Development of small-molecule probes that selectively kill cells induced to express mutant *RAS*

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ABSTRACT

Synthetic lethal screening is a chemical biology approach to identify small molecules that selectively kill oncogene-expressing cell lines with the goal of identifying pathways that provide specific targets against cancer cells. We performed a high-throughput screen of 303,282 compounds from the National Institutes of Health-Molecular Libraries Small Molecule Repository (NIH-MLSMR) against immortalized BJ fibroblasts expressing HRAS^{G12V} followed by a counterscreen of lethal compounds in a series of isogenic cells lacking the HRAS^{G12V} oncogene. This effort led to the identification of two novel molecular probes (PubChem CID 3689413, ML162 and CID 49766530, ML210) with nanomolar potencies and 4–23-fold selectivities, which can potentially be used for identifying oncogene-specific pathways and targets in cancer cells.

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The first rat sarcoma (*RAS*) oncogene was discovered as a genetic element from the Harvey and Kirsten rat sarcoma viruses with the ability to immortalize mammalian cells.[1–3] Mutated RAS oncogenes (i.e., *HRAS*, *NRAS*, and *KRAS*) are found in 10–20% of all human cancers and hence are attractive targets for drug development. Despite significant efforts,[4] currently there are no drugs directly targeting mutated *RAS* oncogenes. Thus, several modes of indirect approaches have emerged for targeting RAS including the use of synthetic lethality.

Synthetic lethal screening is a chemical biology approach to identify small molecules that selectively kill oncogene-expressing cell lines with the goal of identifying pathways that provide specific targets against cancer cells. Two distinct approaches have been investigated for identifying compounds exhibiting *RAS* synthetic lethality. In the first approach, RNAi screens led to the identification of *RAS* synthetic lethal genes such as *TBK1*, *CCNA2*, *KIF2C*, *PLK1*, *APC/C*, and *CDK4*, and *STK33*.[5–9] Disappointingly, small molecules that target the encoded proteins have not been found to show the same pattern of synthetic lethality.[10] In the second approach, phenotypic screens have been used to identify inhibitors that are selectively lethal to cell lines expressing a RAS oncogene. This approach necessitates further studies to identify the target, but
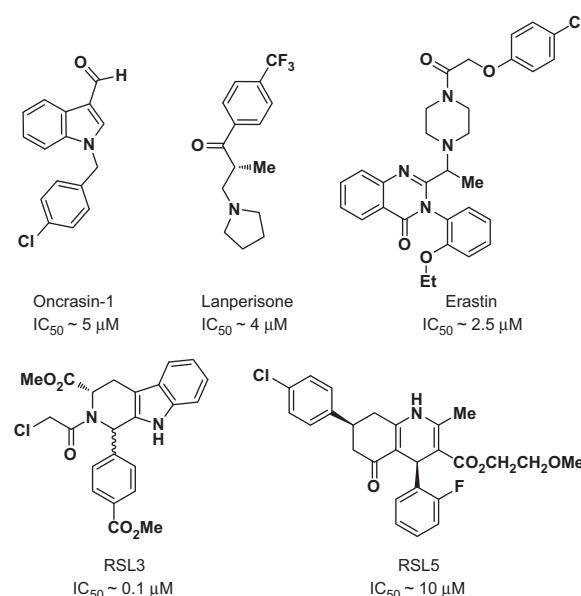
Figure 1. Previously reported small molecules that are lethal to RAS mutant cell lines.

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nonetheless is an alternative approach towards identifying small molecules with novel cellular properties.

Using a phenotypic approach, Guo and colleagues identified oncrasin-1 (Fig. 1), which is selectively toxic to cell lines harboring a *KRAS* oncogene and ineffective in *HRAS* mutant cell lines.[11] More recently, Shaw et al. reported a synthetic lethal screen using mouse embryonic fibroblast harboring an oncogenic $KRAS^{G12D}$ allele and identified lanperisone (Fig. 1) as the most promising candidate.[4] Stockwell and co-workers have identified inhibitors (erastin, RSL3 and RSL5, Fig. 1) that are synthetically lethal with cell lines expressing mutant HRAS and KRAS.[12,13] In this paper, we describe the discovery and development of novel molecular probes exhibiting *RAS* synthetic lethality that could be used to identify new pathways and targets for the development of novel cancer therapeutics.[14]

A high-throughput screen using the National Institutes of Health-Molecular Libraries Small Molecule Repository (NIH-MLSMR) comprising 303,282 compounds (PubChem AID 1832) was performed. Hits were triaged based on activities on four cell lines: BJeLR (expressing $HRAS^{G12V}$), BJeH-LT (isogenic to BJeLR without $HRAS^{G12V}$), DRD (expressing $HRAS^{G12V}$ with alternative oncogenic constructs), BJeH (immortalized background cell line).[15] The hits that emerged from these secondary screens were evaluated for cell viability, which led to the identification of 73 compounds that had IC_{50} <2.5 μM against BJeLR and >4-fold selectivity against the isogenic BJeH-LT cell line. Two hit clusters, an α-chloroacetamide and a nitroisoxazole, were identified and prioritized for optimization. The structures of lead compounds (**1a** and **2a**) from the two series are shown in Figure 2 and the structure/activity relationships (SAR) of these two lead compounds are the subject of this paper.

The first cluster represented by compound **1a** features an α-chloroacetamide that can potentially act as an electrophile. Although covalent inhibitors are rarely considered when initiating a drug discovery program, they have proved to be successful in various disease areas.[16,17] Particularly, in the context of phenotypic screens, where the target is unknown, covalent inhibitors can facilitate target identification through pull-down experiments. RSL3, a previously reported RAS synthetic lethal molecule is also an α-chloroacetamide with an IC_{50} of 100 nM in BJeLR and approximately eightfold selectivity versus BJeH-LT.[14] Compound **1a** also possesses a α-chloroamide sub-unit but shows increased activity and selectivity compared to RSL3 and was thus considered a superior scaffold for SAR studies. To investigate the SAR of **1a**, several analogs were synthesized using the Ugi 4-component reaction (Scheme 1). The influence of all four components was investigated, and the results are presented in Tables 1–3.

Our initial goal was to determine if the α-chloroamide moiety was essential for activity. Thus, analogs where the chloro functional group of **1a** was replaced with a methyl, a methoxy, a hydroxyl moiety, and a heterocyclic leaving group[18] (**1b–e**, Fig. 3) were prepared. All of these analogs were found to be inactive, suggesting that the α-chloroamide moiety is required for activity.

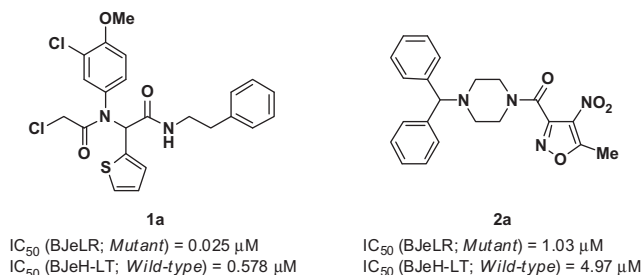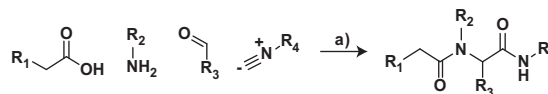**Scheme 1.** Synthesis of α-chloroamide analogs using the Ugi 4-component reaction. Reagent and conditions: (a) MeOH, 23 °C, 48 h.

Table 1
SAR of the aniline portion

Compd	R^1	R^2	IC_{50}^a (μM)		Selectivity[b]
			BJeLR	BJeH-LT	
1a	Cl	OMe	0.025	0.578	23
1f	H	OMe	0.22	1.7	7.8
1g	Cl	H	0.156	0.331	2.1
1h	OMe	H	0.17	2.4	14

[a] Values are means of experiments performed in triplicate.
[b] BJeH-LT IC_{50}/BJeLR IC_{50}.
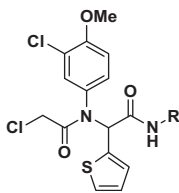
Table 2
SAR on the thiophene portion

Compd	R^1	R^2	IC_{50}^a (μM)		Selectivity[b]
			BJeLR	BJeH-LT	
1a	thienyl	H	0.025	0.578	23
1i	cyclohexyl	H	0.505	1.18	2.3
1j	isopropyl	H	0.895	1.3	1.5
1k	methylthiazolyl	H	8.26	19.8	2.4
1l	chlorothienyl	H	0.061	0.133	2.2
1m	Me	Me	0.058	0.127	2.2
1n	H	H	0.231	0.531	2.3
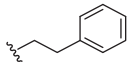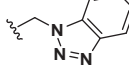
[a] Values are means of experiments performed in triplicate.
[b] BJeH-LT IC_{50}/BJeLR IC_{50}.

In order to assess the stability of **1a** as a nonselective alkylating agent, it was subjected to the presence of glutathione (GSH). After 48 h of incubation of **1a** in PBS/DMS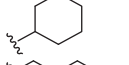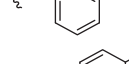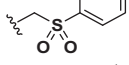O (1:2) at room temperature, in the presence of a physiologically relevant amount of GSH (5 mM), only 20% of the corresponding GSH adduct was observed. This indicates that **1a** is not highly reactive toward thiols and is relatively stable in the presence of nucleophiles such as GSH. When a

1a
IC_{50} (BJeLR; *Mutant*) = 0.025 μM
IC_{50} (BJeH-LT; *Wild-type*) = 0.578 μM

2a
IC_{50} (BJeLR; *Mutant*) = 1.03 μM
IC_{50} (BJeH-LT; *Wild-type*) = 4.97 μM

Figure 2. Hit compounds from the high throughput screening campaign.

Table 3
SAR of the phenethylamine portion

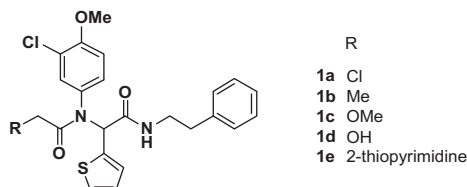| Compd	R	IC$_{50}$^{a} (μM)		Selectivity^b
		BJeLR	BJeH-LT	
1a		0.025	0.578	23
1o		0.113	0.27	2.4
1p		0.075	0.179	2.4
1q	Me	0.089	0.208	2.3
1r		0.102	0.242	2.4

^{a} Values are means of experiments performed in triplicate.
^{b} BJeH-LT IC$_{50}$/BJeLR IC$_{50}$.

	R
1a	Cl
1b	Me
1c	OMe
1d	OH
1e	2-thiopyrimidine

Figure 3. SAR on the α-chloroamide portion.

base (triethylamine) was added, the formation of the GSH adduct was observed in 1 h.

Compound **1a** is a racemate and was thus, subjected to chiral HPLC separation to investigate the importance of the stereogenic center on activity and selectivity. Both enantiomers were obtained with >99% ee (see Supplementary data) and were tested in the cell assays. They were shown to have similar activity and selectivity for HRAS^{G12V} cell lines. To establish whether the enantiomers could racemize under the assay conditions, each enantiomer was subjected to a PBS stability assay. After 48 h incubation in PBS (0.1% DMSO), the stereochemical integrity of the compound was assessed by chiral HPLC/MS. No detectable racemization was observed (see Supplementary data). Given these results, all analogs prepared for SAR studies were synthesized as racemates.

The influence of the substitution on the aniline ring was then examined (Table 1). Both the *meta*-chloro substitutent and the *para*-methoxy substitutent were found to be critical for activity, as removal of either one led to a 10-fold decrease in activity (**1f–g**). A *meta*-methoxy (**1h**) led to a decrease in activity.

Different aldehydes were used in the Ugi reaction to investigate the SAR at the thiophene position (Table 2). Replacing the thiophene ring with hydrophobic group such as a cyclohexyl or an isopropyl group (**1i–j**) led to a decrease in activity and selectivity. The thiophene was also replaced with a thiazole (**1k**) or a 2-chlorothiophene (**1l**) in order to deactivate the 3-position. The thiazole analog **1k** was shown to be inactive, but the 2-chlorothiophene

analog **1l** was found to be very potent with an IC$_{50}$ of 61 nM in BJeLR cell line. However, its selectivity was only 2.2-fold. The thiophene ring was removed in order to address the presence of a stereogenic center in the molecule. Acetone or formaldehyde was used in the Ugi reaction in order to generate the gem-dimethyl moiety (**1m**) or a methylene moiety (**1n**), respectively, in place of the thiophene substituent. The gem-dimethyl analog (**1m**) was equipotent to the initial hit (**1a**) with an IC$_{50}$ of 58 nM in BJeLR cell line but lacked the selectivity. Compound **1n** showed decrease in potency and selectivity.

The influence of the phenethylamine portion of the hit compound was investigated by using different isocyanide components in the Ugi reaction (Table 3). Replacement of the phenethylamine group by shorter hydrophobic amines (**1o–p**) afforded potent compound but the selectivity was eroded.

In addition to investigating the SAR, attempts were made to improve the aqueous solubility of **1a** by the introduction of a sulfone group (**1q**). Although this approach improved the aqueous solubility (16 μM vs <1 μM) it led to a decrease in activity and a 10-fold reduction in selectivity. Introduction of a benzotriazole group (**1r**) did not improve solubility and also led to a decrease in activity and selectivity.

Compound **1a** was found to be the most potent and selective analog in the series. The α-chloroamide portion is required for activity, and the 3-chloro-4-methoxyaniline, the thiophene ring and the phenethylamine portion were found to be optimal for activity and selectivity. Both activity and selectivity of **1a** were confirmed using another pair of HRAS-mutant and wild-type cell lines; HRAS^{G12V} cell line (DRD, IC$_{50}$ = 34 nM) and HRAS wild-type cell line (BJeH, IC$_{50}$ = 2400 nM).

The SAR of the second cluster, the nitroisoxazole hit **2a** (Fig. 2), was then investigated. The synthesis of the analogs was accomplished in six steps using the general scheme developed for the synthesis of **2a** (Scheme 2). Nitration of the 5-methylisoxazole-3-carboxylic acid **3** was accomplished using concentrated sulfuric acid and potassium nitrate. The obtained compound **4** was converted to the corresponding acid chloride **5** in quantitative yield. Benzophenone derivatives **6** were reduced using sodium borohydride to the corresponding alcohol **7**. Treatment of **7** with oxalyl chloride provided **8** and was followed by treatment with an excess of piperazine in refluxing acetonitrile to afford **9**. Coupling of secondary amines **9** with the acid chloride **5** in dichloromethane afforded the final compounds **2**.
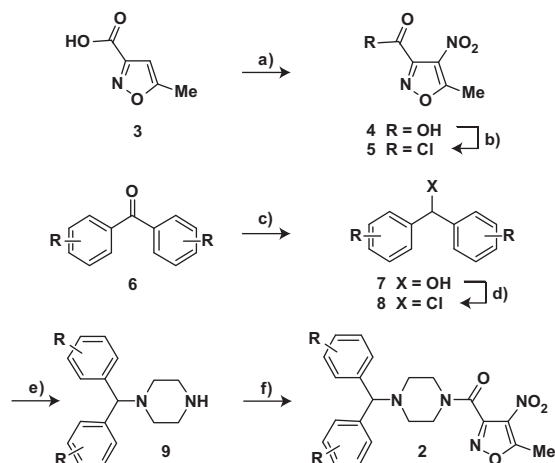
Scheme 2. Representative synthesis of the nitroisoxazole compounds **2**. Reagents and conditions: (a) H$_2$SO$_4$, KNO$_3$; (b) Oxalyl chloride, CH$_2$Cl$_2$; (c) NaBH$_4$, THF–MeOH 1:1; (d) oxalyl chloride, CH$_2$Cl$_2$; (e) piperazine, CH$_3$CN; (f) **5**, CH$_2$Cl$_2$.

Even though the nitro group is present in several FDA–approved drugs, it can be a liability in many instances.[16] Thus, an attempt was made to replace the nitro group with other functional groups (Fig. 4).

Replacement of the nitro group by a primary amine, acetamide, sulfonamide, bromine, methyl, and hydrogen (**2b–g**, Fig. 3) led to inactive compounds, suggesting the importance of the nitro group for activity.

The influence of the nitroisoxazole ring on the activity of the compound (Table 4) was addressed. Replacing the nitroisoxazole ring with other nitroaromatics, such as 4-nitropyrazole (**2h–j**), led to inactive compounds. A nitropyridyl analog (**2k**) was found to be a weak inhibitor and demonstrated poor selectivity. Nitrophenyl (**2l**), as well as a nitrofuran analog (**2m**) were also found to be inactive. Thus, the nitroisoxazole moiety of the molecule was found to be important for activity and was conserved intact for further SAR studies.

The influence of the benzhydryl-piperazine portion of the molecule was then investigated (Table 5). Replacing the benzhydryl-
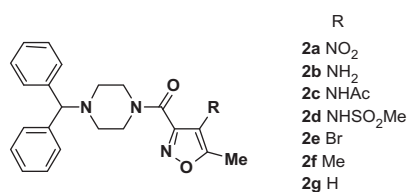
piperazine portion with morpholine (**2n**) or a shorter amide like the *p*-fluorobenzyl analog (**2o**) provided inactive compounds. Keeping the piperazine ring in place and only replacing the benzhydryl portion with an ethylcarbamate (**2p**) or an *o*-methoxyphenyl ring (**2q**) also led to inactive compounds. Even the removal of one of the two phenyl rings of **2a** (**2r**) was sufficient to eliminate activity demonstrating that the benzhydryl portion is necessary for activity. Replacing the piperazine linker with homopiperazine (**2s**) led to a modest improvement in activity but also resulted in a modest decrease in selectivity. Hence, suggesting that homopiperazine is tolerated at that position. The ethylene diamine analog (**2t**) afforded a dramatic decrease in activity indicating the importance of rigidity in this quadrant as well as the importance of hydrogen bonding acceptors.

To improve activity and solubility of the lead, the substitution on both phenyl ring of the benzhydryl group was studied (Table 6). Replacement of both phenyl groups by 2-pyridyl (**2u**) or 4-pyridyl groups (**2v**) increased aqueous solubility to afford analogs with solubility greater than 500 μM. Unfortunately, both of these compounds were found to be inactive.

Introducing *p*-fluoro substituents on both phenyl rings (**2w**) led to a fivefold increase in activity with a slight decrease in selectivity. Introduction of *p*-methoxy substituents on both phenyl groups (**2x**) led to a decrease in activity and selectivity. The introduction of *p*-chloro substituents on both phenyl rings (**2y**) led to a 14-fold increase in activity at the cost of a slight decrease in selectivity. Taking clues from the structure of the drug cetirizine, we made an analog that contains an unsymmetrical benzhydryl unit where only one phenyl ring has a 4-chloro substituent (**2z**). Unfortunately, this modification led to a decrease in activity. The amide function of **2y** was also reduced to the corresponding amine (**2y′**, see Supplementary data), which led to a decrease in activity

R

2a NO$_2$
2b NH$_2$
2c NHAc
2d NHSO$_2$Me
2e Br
2f Me
2g H

Figure 4. SAR at the 4-position of the nitroisoxazole ring.

Table 4
SAR on the nitroisoxazole portion

Compd	R	IC$_{50}$[a] (μM)		Selectivity[b]
		BJeLR	BJeH-LT	
2a		1.03	4.97	4.8
2h		IA	IA	NA[c]
2i		IA	IA	NA
2j		IA	IA	NA
2k		37.3	49.7	1.33
2l		IA	IA	NA
2m		IA	18.9	NA

[a] Values are means of experiments performed in triplicate.
[b] BJeH-LT IC$_{50}$/BJeLR IC$_{50}$.
[c] IA = Inactive, highest concentration tested was 20 μg/ml. NA = Not applicable.

Table 5
SAR on the benzhydrylpiperazine portion

Compd	R	IC$_{50}$[a] (μM)		Selectivity[b]
		BJeLR	BJeH-LT	
2a		1.03	4.97	4.8
2n		IA	IA	NA[c]
2o		IA	IA	NA
2p		IA	IA	NA
2q		IA	IA	NA
2r		IA	IA	NA
2s		0.773	2.79	3.6
2t		21.6	IA	NA

[a] Values are means of experiments performed in triplicate.
[b] BJeH-LT IC$_{50}$/BJeLR IC$_{50}$.
[c] IA = Inactive, highest concentration tested was 20 μg/ml. NA = Not applicable.

Table 6
SAR on the benzhydryl portion
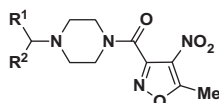
Compd	R^1, R^2	IC_{50}^a (μM)		Selectivity[b]
		BJeLR	BJeH-LT	
2a	$R^1 = R^2 = $ Ph	1.03	4.97	4.8
2u	$R^1 = R^2 = $ 2-Pyridyl	IA	IA	NA[c]
2v	$R^1 = R^2 = $ 4-Pyridyl	IA	IA	NA
2w	$R^1 = R^2 = $ 4-F-Ph	0.183	0.757	4.1
2x	$R^1 = R^2 = $ 4-OMe-Ph	1.57	5.32	3.4
2y	$R^1 = R^2 = $ 4-Cl-Ph	0.071	0.272	3.9
2z	$R^1 = $ Ph, $R^2 = $ 4-Cl-Ph	0.129	0.51	4.0

[a] Values are means of experiments performed in triplicate.
[b] BJeH-LT IC_{50}/BJeLR IC_{50}.
[c] IA = Inactive, highest concentration tested was 20 μg/ml. NA = Not applicable.

(26.4 μM in BJeLR). Homologation of **4** to produce **2w′** was also accomplished (see Supplementary data) but led to a less potent compound (2.99 μM in BJeLR).

Overall, studying the effect of structural changes of the benzhydryl portion of the molecule on activity turned out to be productive, and led to the identification of **2y** with the bis-4-chlorophenyl substituent as the most potent compound in the nitroisoxazole series. Compound **2y** was also found to have an IC_{50} of 107 nM in the DRD cell line, which represents a 16-fold increase in activity compared to the hit compound **2a**. It also shows an IC_{50} of 628 nM in the BJeH cell line. This represents a sixfold selectivity for the DRD (HRAS^{G12V}) versus the BJeH (non expressing HRAS^{G12V}) cell line.

In conclusion, HTS of the MLPCN library followed by SAR investigation led to the identification of two HRAS synthetic lethal compounds **1a** and **2y** with nanomolar potencies against two HRAS^{G12V} expressing cell lines and 4–23-fold selectivities against two control cell lines not expressing HRAS^{G12V}. Because these two probes were identified through a phenotypic screen, their target is still unknown. Future studies will be initiated in order to understand their mode of action and to shed light on RAS-dependent cancers. Compounds **1a** and **2y** have been registered with NIH Molecular Libraries Program (ML 162 and ML 210, respectively) and are available upon request.

Supplementary data

Supplementary (protocols for the synthesis of analogs and bioassays, ^{1}H and ^{13}C NMR and LC–MS) data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.09.047.

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