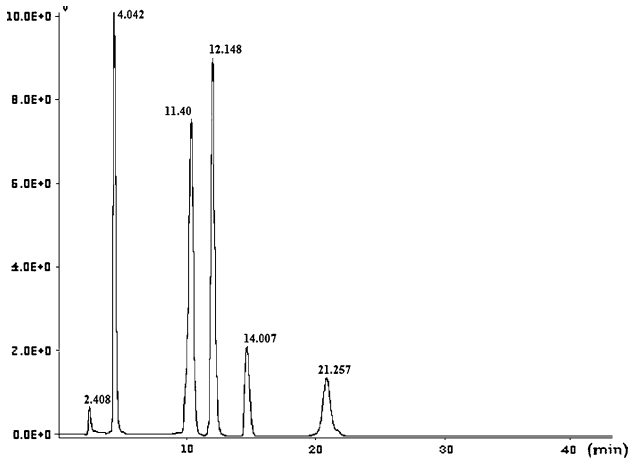


Fig. 1 Figure showing the separation of the HPLC analysis of the mini library

molecular ion peaks in the mass spectra with the molecular weights expected for each model library, and thus confirm which compounds had been formed and which had not. Molecules were considered present if the signal-to-noise ratios of their molecular ion peaks exceeded 3:l. Results of the HPLC analysis of a model library is compiled in Fig. 1. In mass spectrum of the mini-library, all expected six components in mini-library were detected well within the threshold signal to noise ratio. In the synthesis of a large mixture of molecules, the generation of some side products was inevitable. LC-MS analyses of the model libraries indicate that relatively few side products were formed in the course of synthesis of mini-libraries. Very low concentrations of 4-amino-5-benzylthio-(4H)-1,2,4-triazoles could also be detected. Overall, however, we estimate a level of side products below 5–10% of the total number of compounds in a given library.

Biological activity

The synthesized mini libraries were screened for antifungal activity against *Aspergillus niger* (NCIM-945), *Aspergillus flavus* (NCIM-536), *Fusarium oxysporum* (NCIM-1008), *Penicillium chrysogenum* (NCIM-725). The cup plate agar diffusion method was used for antifungal activity; MIC was calculated using serial dilution method14. The tested compounds were dissolved in distilled water to get a solution of 1000, 500, 250, 125, 62, 31.5 μg/ml. Distilled water was used as control. Commercial antifungal fuconazole (100 μg/ml) was also tested under similar conditions for comparison. These pools were deconvoluted by 2D, and leads were identified based on the activity data. The identified leads were synthesized and screened individually for activity. The results of antifungal activity testing of mini-libraries are given in Table 2.

Acknowledgment The authors are thankful to the Principal, Bharati Vidyapeeth College of Pharmacy, Kolhapur for providing necessary facilities for this work.

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