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Discovery and optimization of *N*-acyl and *N*-aroylpyrazolines as B-Raf kinase inhibitors

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ABSTRACT

A high throughput screen identified *N*-aroylpyrazoline **1** as a selective inhibitor of the V600E mutant of B-Raf kinase. Parallel synthesis of acyl, aroyl, and sulfonyl derivatives led to the identification of several potent inhibitors in both enzymatic and cellular (pERK) assays such as compound **42**.

Raf, a serine/threonine protein kinase that is part of the Ras-Raf-MEK-ERK signal transduction pathway[1] regulating cellular growth and proliferation is frequently mutated in many types of cancer.[2] The most common activating mutation is a valine substitution by glutamic acid at amino acid position V600E in the isoform B-Raf resulting in the constitutive activation of the MAP kinase pathway and uncontrolled proliferation of tumor cells. Conversely, suppression of B-Raf (V600E) in human melanoma cells leads to down-regulation of the MAP kinase signaling pathway and apoptosis.[3] Pharmacological inhibition of the B-Raf mutant in melanoma patients by administration of a selective B-Raf inhibitor (PLX-4032) also showed evidence of antitumor activity.[4] Thus, there has been considerable recent interest in developing small molecule inhibitors of mutated B-Raf as therapeutic agents for melanoma and a number of other cancers.[5] Of particular note are sorafenib[6] and a triarylimidazole derivative.[7] As part of our program to identify selective inhibitors of B-Raf, we configured an HTS to assess the effect of our screening compounds on the kinase activity of the mutant V600E form of the protein.[8] The HTS identified an *N*-aroylpyrazoline inhibitor (**1**) of B-Raf (V600E), with an IC_{50} value of 200 nM (Fig. 1). The novelty of the pyrazoline scaffold, which has not previously been associated with kinase inhibition, coupled with the selectivity profile of compound **1**[9] prompted us to use automated parallel synthesis to prepare hundreds of analogs as compound libraries. In this Letter we report the optimization of **1** to give several analogs with single-digit nM IC_{50} values and inhibitory activities in cells approaching 100 nM. In the accompanying article[10] we report on the further optimization of this series for potency, physicochemical and PK properties.

Since there were few examples of compounds related to **1** in our screening library, we prepared a diverse set of substitutions using automated solution-phase synthesis initially investigating acylations and aroylations as shown in Scheme 1. Pyrazolines **2** were prepared by condensation of the appropriate chalcone with hydrazine[11,12] and isomer **2a** subjected to a series of diimide-mediated automated parallel acylations and aroylations.[13] Focusing initially on analogs derived from a diverse set of carboxylic acids, our initial

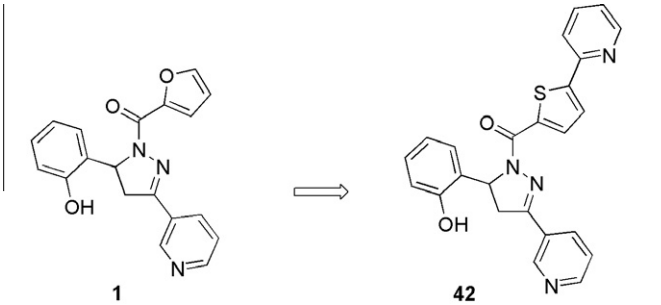

Figure 1. Optimization of a high throughput screening hit for inhibitory activity of V600E BRaf.

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E-mail address: blackburn@mpi.com (C. Blackburn).

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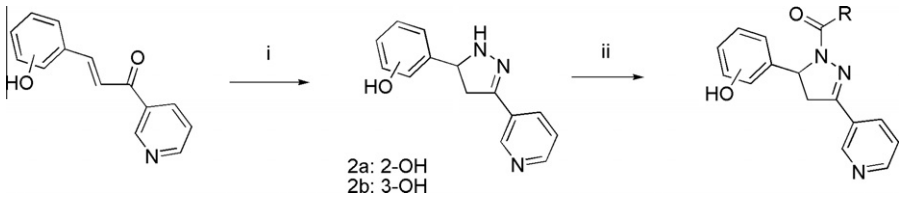
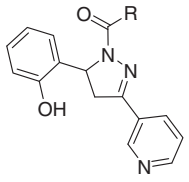
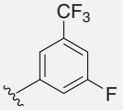
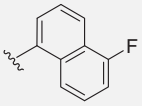
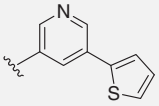
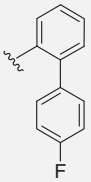
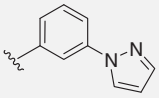
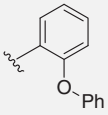
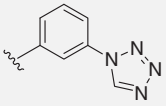
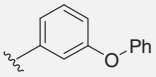
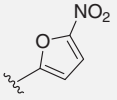
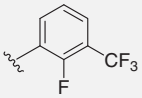
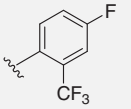
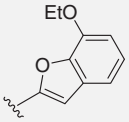
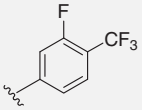
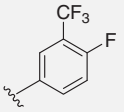


Scheme 1. Reagents and conditions: (i) hydrazine hydrate, EtOH, 80 °C, 3 h; (ii) RCOOH, EDCI, DCM, DMF.

Table 1
B-Raf inhibitory activity of selected compounds from a diverse screening library of *N*-acyl and *N*-aroylpyrazolines

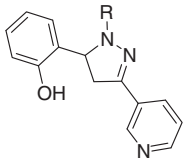


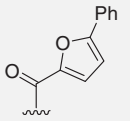
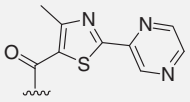
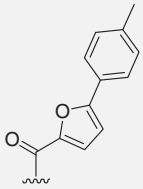
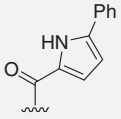
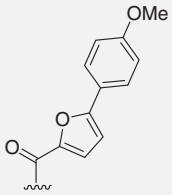
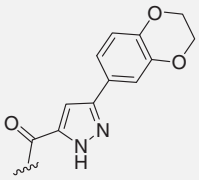
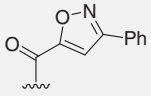
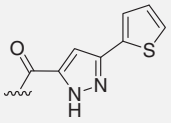
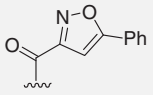
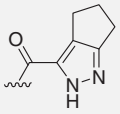
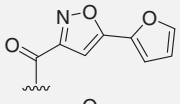
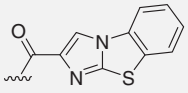
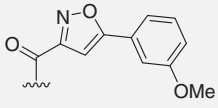
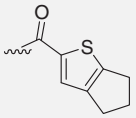
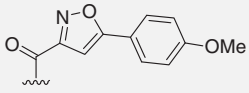
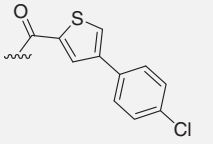
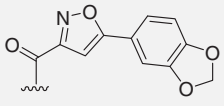
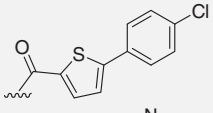
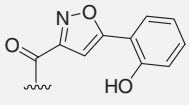
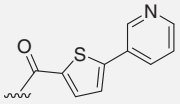
	R	V600E IC_{50} (nM)	pERK IC_{50} (nM)		R	V600E IC_{50} (nM)	pERK IC_{50} (nM)
3		83 ± 8	8000 ± 2200	**12**		110 ± 11	9500 ± 1000
4		>10,000	ND[a]	**13**		260 ± 5	>10,000
5		>10,000	ND	**14**		42 ± 6	8700 ± 1000
6		>10,000	ND	**15**		28 ± 8	>10,000
7		156 ± 10	ND	**16**		130 ± 2	ND
8		>10,000	ND	**17**		7.9 ± 0.9	1400 ± 650
9		>10,000	ND	**18**		25.7 ± 1.7	3500 ± 1000
10		660 ± 20	ND	**19**		22.6 ± 3.0	2000 ± 600
11		200 ± 31	3800 ± 900	**20**		24.2 ± 1.0	1500 ± 500

[a] Not determined.

library of 184 compounds included aliphatic, alicyclic, substituted benzoic, heteroaromatic, biaryl, and bicyclic examples. Compounds were tested for their ability to inhibit the phosphorylation of a biotinylated peptide by B-Raf V600E.[8] Inhibition of cellular activity

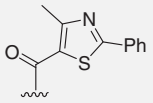
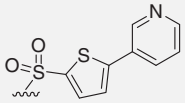
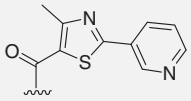
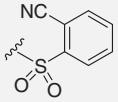
Table 2
Optimization of B-Raf inhibitory activity of *N*-aroyl pyrazolines



	R	V600E IC_{50} (nM)	pERK IC_{50} (nM)		R	V600E IC_{50} (nM)	pERK IC_{50} (nM)
21		16.0 ± 3.0	1700 ± 150	**33**		6.5 ± 0.15	710 ± 100
22		58.0 ± 4.0	2400 ± 15	**34**		41.5 ± 11.5	1100 ± 200
23		18.0 ± 1.0	3700 ± 30	**35**		10.7 ± 3.4	240 ± 40
24		8.0 ± 3.0	1100 ± 100	**36**		10.3 ± 0.6	410 ± 200
25		8.0 ± 2.0	810 ± 300	**37**		7.0 ± 0.1	350 ± 100
26		5.6 ± 0.3	970 ± 300	**38**		9.1 ± 0.7	61 ± 25
27		6.7 ± 1.3	930 ± 400	**39**		6.9 ± 0.7	920 ± 175
28		15.4 ± 5.6	460 ± 180	**40**		216 ± 55	ND[a]
29		8.6 ± 1.6	600 ± 250	**41**		140 ± 9	ND
30		14.3 ± 1.6	130 ± 5	**42**		18.7 ± 7.1	180 ± 40

(*continued on next page*)

Table 2 (*continued*)

	R	V600E IC_{50} (nM)	pERK IC_{50} (nM)		R	V600E IC_{50} (nM)	pERK IC_{50} (nM)
31		37.9 ± 4.2	1300 ± 40	**43**		2000 ± 50	>10,000
32		8.1 ± 2.8	940 ± 110	**44**		450 ± 15	ND

[a] Not determined.

was assessed by determining the decrease in phosphorylation of the Raf kinase substrate pERK in A375 cells.[14]

Over half the compounds from the first library exhibited IC_{50} values <1 μM in the enzymatic assay for B-Raf V600E activity;[15] general SAR trends are illustrated by the compounds listed in Table 1. Small acyl groups such as cyclopropyl were tolerated, (compound **3**), but these derivatives showed little cellular activity. In the case of analogs derived from aryl carboxylic acids, a wide range of activity was observed largely determined by conformational effects. Thus, while numerous active compounds were identified, 1-naphthyl derivative **4** and related quinolines (not shown) were found to be inactive. Similarly, compounds **5** and **6** with large *ortho*-substituents, showed no inhibitory activity of B-Raf V600E, while aryl ether **7**, with the phenoxy group *meta* to the carbonyl, showed improved enzyme inhibitory activity in comparison to the original hit **1**. The potencies of five isomeric fluoro-trifluoromethyl benzoic acid derivatives (**8–12**) that were represented in the library further illustrate this steric effect. While compounds **8** and **9** with substituents *ortho* to the carbonyl group were inactive, the other three isomers showed sub-μM activity in the enzyme assay with the more potent compounds **11** and **12** having the larger trifluoromethyl group in the *meta* (or 3′)-position. Compounds **11** and **12** are comparable to **1** in enzyme inhibitory activity albeit considerably less active in the cellular assay. Interestingly, compounds **13** to **15** with larger substituents *meta* to the carbonyl, also showed comparable or improved potency compared to the original hit (**1**) but cellular activity was again negligible in each case. Substituted furans and benzofurans also featured in the library. Derivatives **16** to **18**, for example, all had improved activities compared to unsubstituted furan **1**, bromo derivative **17** being particularly noteworthy with an IC_{50} in the enzyme assay of 8 nM accompanied by the best cellular activity observed for members of the first library. Pyrrole carboxylic acid derivative **19** and thiophene compound **20** were also found to be superior to **1** in enzyme inhibitory activity without attendant improvements in cellular activity. In summary, the first library identified a large number of compounds with improved B-Raf V600E inhibitory activity, ten of which are listed in Table 1. However, only two compounds, furan **17** and thiophene **20** showed minor improvements to cellular activity compared to the original hit.

We next focused our attention on improving cellular activity by preparing targeted libraries encompassing a selection of five-membered heterocyclic carboxylic acids with additional ring substitutions or fusions (Table 2). 5-Phenyl furan **21** showed 16 nM enzymatic and 1.7 μM cellular activity but substitutions in the phenyl ring (e.g., compounds **22** and **23**) did not lead to further improvements in cellular activity. Phenyl-substituted isoxazoles **24** and **25** also showed high inhibitory activities and several additional analogs (**26–30**) showed promising enzyme and cell potencies with the 4-methoxy (**28**) or fused piperonyl (**29**) derivatives having the best combinations of enzyme and cellular activities. We also identified *ortho*-hydroxy derivative **30**, one of the few compounds with inhibitory activity in cells approaching 100 nM. Although phenyl-thiazole **31** showed modest enzyme and cellular potencies, incremental improvements resulted from incorporation of pyridyl nitrogens (compounds **32** and **33**). Phenylpyrrole **34** proved to be slightly superior to furan **21** in cells even though the enzymatic activity was lower. Several potent pyrazoles were identified such as **35** and **36**, the more active with the fused dioxane ring system and fusion of an alicyclic ring onto the pyrazole was also beneficial as in compound **37**. A tricyclic compound **38** was also identified that showed the highest cellular potency among this series of library compounds. In the thiophene series, a fused analog **39** with promising properties was identified but isomeric chlorophenyl derivatives **40** and **41** were not very active. Pyridyl derivative **42**, however, showed significant improvements compared to the corresponding compound **13** with the pyridyl and thiophene rings transposed.

To assess the effect of the orientation of the hydroxyl group on B-Raf inhibitory activity, the *meta* hydroxyl pyrazoline **2b** was converted to a series of acyl and aroyl derivatives. Of the approximately 100 compounds prepared, the only compound to exhibit any significant enzymatic activity was the analog of lead compound **42**, which was some 35-fold less active in the enzymatic assay with no cellular activity.[10] Lastly we prepared a library of sulfonyl derivatives by reaction of **2a** with one hundred sulfonyl chlorides in pyridine solution. The inputs included aliphatic, benzylic, and arylsulfonyl halides with a diverse range of substituents including heteroaryl derivatives yet most of the compounds showed no significant B-Raf V600E inhibitory activity. Sulfonyl analog **43** of the lead compound **42** from the carboxamide series had an IC_{50} value of 2 μM in the enzymatic assay and showed no cellular activity; only compound **44**, had sub-μM activity (Table 2).

In conclusion, we have used automated parallel solution-phase synthesis to prepare over four hundred pyrazoline derivatives that were screened in enzyme and whole cell assays measuring the inhibition of B-Raf V600E. We have discovered a series of novel and selective[16] inhibitors of mutant B-Raf (V600E) many of which show low nM activity in the enzymatic assay. Of the three compounds identified with promising cellular activities (**30**, **38**, and **42**), arylthiophene **42** was judged to be the most attractive analogue from this series of chiral compounds[17] for further optimization. Docking into a homology model of the active conformation of wild-type B-Raf suggests that the phenol of compound **42** forms an intramolecular hydrogen bond to the carbonyl oxygen with the nitrogen of the 3-pyridyl ring forming a hydrogen bond to the backbone N–H of C532 in the hinge region. This binding mode and optimization of the series for cellular activity and PK properties are described further in the accompanying Letter.[10]

References and notes

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8. The Raf enzyme inhibition assay was conducted in a Flash Plate® format in streptavidin coated 384 well plates monitoring the phosphorylation by ^{33}P ATP (0.5 μCi/reaction) of a biotinylated surrogate peptide at 4 μM concentration by 20 nM Raf mutant V600E at pH 7.5 buffered by 50 mM HEPES in the presence of 10 mM DTT and 50 mM $MnCl_2$ at 30 °C. After 3 h the reaction was stopped by addition of 100 mM EDTA and the reaction mixture transferred to a Flash Plate®, incubated for 2 h and then read on a Topcount analyzer.
9. Pyrazoline **1** was found to have inhibitory IC_{50} values >10 μM for a panel of kinases that included CAMKII, CDK1, CDK2E, CHK1, CHK2, CKII, KDR2, FLT3, CSF1R, KIT2, LCK2, PKA2, and FGFR1.
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13. Each carboxylic acid (110 μmol), in DMF solution (0.5 mL) in a sealed tube, was activated by addition of a solution of EDCI (110 μmol) in DMF (1 mL) delivered by a Tecan liquid handler. A solution of **2** (100 μmol) in a mixture of DMF (0.5 mL) and dichloromethane (0.5 mL) was then added to each vessel and agitated for 24 h. Each reaction mixture was then partitioned between dichloromethane and water with the solvents dispensed and mixed by the liquid handler. The organic phase from each extraction was collected and evaporated from deep well plates to give the crude products that were purified by preparative reverse phase HPLC using mass-directed fraction collection conducted on an Agilent 1100 series LC/MSD instrument using a Waters SunFire C18 5 μm Prep OBD column (19 × 150 mm). The compounds were eluted with a water–MeCN gradient (0.1% formic acid) optimized by the A2Prep Agilent software. All compounds described herein were further characterized by ^{1}H NMR spectroscopy and mass spectrometry.
14. Inhibition of Raf kinase activity in whole cells was assessed by determining the decrease in phosphorylation of the Raf kinase substrate pERK. This whole cell ELISA assay utilized a human melanoma cell line (A375) possessing the mutation B-Raf V600E. A375 cells seeded overnight were incubated with Raf inhibitors for 3 h at 37 °C. At the end of the incubation, the cells were fixed, permeabilized, blocking buffer added and the plates were incubated overnight. After the blocking buffer was discarded, the plates were incubated with anti-phospho-ERK antibody for 1 h followed by treatment with anti-horse radish peroxidase. Optical density was read at 650 nm for the substrate tetramethybenzidine.
15. In general, inhibitory IC_{50} values for the wild-type (WT) enzyme B-Raf were found to be comparable (within twofold) to those determined for the mutant V600E. Compounds **20** (Table 1) and **42** (Table 2), for example, showed IC_{50} values of 17 nM and 18 nM, respectively, for inhibition of the WT enzyme.
16. Compounds **14**, **17**, **18**, **19**, and **20**, for example, were found to have inhibitory IC_{50} values >10 μM for a panel of kinases that included KDR2, FLT3 and CSF1R, KIT2, AKT2, and FGFR1.
17. Separation of compound **42** into its component enantiomers could be effected by chiral chromatography on a Chirobiotic V column eluting with 67% methanol, 33% water containing 0.1% TFA. The early eluting isomer showed IC_{50} values of 1.2 and 14 μM in the enzymatic and cellular assays, respectively. The later eluting isomer was considerably more active (15 nM in the enzymatic assay and 280 nM in the cellular assay).