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Synthesis of novel Ral inhibitors: an *in vitro* and *in vivo* study

Chao Yan^{a,†}, Dan Theodorescu^{a,b}, Bettina Miller^c, Amit Kumar^c, Vijay Kumar^c, David Ross^c, and Michael F. Wempe^{c,*}

^aDepartments of Pharmacology and Surgery, University of Colorado, Aurora, Colorado 80045, USA

^bUniversity of Colorado Comprehensive Cancer Center, Aurora, Colorado 80045, USA

^cDepartment of Pharmaceutical Sciences, University of Colorado, Aurora, Colorado 80045, USA

Abstract

Chemical synthesis was performed to produce a series of 6-amino-1,3-disubstituted-4-phenyl-1,4-dihydro pyrano[2,3-*c*]pyrazole-5-carbonitrile compounds (**14** – **57**) which were characterized by ¹H-NMR, ¹³C-NMR and LC/MS-MS. These compounds were assessed for their effect on the *in vitro* anchorage independent growth of human lung cancer cell line H2122 and IC₅₀ values calculated. Two of the more potent compounds, BQU057 **40** and BQU082 **57** also displayed a dose dependent effect on RalA and RalB activity in H2122 spheroids using the common RalBP1 pull-down assay. Mouse PK and tissue distribution studies on **40** and **57** were performed and demonstrated that parent drug was present in tumor 3.0 h post i.p. (50 mg/Kg) dose.

Graphical Abstract

Keywords

Ral Inhibitor; Chemical Synthesis; Tissue Distribution

The Ras family of evolutionary related proteins is ubiquitously expressed in cells and organs. The Ras genes (H-, K-, R-, N-)– a prototypical Ras superfamily member belongs to the class of protein known as ‘small GTPase’ and are involved in cellular signal transduction.¹ Ras is frequently mutated in human cancer² and even when not mutated, over-

*Corresponding Author: michael.wempe@ucdenver.edu; 303-724-8982.

[†]Chao Yan's new contact information: State Key Laboratory of Pharmaceutical Biotechnology, College of Life Sciences, Nanjing University, Nanjing, China

Supplementary Material

Supplementary materials associated with this article can be found in the online version, at _____.

active (from upstream mediators) Ras signaling can drive cancer growth.³ While efforts to develop targeted therapy against Ras have not yet been successful, targeting Ras effectors is a viable alternative approach. Examples include targeting the Raf-MEK-ERK mitogen activated protein kinase (MAPK) cascade and phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathways.⁴⁻⁵ However, until recently the third canonical effector pathway, RalGEF-Ral has not been targeted.⁶ Ral (Ras-like) is an important member of the Ras small GTPase superfamily and shares a high structural similarity with the Ras protein. Like all GTPases, Ral cycles between an inactive GDP-bound state and an active GTP-bound state. Ral gets activated by guanine exchange factors (RalGEFs) which are effectors of Ras. Once activated, Ral will bind to its own specific downstream effectors such as RalBP1⁷ and Sec5/Exo84,⁸ which mediate the biological functions of Ral in cells. The contributions of Ral signaling to tumorigenesis, progression and metastasis in cancer have been well established by us⁹⁻¹⁰ and others.¹¹⁻¹⁵

We sought to identify potential small molecular weight Ral inhibitors. An *in silico* search (~500,000 compounds) was performed and was followed by in vitro ELISA screening (88 zecomponents) to probe selective binding of active RalA-GTP to its effector protein RALBP1.¹⁶ From these data, two compounds (RBC8 and RBC10, Figure 1) were identified as potential “lead” templates and these two compounds were further studied via tumor xenograft *in vivo* model (H2122 human lung cancer cell line).

We were not only interested in the ability to decrease *in vivo* tumor growth, but also wanted to define tumor and tissue distribution for both compounds. The tissue distribution of RBC8 and RBC10 (3.0 hr post i.p. dose; Table 1) in a mouse model illustrated that drugs were detectable in all tissues tested (brain, heart, kidney, liver and lung) with liver demonstrating the highest concentrations. As the liver is a major site of drug metabolism, we sought to probe mouse liver metabolism. Mouse (CD-1) liver microsomes were used to probe CYP catalyzed biotransformation of RBC8 (Figure 2). LC/MS-MS methods were used to illustrate the formation of three mono-oxidation products (**M1**, **M3**, and **M5**), and two dealkylation (demethylation) products (**M2** and **M4**); metabolites numbers are listed in order of retention time. These *in vitro* generated metabolites were also observed *in vivo* (Supplementary materials section, S-Table 1). Overall **M1** and **M4** were the two major *in vivo* metabolites. In an analogous fashion, RBC10 mouse liver Phase I metabolism was probed. Two major metabolites were produced *in vitro* (**M6** and **M7**) and these two metabolites were confirmed in the *in vivo* samples (Supplementary materials section, S-Table 2). The distribution and metabolism data illustrate that parent drug was distributed and that parent drug was present in the tumors. Consequently, we decided to probe the RBC8 chemical template. We sought to prepare a series of RBC8 analogs and probe how the change in structure might alter *in vitro* biological activity (i.e. Structure-Activity Relationship (SAR)).

The chemical synthesis to prepare RBC8 analogs begins with the reaction of a hydrazine (**1**) with a 3-oxo-ester (**2**) to produce various 1,3-disubstituted-1*H*-pyrazol-5(4*H*)-one compounds (**3–11**; Table 2). This allows control of R1 and R2 substitutions in the final compound.

The various 1,3-disubstituted-1*H*-pyrazol-5(4*H*)-one compounds (**3–11**) were then utilized in the next reaction which used various substituted aromatic aldehydes (**12**). The aromatic aldehydes (Figure 3) reacted with malononitrile to give the corresponding alkene; these alkenes then reacted in tandem with the 1,3-disubstituted-1*H*-pyrazol-5(4*H*)-one compound to produce the corresponding 6-amino-1,3-disubstituted-4-phenyl-1,4-dihydro pyrano[2,3-*c*]pyrazole-5-carbonitrile (**14 – 57**; Table 3). The general synthesis has been previously described in the literature;¹⁷ the chemical synthesis and chemical characterization for the prepared analogs has been summarized in the Supplementary Materials Section. The compounds were then tested *in vitro* to produce IC₅₀ growth values.

The various synthesized analogs demonstrated IC₅₀ values from 1.1 to > 10 μM in H2122 cells and provided limited SAR information. For example, if we compare compound **14** (R1 and R2 = methyl) to compound **29**, the data supports that size rather than electronics on the aromatic ring are important factors; this appears to also be present when one compares **32** to **37** and **38**, the bulkier **38** being more potent in the *in vitro* growth assay. Comparing **18** to **35** illustrates that subtle chemical modifications can give rise to significant changes; from this series, compounds such as **35**, **38**, **40** and **57** were appealing. Consequently, we choose two analogs for further study (BQU057 **40** and BQU082 **57**, Figure 4).

Compound **40** and **57** were then used to determine the Ral activity inhibition in cells. As presented in Figure 5, both compounds displayed a dose dependent *in vitro* effect on RalA and RalB activity.

Lastly, we also performed mouse PK experiments (i.p. dosing of BQU057 and BQU082) to obtain plasma concentration versus time profile data 5 h post-dose. These data illustrate that the two compounds have very short plasma half-lives, T_{1/2} < 1.5 h.

As summarized in Table 4, tissue distribution was probed at 3.0 h post i.p. dose. BQU057 and BQU082 were found to be well distributed in the mouse xenograft model and similar to RBC8 and RBC10 were drug was detected in tumor.

In conclusion, we have prepared novel compounds, which display reasonable Ral inhibitory potential (*i.e.* approaching 1.0 μM). While this initial chemistry and biology effort has produced interesting analogs, we continue to seek compounds which are more potent and have improved ADME properties (*i.e.* lower IC₅₀, improved metabolic stability, longer half-life) through structure based design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
RBC8 and RBC10

Figure 2.
RBC8 proposed Phase I biotransformation

Figure 3.
Synthesis scheme for RBC8 analogs

Figure 4.
Chemical Structures of BQU057 and BQU082

Figure 5.
BQU057 and BQU082 Ral Activity Inhibition data

Table 1

RBC8 and RBC10 tissue distribution data

Tissue	RBC8	RBC10
Brain	0.57 ± 0.37	0.21 ± 0.09
Heart	13.73 ± 9.46	3.54 ± 0.60
Kidney	24.62 ± 12.72	5.73 ± 1.80
Liver	39.68 ± 14.67	11.47 ± 1.02
Lung	21.61 ± 10.84	3.18 ± 0.98
Tumor	5.83 ± 3.46	4.17 ± 1.45
Plasma *	3.91 ± 2.29	3.95 ± 0.30

* plasma expressed as µg/mL while tissues are µg/g;

Data n = 3 ± SD; 3.0 h post i.p. dose (50 mg/Kg)

Table 2

1,3-disubstituted-1H-pyrazol-5(4H)-one analogs

Compound #	R1	R2
3	Ph	Me
4	Me	Me
5	Me	Ph
6	Ph	Ph
7	CH ₂ -Ph	Ph
8	Me	3,4-Di-OMe-Ph
9	Ph	3,4-Di-OMe-Ph
10	Ph	4-OMe-Ph
11	Me	4-OMe-Ph

Table 3

RBC8 analogs prepared and tested *in vitro*

Compound #	R1	R2	R3	R4	R5	R6	R7	IC ₅₀ (μM)
14	Me	Me	H	H	H	H	H	9.1 ± 0.3
15	Me	Ph	H	H	H	H	H	> 10
16	Ph	Me	H	H	H	H	H	2.7 ± 0.1
17	Ph	Ph	H	H	H	H	H	4.1 ± 0.3
18	Ph	4-OMe-Ph	H	H	H	H	H	7.2 ± 0.6
19	Me	4-OMe-Ph	H	H	H	H	H	> 10
20	Me	3,4-DiOMe-Ph	H	H	H	H	H	> 10
21	Me	Me	F	H	H	H	H	> 10
22	Ph	Me	F	H	H	H	H	6.6 ± 0.5
23	Me	Ph	F	H	H	H	H	> 10
24	Ph	Ph	F	H	H	H	H	4.1 ± 0.3
25	Me	3,4-DiOMe-Ph	F	H	H	H	H	> 10
26	Ph	4-OMe-Ph	F	H	H	H	H	6.0 ± 0.5
27	Ph	3,4-DiOMe-Ph	F	H	H	H	H	7.6 ± 0.3
28	Me	4-OMe-Ph	F	H	H	H	H	> 10
29	Me	Me	H	F	H	H	F	4.2 ± 0.1
30	Me	Ph	H	F	H	H	F	6.6 ± 0.4
31	Ph	Me	OMe	H	H	H	H	4.1 ± 0.5
32	Me	Me	OMe	H	H	H	H	8.6 ± 1.1
33	Me	Ph	OMe	H	H	H	H	> 10
34	Ph	Ph	OMe	H	H	H	H	3.1 ± 0.7
35	Ph	p-OMe-Ph	OMe	H	H	H	H	1.1 ± 0.2
36	Me	p-OMe-Ph	OMe	H	H	H	H	> 10
37	Me	Me	OMe	F	H	H	H	5.8 ± 0.3
38	Me	Me	OMe	H	OMe	OMe	H	1.6 ± 0.4
39	Me	Ph	OMe	F	H	H	H	> 10
40	Me	Me	CF ₃	H	H	H	H	2.2 ± 0.3
41	Me	Ph	CF ₃	H	H	H	H	4.1 ± 0.9

Compound #	R1	R2	R3	R4	R5	R6	R7	IC ₅₀ (μM)
42	Me	Me	O-CF ₃	H	H	H	H	2.9 ± 0.4
43	Me	Ph	O-CF ₃	H	H	H	H	5.5 ± 1.0
44	Me	Me	CN	H	H	H	H	5.6 ± 0.5
45	Me	Ph	CN	H	H	H	H	5.2 ± 0.3
46	Me	Me	CH(CH ₃) ₂	H	H	H	H	4.0 ± 0.5
47	Me	Ph	CH(CH ₃) ₂	H	H	H	H	5.4 ± 0.6
48	Me	Me	O-CH ₂ -	CH ₂ -O-	H	H	H	8.6 ± 0.5
49	Me	Ph	O-CH ₂ -	CH ₂ -O-	H	H	H	8.4 ± 0.9
50	Me	Me	N: *	H	H	H	H	4.1 ± 0.8
51	Me	Ph	N: *	H	H	H	H	5.2 ± 0.6
52	Me	Me	H	N: *	H	H	H	8.8 ± 0.4
53	Me	Ph	H	N: *	H	H	H	> 10
54	Me	Me	H	Br	N: *	H	H	2.4 ± 0.4
55	Me	Ph	H	Br	N: *	H	H	1.7 ± 0.1
56	Me	Me	Imidazole	H	H	H	H	1.7 ± 0.1
57	Me	Ph	Imidazole	H	H	H	H	1.3 ± 0.3

n = 3–4 ± SD;

* attached to the aromatic ring

Table 4

BQU057 and BQU082 mouse tissue distribution data

Tissue	BQU057 (40)	BQU082 (57)
Brain	1.39 ± 0.42	0.17 ± 0.07
Heart	2.08 ± 0.42	0.93 ± 0.24
Kidney	2.40 ± 0.91	0.63 ± 0.26
Liver	4.07 ± 1.76	0.98 ± 0.43
Lung	1.87 ± 0.63	1.09 ± 0.41
Tumor	1.22 ± 0.27	0.38 ± 0.21
Plasma *	5.64 ± 0.64	0.49 ± 0.19

* plasma expressed as µg/mL while tissues are µg/g.

Data n = 4 ± SD; 3.0 h post i.p. dose (50 mg/Kg)