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Structure-Activity Relationship Studies of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N'*-Phenyl-Acetohydrazonoyl Cyanide Analogues: Identification of Potent Exchange Proteins Directly Activated by cAMP (EPAC) Antagonists

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ABSTRACT:

Exchange proteins directly activated by cAMP (EPAC) as guanine nucleotide exchange factors mediate the effects of the pivotal second messenger cAMP, thereby regulating a wide variety of intracellular physiological and pathophysiological processes. A series of novel 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide EPAC antagonists were synthesized and evaluated in an effort to optimize properties of the previously identified high-throughput (HTS) hit **1** (ESI-09). Structure–activity relationship (SAR) analysis led to the discovery of several more active EPAC antagonists (e.g. **22** (**HJC0726**), **35** (**NY0123**), and **47** (**NY0173**)) with low micromolar to submicromolar inhibitory activity. These inhibitors may serve as valuable pharmacological probes to facilitate our efforts in elucidating the biological functions of EPAC and developing potential novel therapeutics against human diseases. Our SAR results have also revealed that further modification at the 3-, 4-, and 5-positions of the phenyl ring as well as the 5-position of the isoxazole moiety may allow for the development of more potent EPAC antagonists.

INTRODUCTION

The pivotal second messenger cAMP can regulate a wide range of physiological and pathological processes via signaling pathways which are involved in many disease states including cancer, diabetes, inflammation, heart failure, and neurological disorders.[1] The physiological effects of cAMP are mainly transduced by three different downstream effectors, including protein kinase A (PKA),[2] cyclic nucleotide-activated ion channels,[3, 4] and the most recently discovered exchange proteins directly activated by cAMP (EPAC).[5-10] After activated by the elevation in intracellular concentration of cAMP, EPAC proteins act as guanine nucleotide exchange factors (GEFs) to catalyze the exchange of GDP for GTP on the Ras-like small GTPases, Rap1 and Rap2,[5, 6, 11] thereby controlling the Rap-mediated biological processes. Two isoforms of EPAC proteins have been identified, EPAC1 and EPAC2,[5, 6, 11] which are coded by two independent genes in both mature and developing tissues of mammals with distinct expression patterns. EPAC1, also known as cAMP-GEF-I (RAPGEF3 in humans), is nearly ubiquitously expressed, whereas the expression of EPAC2, also known as cAMP-GEF-II (RAPGEF4 in humans), is relatively restricted. At least three splice variants of human EPAC2 have been found to exhibit a confined expression pattern.[12] Epac2A is somewhat the most broadly expressed, and largely found in brain, pituitary and pancreatic islets.[6, 13, 14] By contrast, Epac2B has so far only been detected in the adrenal and testis,[13, 15] while Epac2C expression is restricted to the liver.[16]

Since their discovery in 1998, EPAC proteins have evoked significant interest toward elucidating their roles in physiological processes and pathogenesis, as well as the molecular mechanisms of EPAC activation. Accumulating evidence has demonstrated that EPAC proteins play a critical role in insulin secretion, cardiac contraction, osteoclast differentiation, vascular

permeability, neurotransmitter release, Treg-mediated immune suppression, integrin-mediated cell adhesion, cell migration and proliferation, cell exocytosis and apoptosis, as well as gene transcription and chromosomal integrity.[17-23] Meanwhile, a substantial number of small-molecule EPAC ligands including various cAMP analogues and newly discovered EPAC-specific antagonists have been discovered and exploited for uncovering the biological functions of EPAC.[10] Various cAMP derivatives have been reported,[24, 25] such as 8-pCPT-2'-*O*-Me-cAMP (007),[26-28] 8-pCPT-2'-*O*-Me-cAMP-AM (007-AM),[29] and Sp-8-pCPT-2'-*O*-Me-cAMPS,[29-31] which have been used as powerful pharmacological tools in EPAC related research. However, these cAMP analogues as EPAC ligands are limited in their use due to a lack of selectivity between the isoforms of EPAC and potential off-target effects. Recently, substantial effort has been made to focus on non-nucleotide small molecules as EPAC-selective or isoform-specific antagonists, such as 4-cyclopentyl-2-((2,5-dimethylbenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (HJC0197) and 4-cyclopropyl-2-((2,5-dimethylbenzyl)thio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (HJC0198) as EPAC antagonists,[32] 5,7-dibromo-6-fluoro-3,4-dihydro-2-methyl-1(2*H*)-quinolinecarboxaldehyde (CE3F4) as an EPAC1 selective inhibitor,[33, 34] as well as 1,3,5-trimethyl-2-tosylbenzene (ESI-05) and 1-(mesitylsulfonyl)-2,4-dimethyl-1*H*-pyrrole (HJC0350) as specific EPAC2 antagonists.[35]

To identify new pharmacological probes with high capability of specifically inhibiting EPAC activity, our team developed an automated, robust and sensitive fluorescence-based high-throughput screening (HTS) assay, which is based on detecting the dose-dependent change in fluorescence intensity of a fluorescent cyclic nucleotide analog, 8-NBD-cAMP with purified full-length EPAC2 protein.[36, 37] After analyzing the EPAC2 8-NBD-cAMP competition activity, selected compounds were evaluated by their ability to inhibit cAMP-mediated EPAC1 and

EPAC2 GEF activity using purified recombinant full-length EPAC1 and EPAC2 proteins. A Maybridge Hitfinder compound library of 14,400 structurally diverse small molecules has been screened.[32, 35, 38] Among these, 2-(5-*tert*-butylisoxazol-3-yl)-*N*'-(3-chlorophenyl)-2-oxoacetohydrazonoyl cyanide (ESI-09, **1**) has been identified and characterized as a novel non-cyclic nucleotide EPAC-specific antagonist with no effect towards PKA (Figure 1).[38] NMR spectroscopy experiments[39] have also verified that **1** specifically interacts with the cAMP binding domain (CBD) of human EPAC1 (aa. 148-318).[40-49] **1** dose-dependently competes with 8-NBD-cAMP in binding to EPAC2, with an IC_{50} value of 9.0 μM. In the presence of 20 μM of cAMP, **1** also dose-dependently displays cAMP-mediated the GEF inhibitory activity of full-length EPAC2 and EPAC1 with apparent IC_{50} values of 4.4 μM and 10.8 μM, respectively.[39]

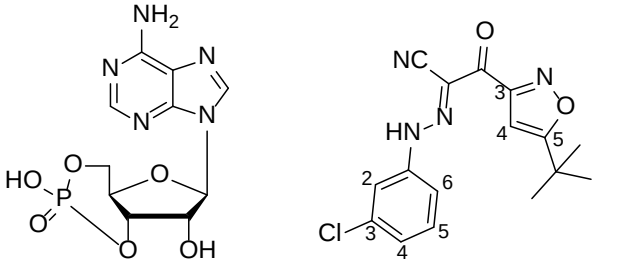


Figure 1. Structures of cAMP and HTS hit **1**

1 as a specific EPAC inhibitor has been a useful tool compound in revealing the roles of cAMP in regulation of *Plasmodium falciparum* merozoite invasion of human erythrocytes[50] and restoration of endothelial barrier after thrombin-induced hyperpermeability,[51] uncovering the functions of EPAC in osteoclast formation,[23] cAMP dependent regulation of Schwann cell proliferation and differentiation,[52] in addition to other fields.[53-55] Further biological functional investigations have also demonstrated that **1** can specifically block intracellular Akt phosphorylation, EPAC-mediated Rap1 activation and insulin secretion in pancreatic β cells.

Treatment of pancreatic ductal adenocarcinoma (PDA) cells with **1** has no obvious effect on cell proliferation and viability, but can give rise to a significant decrease in cell migration and invasion.[38] Further, by employing an orthotopic metastatic PDA mouse model, **1** was found to reduce local and distant spread of MIA PaCa-2 cells and significantly decrease metastasis to the liver at a dose of 10 mg/kg via i.p. injection for 3 weeks.[20] Compound **1** also enhances leptin signaling in organotypic hypothalamic slice culture system. Administration of **1** in wild-type mice at a dose of 50 mg/kg by oral gavage for 3 weeks significantly reduces plasma leptin.[56] Moreover, treatment of **1** at a nontoxic concentration can attenuate the formation of cytopathic effects, significantly reduce viral yields, and effectively protect permissive cells against Middle East respiratory syndrome coronavirus (MERS-Cov) infection by inhibiting viral RNA replication and protein expression of MERS-CoV without affecting the expression and localization of EPAC protein.[57] It was also shown that hit **1** can completely recapitulate the EPAC1 knockout phenotype *in vivo* via pharmacological inhibition of EPAC1, and significantly block the early stage of rickettsial attachment and invasion into the nonphagocytic host cells.[58] Treatment with **1** at a dose of 10 mg/kg via i.p. injection for 12 days significantly protects wild-type mice against rickettsial infection with much milder disease manifestations and dramatically improved survival.[58] Taken together, these findings support the proof-of-concept study that compound **1** is a selective pharmacological probe in unraveling the *in vivo* functions of EPAC, and may provide potential novel therapeutics for the prevention and treatment of various human diseases including pancreatic cancer, diabetes, obesity, Middle East respiratory syndrome coronavirus infections, and fatal rickettsioses.

Compound **1** displays excellent bioavailability,[58] low toxicity to animals,[58] good membrane permeability, no significant inhibitory effects on PDEs,[10] as well as very weak

inhibitory activities towards hERG and CYP450 enzymes.[10] All these combined observations support that non-nucleotide small molecule **1** may have superior advantages in terms of off-target effects, selectivity and toxicities over the traditional cAMP analogues. Despite a potential concern associated with its protein denaturing properties at high concentrations,[59] our extensive biochemical and pharmacological study[39] has defined its therapeutic window and validated that **1** indeed acts as an EPAC specific antagonist. Therefore, it is imperative to further optimize **1** through rational drug design approaches to develop advanced leads with enhanced activity, specificity, and better druglike properties.

Herein, we report our chemical optimization efforts using the HTS hit **1** as the chemical lead and the detailed structure-activity relationship (SAR) studies on a series of substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues. Several new molecules such as 2-(5-(*tert*-butyl)isoxazol-3-yl)-*N*-(3,5-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (**22**, HJC0726),[60] 2-(5-(*tert*-butyl)isoxazol-3-yl)-2-oxo-*N*-(3,4,5-trichlorophenyl)acetohydrazonoyl cyanide (**35**, NY0123),[60] and 2-(5-cyclohexylisoxazol-3-yl)-2-oxo-*N*-(3,4,5-trichlorophenyl)acetohydrazonoyl cyanide (**47**, NY0173)[60] have been identified as potent EPAC antagonists with low micromolar to submicromolar inhibitory activity.

RESULTS AND DISCUSSION

Design. Given the well characterized X-ray crystal structures of inactive[61] and active[62] forms of EPAC2 proteins, we examined the predicted the binding mode via molecular docking studies of **1** into the cAMP binding domain B of active EPAC2 protein using the AutoDock Vina algorithm,[10] thereby understanding an effective interaction mode of the 2-(isoxazol-3-yl)-2-oxo-

N'-phenyl-acetohydrazonoyl cyanide-based chemotype. Results suggest that the binding of the inhibitor to the EPAC2 protein may primarily occur through two terminal hydrophobic interactions and a unique linker. In the present work we focus our new molecular design on the optimization of the aforementioned two main terminal hydrophobic pharmacophores: the 3-chlorophenyl moiety (P1) and the 5-*tert*-butyl group on the isoxazole (P2), as well as the exploration of the linker (P3) for systematic chemical modifications as depicted in Figure 2.

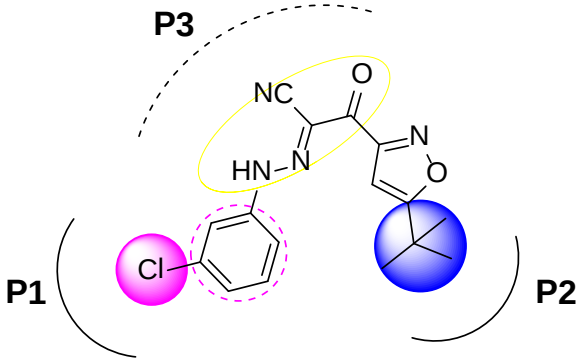
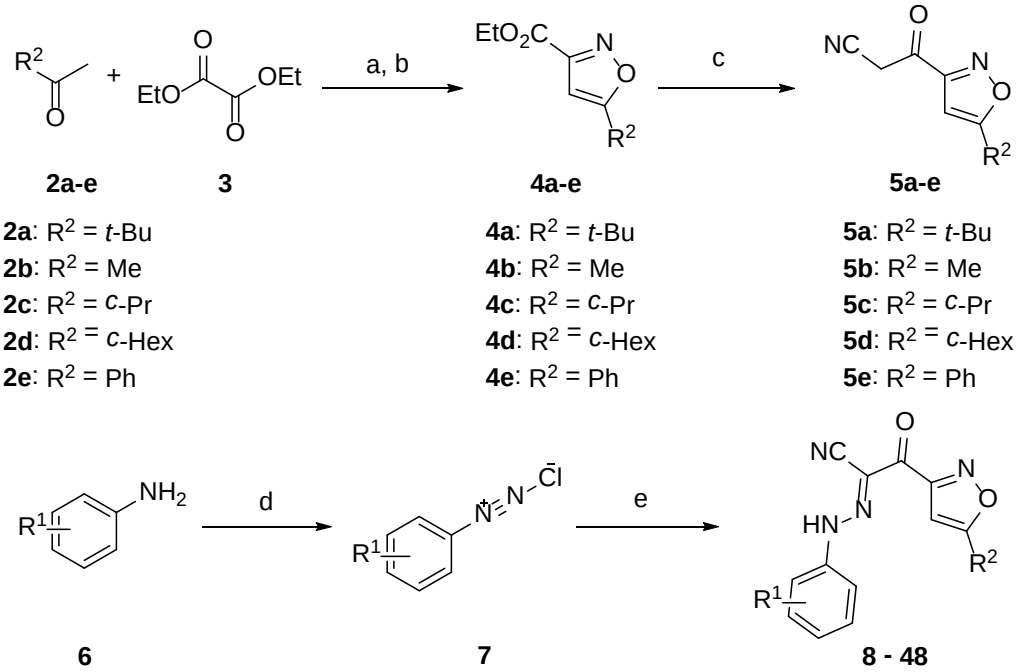


Figure 2. Proposed structural modifications on **1**

Chemistry. As outlined in Scheme 1, the key intermediates **4a-e** were generated by condensation of the commercially available pinacolone **2a-e** with oxalic acid diethyl ester **3** in the presence of NaH (60%) followed by oximation with hydroxylamine hydrochloride and subsequent cyclization by heating in one-pot procedure in 30-87% yields.[63] Ethyl esters **4a-e** were then converted into the corresponding ketonitriles **5a-e** by the treatment with MeLi as the base and CH_3CN as cyanide source under our modified Kowalsko's protocol.[63] Requisite aromatic amines **6** were treated with sodium nitrite and 2 N hydrochloric acid to give the corresponding aryldiazonium chlorides **7**. Direct coupling of the aryldiazonium salts **7** with the

crude cyanomethyl ketones **5a-e** in the presence of NaOAc as the catalyst at 0 °C afforded new substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide derivatives **8** – **48** in 24-76% yields in two steps from **4a-e** (Scheme 1).

Scheme 1. Synthesis of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Analogues with Modification on the Phenyl Ring and the Isoxazol Ring[a]

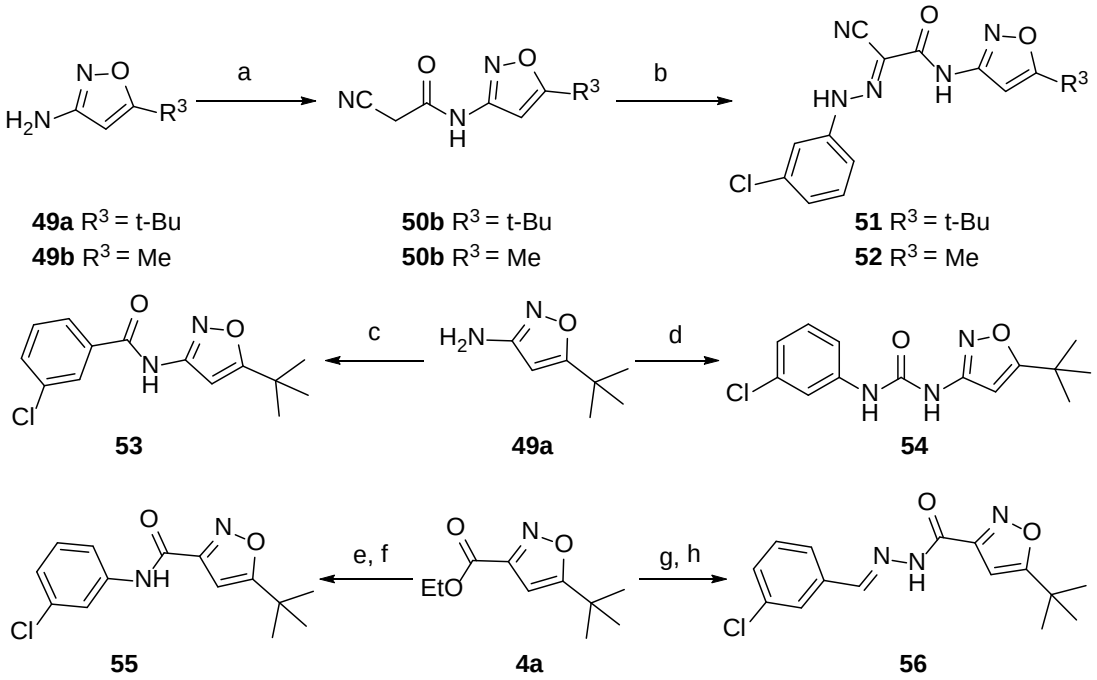


For compounds **8** - **48**: **R1** = H; 2-Cl; 4-Cl; 3-Br; 3-F; 3-CF_3; 3-NO_2; 3-COOEt; 3-CN; 3-$COCH_3$; 3-OMe; 3-CH_2OH; 3-Me; 2,5-di-Cl; 3,5-di-Cl; 3,4-di-Cl; 2,3-di-Cl; 3,5-di-CF_3; 3-F, 5-CF_3; 3,5-di-F; 3-Cl, 5-CF_3; 3-F, 5-Cl; 3-F, 4-Cl; 3-Cl, 4-F; 3-CF_3, 4-Cl; 3,4-di-F; 3-Cl, 4-CF_3; 3,4,5-tri-Cl; 3,4,5-tri-F; 2,4,6-tri-Cl. **R2** = *t*-Bu; Me; *c*-Pr; *c*-Hex; Ph.

[a]Reagents and conditions: (a) NaH, THF, 0 °C to rt; (b) NH_2OH•HCl, EtOH/THF, rt to reflux, 30-87% for two steps (c) CH_3CN, MeLi, THF, -78 °C; (d) 2N HCl, $NaNO_2$, H_2O, 0 °C; (e) **5a-e**, NaOAc, EtOH, 24-76% for three steps.

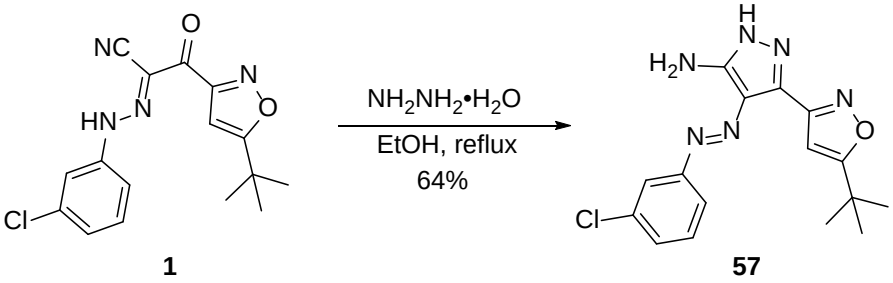
As depicted in Schemes 2 and 3, amides **51**-**53** and **55**, urea **54**, carbohydrazide **56** and pyrazole **57** were prepared as novel substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues bearing different linkers between isoxazole and chlorobenzene of **1**. Condensation of the commercially available isoxazol-3-ylamines **49a-b** with 2-cyanoacetic acid in the presence of EDCI and DIPEA yielded the corresponding intermediates **50a-b**, which were then converted to **51** and **52** by coupling with 3-chlorophenyldiazonium salt upon treatment with NaOAc, in 87% and 53% yields over two steps, respectively. Amide **53** and urea **54** were prepared by condensation of **49a** with 3-chlorobenzoic acid and 1-isocyanato-4-chlorobenzene, in 33% and 17% yields, respectively. Compound **55**, the reverse amide of **53**, was prepared first by removing the protection group of the ester **4a**, following by condensation with 3-chloroaniline in the presence of HBTU and DIPEA, in 48% yield for two steps. Hydrazinolysis of ester **4a** and then condensation with 3-chlorobenzaldehyde provided carbohydrazide **56** with a unique linker in 61% yield over two steps.[64] To mask the ketone group, pyrazole **57** was prepared by cyclization of the linker of **1** with hydrazine hydrate in refluxing ethanol in 64% yield.[65]

Scheme 2. Synthesis of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Analogues with Modification on the Linker[a]



[a]Reagents and conditions: (a) 2-cyanoacetic acid, EDCI, DIPEA, DCM, rt; (b) **7**, NaOAc, EtOH, 0 °C to rt, 53-87% yields for two steps. (c) 3-chlorobenzoic acid, $(COCl)_2$, Et_3N, DCM, 0 °C to rt, 33%; (d) 1-isocyanato-3-chlorobenzene, DCM, rt, 17%; (e) LiOH, THF/H_2O, rt; (f) 3-chloroaniline, HBTU, DIPEA, rt, 48% for two steps (g) NH_2NH_2, EtOH, reflux; (h) 3-chlorobenzaldehyde, EtOH, rt, 61% for two steps.

Scheme 3. Synthesis of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Analogue 57 with Cyclization of the Linker

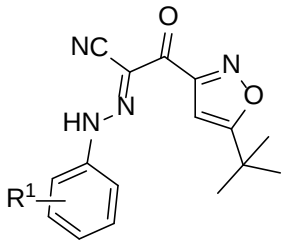


***In Vitro* Evaluation of EPAC2 Inhibition.** All newly synthesized compounds have been evaluated for their ability to compete with 8-NBD-cAMP binding to recombinant EPAC2 proteins to determine IC_{50} values.[36] The previous hit **1** and cAMP were used as the reference

compounds and our data were almost identical to those previously reported,[10, 39] with IC_{50} values of 8.9 and 32.0 μM in inhibiting EPAC2, respectively.

As shown in Table 1, we initially focused on investigating the effect of various substitutions on the phenyl ring of our previous hit **1**. To evaluate the importance of substitutions, we removed 3-chloro on the phenyl ring of **1** leading to compound **8**, which has a about 7-fold loss of binding affinity with an IC_{50} of 59.8 μM. Adjustment of 3-chloro to either 2-position or 4-position (as in compound **9** or **10**) resulted in a 3-fold decreased inhibitory activity in comparison with **1**, suggesting that a proper substitution at 3-position is beneficial to its activity. We next explored the electronic effect of substitutions at the 3-position of the phenyl ring in detail. Replacement of 3-chloro with its congeners bromo and fluoro (as in compounds **11** and **12**) led to a slight loss of activity compared to that of **1**, while increased activity by about 3-4 fold compared to that of non-substituted analog **8**, with IC_{50} values of 14.0 μM and 22.4 μM, respectively. Compounds **13** and **14** with other electron-withdrawing groups such as trifluoromethyl and nitro led to approximately 5-fold and 2-fold increase in potency when compared to that of **8**, with IC_{50} values of 11.9 μM and 31.3 μM, respectively. Compounds **15** with ethyl carboxyl and **16** with cyano group both retained a similar inhibitory activity to that of **8**. However, compound **17** with acetyl group displayed a dramatic loss of activity. Introduction of electron-donating groups such as methoxy and its isomer hydroxymethyl afforded compounds **18** and **19** which also resulted in a significant loss of activity. Interestingly, compound **20** with a methyl group was about 2-fold more potent than non-substituted analog **8**, with an IC_{50} of 25.1 μM. These results suggest that 3-position of phenyl ring of **1** can tolerate chemical modifications with either electron-withdrawing or electron-donating groups.

Table 1. Apparent IC_{50} Values of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Scaffolds with Modifications on the Phenyl Ring for Competing with 8-NBD-cAMP in Binding to EPAC2



compd	R^1	IC_{50} (μM)[a]	Relative affinity (RA)[b]
cAMP		32.0 ± 5.9	1.0
1	3-Cl	8.9 ± 1.2	3.6
8	H	59.8 ± 9.4	0.5
9	2-Cl	28.3 ± 4.0	1.1
10	4-Cl	26.5 ± 4.1	1.2
11	3-Br	14.0 ± 2.4	2.3
12	3-F	22.4 ± 2.8	1.4
13	3-CF_3	11.9 ± 1.7	2.7
14	3-NO_2	31.3 ± 5.5	1.0
15	3-COOEt	71.4 ± 23.8	0.4
16	3-CN	67.8 ± 23.5	0.5
17	3-$COCH_3$	NE[c]	-
18	3-OMe	NE	-
19	3-CH_2OH	NE	-
20	3-Me	25.1 ± 4.8	1.3
21	2,5-di-Cl	7.3 ± 1.2	4.4
22	3,5-di-Cl	1.9 ± 0.3[39]	16.8
23	3,4-di-Cl	3.0 ± 0.6	10.7
24	2,3-di-Cl	35.6 ± 7.9	0.9
25	3,5-di-CF_3	6.4 ± 0.9	5.0
26	3-F, 5-CF_3	5.8 ± 0.9	5.5
27	3,5-di-F	5.3 ± 0.8	6.0
28	3-Cl, 5-CF_3	1.6 ± 0.3	20.0
29	3-F, 5-Cl	2.5 ± 0.4	12.8
30	3-F, 4-Cl	4.3 ± 0.5	7.4

31	3-Cl, 4-F	2.7 ± 0.3	11.9
32	3-CF_3, 4-Cl	2.9 ± 0.5	11.0
33	3,4-di-F	6.6 ± 0.8	4.8
34	3-Cl, 4-CF_3	3.6 ± 0.6	8.9
35	3,4,5-tri-Cl	0.9 ± 0.2	35.6
36	3,4,5-tri-F	1.1 ± 0.2	29.1
37	2,4,6-tri-Cl	19.0 ± 1.7	1.7

[a]The values are the mean ± SE of at least three independent experiments.
[b]RA = $IC_{50,cAMP}$ / $IC_{50,compound}$.
[c]NE: No effect, indicating that a specific IC_{50} cannot be calculated from the data points collected.

We then explored the effect of disubstituted phenyl rings. By keeping 3-chloro and introducing another chloro group on 2, 4, 5, or 6-position (as in compounds **21**-**24**), 3,4- and 3,5-dichloro analogs **22** and **23** bearing the best binding affinity, were about 5- and 3-fold more potent than that of parent compound **1**, with IC_{50} values of 1.9 μM and 3.0 μM, respectively. We then explored fluorinated compounds given their notable successes in drug discovery with an increasing number of fluorine-containing drugs on the market. Due to the unique nature of fluorine, fluorine substitution is commonly used in contemporary medicinal chemistry and drug design to alter the physicochemical and conformational properties, as well as selective reactivity of developing candidates, thereby improving metabolic stability, solubility, bioavailability, and protein–ligand interactions.[66, 67] Moreover, 3-fluoro and 3-trifluoromethyl analogues **12** and **13** from the above mono-substituted SAR studies also exhibited comparable potency to that of 3-chloro (as in compound **1**). Therefore, a series of compounds with fluoro, trifluoromethyl, and chloro groups in 3,4- or 3,5-disubstituted style were designed and synthesized (as in compounds **25**-**34**). All of these compounds were approximately 2-6 folds more potent than hit **1**. Compounds **28**, **29**, **31** and **32** displayed comparable potency to that of compounds **22** and **23**, with IC_{50} values of 1.6-3.0 μM (Figure 3). Inspired by the results from 3,4- and 3,5-disubstituted

design, we attempted to combine their substituted effects and study 3,4,5-trisubstituted analogs. Intriguingly, 3,4,5-trichloro substituted analog **35** and 3,4,5-trifluoro substituted analog **36** demonstrated an enhanced potency with IC_{50} values of 0.9 and 1.1 μM, respectively (Figure 3), while 2,4,6-trichloro substituted analog **37** resulted in a dramatic loss of activity with an IC_{50} of 19.0 μM. These findings suggest that further optimization on the substitutions at the 3-, 4-, and/or 5-positions of the phenyl ring may offer the potential to develop new and more potent EPAC2 inhibitors.

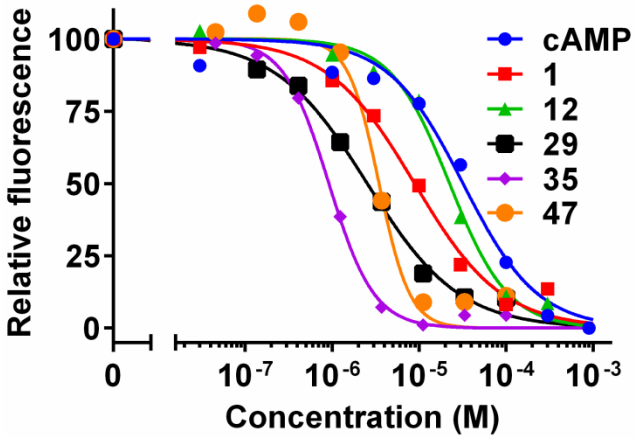
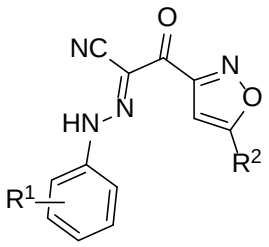


Figure 3. Relative binding affinity of EPAC2 antagonists. Dose-dependent competition of EPAC2 antagonists with 8-NBD-cAMP in binding to EPAC2.

Having identified an optimal substitution for the phenyl ring that maintains good potency, we then directed our effort on tuning the isoxazole scaffold. Compounds **1**, **22** and **35** were chosen as reference compounds. By keeping 3-Cl, 3,5-di-Cl, and 3,4,5-tri-Cl as constant substitutions on the phenyl ring, we embarked on exploring the steric hindrance effects of substitutions on the 5-position of the isoxazole ring via replacing *t*-butyl group of the reference compound with methyl, *c*-propyl, *c*-hexyl, or phenyl group. As shown in Table 2, compounds

38-**41**, **42**-**45**, and **46**-**48** were three small parallel series, which mainly displayed good EPAC2 inhibitory effects in the order of *t*-Bu > *c*-Hex ⩾ *c*-Pr > Me > Ph. Among these, compounds **43**, **44**, **46** and **47** were more potent than hit **1**, with IC_{50} values of 3.45-8.8 μM (Figure 3). Notably, compounds **41** and **48** with phenyl substitutions were totally inactive, indicating that more conformationally constricted biaryl moieties may be unsuitable for fitting into the shallow hydrophobic pockets of EPAC proteins. These results suggest that appropriate bulky alkyl substitutions offering sufficient lipophilicity are more favorable for the ligand to interact with EPAC2 proteins.

Table 2. Apparent IC_{50} Values of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Scaffolds with Modifications on the Isoxazole Ring for Competing with 8-NBD-cAMP in Binding to EPAC2



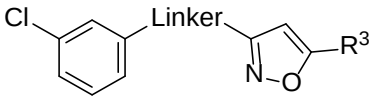
compd	R^1	R^2	IC_{50} (μM)[a]	Relative affinity (RA)[b]
cAMP			32.0 ± 5.9	1.0
1	3-Cl	*t*-Bu	8.9 ± 1.2	3.6
38	3-Cl	Me	NE[c]	-
39	3-Cl	*c*-Pr	60.0 ± 20.9	0.5
40	3-Cl	*c*-Hex	11.3 ± 2.1	2.8
41	3-Cl	Ph	NE	-
22	3,5-di-Cl	*t*-Bu	1.9 ± 0.3[39]	16.8
42	3,5-di-Cl	Me	22.3 ± 0.4	1.4
43	3,5-di-Cl	*c*-Pr	5.5 ± 1.5	5.8
44	3,5-di-Cl	*c*-Hex	4.8 ± 1.2	6.7
45	3,5-di-Cl	Ph	28.0 ± 4.0	1.1
35	3,4,5-tri-Cl	*t*-Bu	0.9 ± 0.2	35.6
46	3,4,5-tri-Cl	*c*-Pr	8.8 ± 2.3	3.6

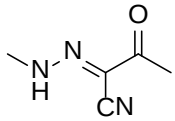
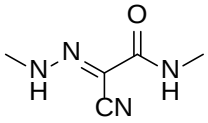
47	3,4,5-tri-Cl	*c*-Hex	3.5 ± 0.8	9.1
48	3,4,5-tri-Cl	Ph	NE	-

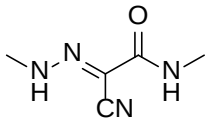
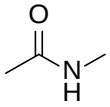
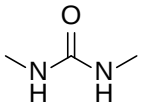
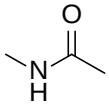
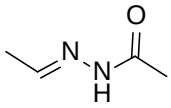
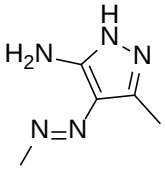
[a]The values are the mean ± SE of at least three independent experiments.
[b]RA = $IC_{50,cAMP}$ / $IC_{50,compound}$.
[c]NE: No effect, indicating that a specific IC_{50} cannot be calculated from the data points collected.

Amides **51**-**53** and **55**, urea **54**, carbohydrazide **56** and pyrazole **57** represented a subseries of novel substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues bearing different linkages between the isoxazole and chlorobenzene by modifications of the linker length and type. Interestingly, compounds **51** and **52** with an additional NH fragment inserted into the linker of **1** and **38** were completely inactive in binding to EPAC2 (Table 3). Other linkages including amide for **53** and **55**, urea for **54**, carbohydrazide for **56** as well as pyrazole for **57** also led to an entire loss of potency towards EPAC2 proteins. These results indicate that the length, shape, and type of the linkages are important for the ligand to interact with EPAC2 proteins.

Table 3. Apparent IC_{50} Values of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Scaffolds with Modifications on the Linker for Competing with 8-NBD-cAMP in Binding to EPAC2



compd	Linker	R^3	IC_{50} (μM)[a]
1		*t*-Bu	8.9
51		Me	NE[b]

52		*t*-Bu	NE
53		*t*-Bu	NE
54		*t*-Bu	NE
55		*t*-Bu	NE
56		*t*-Bu	NE
57		*t*-Bu	NE

[a]The values are the mean ± SE of at least three independent experiments.
[b]NE: No effect, indicating that a specific IC_{50} cannot be calculated from the data points collected.

***In Vitro* Evaluation of EPAC1 Inhibition.** From the biological results discussed above, compounds **22**, **25**, **28**-**29**, **31**-**32**, **34**–**36**, **44** and **46**-**47** were identified as potent EPAC2 inhibitors with IC_{50} values lower than 10 μM. Therefore, these selected compounds together with hit **1** were further evaluated for their ability to inhibit EPAC1-mediated Rap1b-bGDP exchange activity. As shown in Table 4, the previous hit **1** is slightly less potent on EPAC1 inhibition than that on EPAC2, with an IC_{50} value of 10.8 μM. Interestingly, almost all of our selected, newly synthesized analogues exhibited significantly enhanced binding affinity compared to that of **1**. Due to the structural discrepancy between EPAC1 and EPAC2 protein,[10] we found that there is no similar SAR trend towards EPAC1 as observed for EPAC2. Among these, compound **22** and **35** shared the best inhibitory activity as EPAC1 inhibitors, with the IC_{50} values of 2.4 μM

(Figure 4). Compounds **22**, **35**, and **47**, with IC_{50} values lower than 4 μM for both EPAC1 and EPAC2 may serve as valuable pharmacological tools to probe the functions of EPAC in disease, or as potential drug candidates for further preclinical development.

Table 4. Apparent IC_{50} Values of Substituted 2-(Isoxazol-3-yl)-2-Oxo-*N*'-Phenyl-Acetohydrazonoyl Cyanide Scaffolds for Inhibiting EPAC1 GEF activity

compd	EPAC1 IC_{50} (μM)[a]
1	10.8 ± 1.6
22	2.4 ± 0.2
25	5.7 ± 1.7
28	5.7 ± 1.5
29	11.6 ± 3.1
31	7.8 ± 2.0
32	9.7 ± 2.3
34	4.3 ± 0.9
35	2.4 ± 0.3
36	8.9 ± 2.2
44	2.6 ± 0.6
46	4.1 ± 0.7
47	4.0 ± 0.9

[a]The values are the mean ± SE of at least three independent experiments.

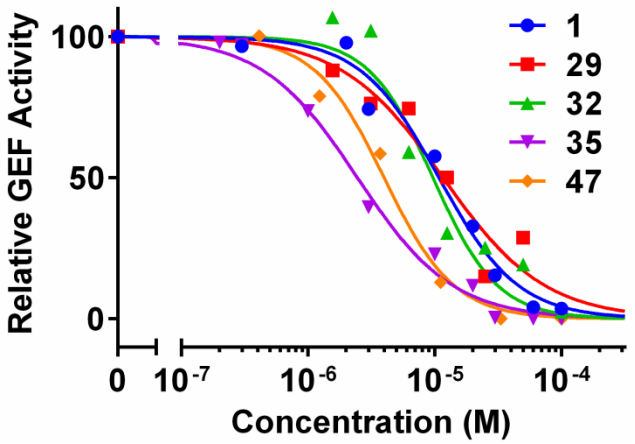


Figure 4. Relative inhibitory activity for EPAC1-mediated Rap1b-bGDP exchange.

Solubility of Selected Ligands in Water and Ethanol. To examine whether the synthesized substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues have better solubility than **1** and to define their therapeutic window, a previously reported HPLC analysis method[68, 69] was employed to measure the solubility of several selected analogues such as **25**, **28**, **31**, **35**, **36**, and **47** (Table 5). One-point calibration was performed against standards with known concentrations of the sample compounds to determine concentrations of the indicated compounds in samples. **1** itself has a limited aqueous solubility with a maximum concentration around 5.95 µg/mL, while possessing an excellent solubility in ethanol (14.98 mg/mL). Addition of another or two chloro atoms onto the phenyl ring of **1** not only enhanced their binding affinity on EPAC1 and EPAC2, but also improved their aqueous solubility. For instance, aqueous solubility of analogue **22** with 3,5-di-Cl on phenyl ring is enhanced with a saturated concentration of 45.4 µg/mL, and that of the 3,4,5-tri-Cl derivatives **35** and **47** is 12.7 and 10.1 µg/mL, respectively. Meanwhile, fluorinated compounds **25**, **28**, **31** and **36** also exhibit improved both potency and aqueous solubility. Particularly, compound **25** with 3,5-di-CF_3 on the phenyl ring of **1** displays a significantly improved solubility both in water and ethanol, with saturated

concentrations of 418.5 μg/mL and more than 150 mg/mL, indicating approximately 70-fold and 10-fold improvement in comparison with that of **1,** respectively (Table 5).

Table 5. Solubility of Selected Compounds in Water and Ethanol

compd	MW	Solubility in Water[a]	Solubility in Ethanol[a]
1[39]	330.769	5.95 ± 0.83 μg/mL (18.0 ± 2.5 μM)	14.98 ± 0.39 mg/mL (45.3 ± 1.2 mM)
22[39]	365.214	45.4 ± 1.4 μg/mL (124.3 ± 3.8 μM)	6.8 ± 0.86 mg/mL (18.6 ± 2.3 mM)
25	432.320	418.5 ± 17.3 μg/mL (968.0 ± 40.0 μM)	> 150 mg/mL (> 347.0 mM)
28	398.770	20.1 ± 0.8 μg/mL (50.4 ± 2.0 μM)	14.6 ± 0.9 mg/mL (36.6 ± 2.3 mM)
31	348.762	82.6 ± 3.6 μg/mL (236.8 ± 10.3 μM)	8.21 ± 0.9 mg/mL (23.5 ± 2.6 mM)
35	399.659	12.7 ± 1.0 μg/mL (31.8 ± 2.5 μM)	4.37 ± 0.52 mg/mL (10.9 ± 1.3 mM)
36	350.301	75.7 ± 7.1 μg/mL (216.0 ± 20.3 μM)	28.6 ± 1.4 mg/mL (81.6 ± 4.0 mM)
47	425.694	10.1 ± 0.79 μg/mL (23.7 ± 1.9 μM)	3.63 ± 0.46 mg/mL (8.5 ± 1.1 mM)

[a]The values are the mean ± SE of at least three independent experiments.

Predicted Docking Mode of Compound 47 with the cAMP Binding Domain (CBD) of EPAC2 Protein.

Since our robust HTS assay is particularly sensitive for identifying compounds that directly compete with 8-NBD-cAMP in binding to EPAC2, we predicted that our compounds may bind to the cAMP binding domain (CBD) of EPAC2. Molecular docking studies were performed to help us better understand the structure-activity relationships of these new compounds toward EPAC2. Using the AutoDock 4.0 method, the docking results revealed that our new compounds

fit nicely into the functional cAMP binding pocket of active EPAC2 (PDB code: 3CF6).[32, 62] To further characterize the binding pose, we selected analogue **47** as a case study for our theoretical investigation. As shown in Figure 5A, the hydrophobic cyclohexylisoxazolyl moiety interacts with the hydrophobic residues of Phe 367, Leu406, Ala407, and Ala415, while 3,4,5-trichlorophenyl fragment forms hydrophobic interactions with residues Val386 and Leu397. Moreover, this binding mode is further stabilized by the occurrence of hydrogen bonds between the NH hydrogen atom of the linker and the oxygen atom of residue Asp402. These results are in full agreement with our established SAR data that hydrophobic groups at the isoxazolyl moiety and phenyl scaffold as well as the types of the linkers are crucial to target EPAC2. The molecular docking studies of **47** also comply with our previous results of hit **1**,[10] through the overlay analysis of two ligands as depicted in Figure 5B. Given the critical role of the linker, these docking findings may also help us understand why amides **53** and **55**, urea **54**, carbohydrazide **56** and pyrazole **57** display no significant activity. Taken together, these studies suggest that optimization of the substitutions at the isoxazolyl moiety and phenyl scaffold while keeping the NH groups on the linker may provide further opportunities to improve the binding affinity and specificity of the substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues towards EPAC proteins.

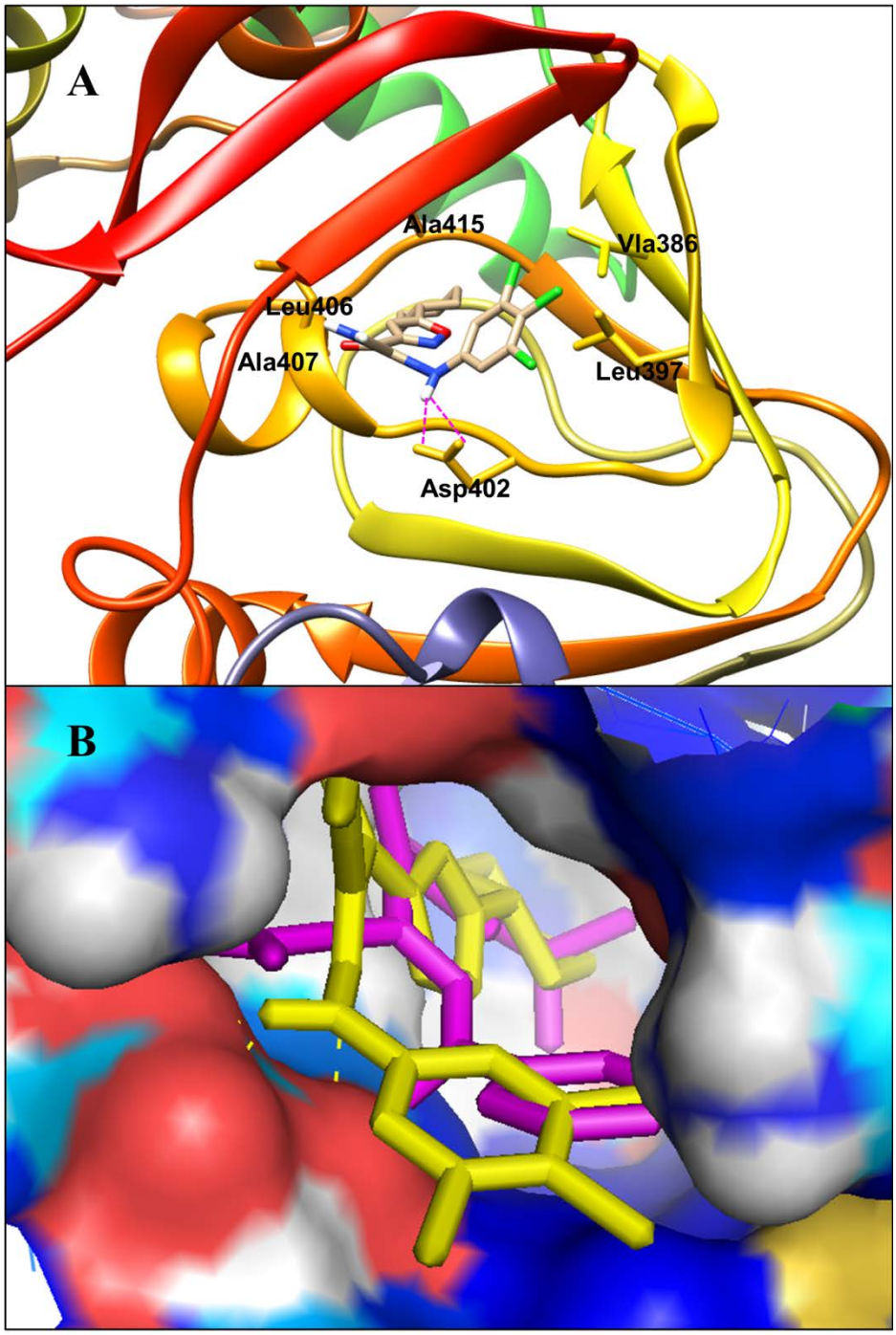


Figure 5. (A) Predicted binding mode and molecular docking of **47** into the cAMP binding domain B (CBD) of EPAC2 protein. Important residues are drawn in sticks. Hydrogen bonds are shown as dashed purple lines. (B) Overlay analysis of ligands **1** and **47. 1** is shown in purple while **47** is depicted in yellow.

CONCLUSION

In summary, a series of substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide derivatives have been designed, synthesized and biologically evaluated with respect to their inhibitory capability towards EPAC proteins. The SAR results based on EPAC2 activity were consistent with our docking studies, indicating that further modifications at the 3-, 4-, 5-positions of the phenyl scaffold and the 5-position of the isoxazole moiety may allow us to tune the original hit **1** to achieve more potent EPAC2 antagonists. Interestingly, various linkers explored led to a significant loss in activity, indicating that the linker in the parent compound plays a critical role in the binding activity towards EPAC. Furthermore, several new molecules such as compounds **22**, **35**, and **47** have been identified as potent EPAC1 and EPAC2 inhibitors with IC_{50} values in the low micromolar to submicromolar range. These compounds may hold promise as potential drug candidates for the preclinical development to IND-enabling studies towards novel therapeutics against human diseases. Additionally, these small-molecule inhibitors may serve as valuable pharmacological probes to facilitate our efforts in elucidating the physiological functions of EPAC in disease and associated signaling pathways. Further studies of selected compounds in various human disease models and a systematic optimization based upon identified advanced chemical leads towards EPAC sub-type selectivity are under way and will be reported in due course.

EXPERIMENTAL SECTION

General Chemistry Information. All commercially available starting materials and solvents were reagent grade, and used without further purification. Reactions were performed under a nitrogen atmosphere in dry glassware with magnetic stirring. Preparative column

chromatography was performed using silica gel 60, particle size 0.063-0.200 mm (70-230 mesh, flash). Analytical TLC was carried out employing silica gel 60 F254 plates (Merck, Darmstadt). Visualization of the developed chromatograms was performed with detection by UV (254 nm). NMR spectra were recorded on a Bruker-600 or Bruker-300 (^{1}H, 600 & 300 MHz; ^{13}C, 150 & 75 MHz) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with TMS as an internal reference. Chemical shifts were expressed in ppm, and *J* values were given in Hz. High-resolution mass spectra (HRMS) were obtained from Thermo Fisher LTQ Orbitrap Elite mass spectrometer. Parameters include the following: Nano ESI spray voltage was 1.8 kV; Capillary temperature was 275 ^{o}C and the resolution was 60,000; Ionization was achieved by positive mode. Melting points were measured on a Thermo Scientific Electrothermal Digital Melting Point Apparatus and uncorrected. Purities of final compounds were established by analytical HPLC, which was carried out on a Shimadzu HPLC system (model: CBM-20A LC-20AD SPD-20A UV/VIS). HPLC analysis conditions: Waters μBondapak C18 (300 × 3.9 mm); flow rate 0.5 mL/min; UV detection at 270 and 254 nm; linear gradient from 10% acetonitrile in water to 100% acetonitrile in water in 20 min followed by 30 min of the last-named solvent (0.1% TFA was added into both acetonitrile and water). All biologically evaluated compounds are > 95% pure.

Ethyl 5-*tert*-butylisoxazole-3-carboxylate (4a). To a solution of pinacolone (500 mg, 5.0 mmol) in THF (10 mL) was added NaH (60%) (220 mg, 5.5 mmol) at 0°C. The reaction mixture was stirred at rt for about 30 min. Diethyl oxalate (730 mg, 5.0 mmol) was added at 0°C and the mixture was then stirred at rt overnight. To the resulting solution, hydroxylamine hydrochloride (380 mg, 5.5 mmol) in ethanol (10 mL) was added dropwise. The mixture was heated at reflux for 16 h. After this time the sodium chloride was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by short column

chromatography on silica gel eluting with hexane/ethyl acetate (4:1) to provide **4a** as a colorless oil (855 mg, 87%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.37 (s, 1H), 4.43 (q, J = 7.2 Hz, 2H), 1.41 (t, J = 7.2 Hz, 3H), 1.37 (s, 9H).

Ethyl 5-methylisoxazole-3-carboxylate (4b). Compound **4b** was prepared in 55% yield (two steps from acetone) by a procedure similar to that used to prepare compound **4a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.36 (s, 1H), 4.38 (q, J = 7.2 Hz, 2H), 2.48 (s, 3H), 1.34 (t, J = 7.2 Hz, 3H).

Ethyl 5-cyclopropylisoxazole-3-carboxylate (4c). Compound **4c** was prepared in 74% yield (two steps from 1-cyclopropylethanone) by a procedure similar to that used to prepare compound **4a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.32 (s, 1H), 4.44 (q, J = 7.1 Hz, 2H), 2.07 (m, 1H), 1.42 (t, J = 7.1 Hz, 3H), 1.18 – 1.10 (m, 2H), 1.06 – 0.98 (m, 2H).

Ethyl 5-cyclohexylisoxazole-3-carboxylate (4d). Compound **4d** was prepared in 30% yield (two steps from 1-cyclohexylethanone) by a procedure similar to that used to prepare compound **4a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.37 (s, 1H), 4.42 (q, J = 7.2 Hz, 2H), 2.91 – 2.78 (m, 1H), 2.12 – 2.04 (m, 2H), 1.91 – 1.62 (m, 3H), 1.49 – 1.25 (m, 8H).

Ethyl 5-phenylisoxazole-3-carboxylate (4e). Compound **4e** was prepared in 73% yield (two steps from acetophenone) by a procedure similar to that used to prepare compound **4a**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (dd, J = 6.5, 3.2 Hz, 2H), 7.45 (dd, J = 5.0, 1.8 Hz, 3H), 6.91 (s, 1H), 4.45 (q, J = 7.1 Hz, 2H), 1.42 (t, J = 7.1 Hz, 3H).

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-phenyl-2-oxoacetohydrazonoyl cyanide (8). To a solution of CH_3CN (410 mg, 10.0 mmol) in anhydrous THF (5 mL) was added 1.6 M methyl lithium in diethyl ether (3.1 mL, 5.0 mmol) at -78 °C under nitrogen. The mixture was stirred at -78 °C for 0.5 h, and **4a** (500 g, 2.5 mmol) in THF (5 mL) was then added dropwise. The solution was stirred at -78 °C for 1 h and then quenched with acetic acid (0.3 mL, 5.0 mmol). The mixture

was warmed to 0 °C and poured onto ice/water (10 mL) and extracted with ethyl acetate (50 mL). The organic lay was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue **5a** (490 mg) was obtained as a yellow oil and directly used for next step without further purification.

To a solution of aniline (22 mg, 0.24 mmol) in H_2O (1 mL) cooled to -5°C was added 0.24 mL of 1 N HCl (aq.). To the resulting acidic aniline solution, 1 mL solution of sodium nitrite (16 mg, 0.24 mmol) in H_2O was added dropwise to generate the aryldiazonium salt solution. To the aryldiazonium salt solution was added sodium acetate (33 mg, 0.4 mmol), followed by 1 mL solution of crude **5a** (38 mg, 0.2 mmol) in ethanol. The reaction mixture was stirred at 0 ^oC for 5 min, and then poured onto H_2O (2 mL) and extracted with ethyl acetate (20 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel eluting with hexane/ethyl acetate (2/1) to provide the desired product **8** (45 mg, 76%, two steps from **4a**) as a yellow solid (mp 118-119 ^oC). HPLC purity 99.4% (t_R = 20.50 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.60 (bs, 1H), 7.49 (d, 2H, J = 7.8 Hz), 7.42 (d, 2H, J = 7.8 Hz), 7.20-7.23 (m, 1H), 6.66 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.8, 179.2, 160.0, 142.1, 129.3, 125.6, 116.8, 112.2, 110.7, 100.2, 32.4, 28.3. HRMS (ESI) calcd for $C_{16}H_{17}N_4O_2$ 297.1346 $(M + H)^+$, found 297.1355.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(2-chlorophenyl)-2-oxoacetohydrazonoyl cyanide (9). Compound **9** was prepared in 76% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 143-144 ^oC). HPLC purity 96.6% (t_R = 22.77 min). ^1H NMR (600 MHz, DMSO-d_6) δ 7.53-7.60 (m, 1H), 7.47-7.52 (m, 1H), 7.37-7.46 (m, 1H), 7.22-7.36 (m, 1H), 6.66 (s, 1H), 1.34 (s, 9H). ^{13}C NMR (150

MHz, DMSO-d_6) δ 180.8, 178.6, 159.9, 139.3, 129.9, 128.1, 126.9, 123.5, 119.6, 114.4, 110.7, 99.9, 32.3, 28.2. HRMS (ESI) calcd for $C_{16}H_{16}ClN_4O_2$ 331.0956 $(M + H)^+$, found 331.0969.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(4-chlorophenyl)-2-oxoacetohydrazonoyl cyanide (10). Compound **10** was prepared in 75% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 142-143 ^oC). HPLC purity 98.1% (t_R = 21.74 min). ^1H NMR (600 MHz, DMSO-d_6) δ 7.46-7.60 (m, 4H), 6.64 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.5, 179.6, 160.8, 142.5, 130.1, 129.9, 119.2, 113.3, 111.6, 100.8, 33.0, 29.0. HRMS (ESI) calcd for $C_{16}H_{16}ClN_4O_2$ 331.0956 $(M + H)^+$, found 331.0963.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-bromophenyl)-2-oxoacetohydrazonoyl cyanide (11). Compound **11** was prepared in 75% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 162-163 ^oC). HPLC purity 98.3% (t_R = 21.93 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.84 (s, 1H), 7.60 (s, 1H), 7.48-7.52 (m, 1H), 7.36-7.40 (m, 2H), 6.69 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.0, 179.3, 160.0, 143.8, 131.4, 127.9, 122.3, 119.0, 116.2, 113.3, 110.5, 100.3, 32.5, 28.4. HRMS (ESI) calcd for $C_{16}H_{16}BrN_4O_2$ 375.0451 $(M + H)^+$, found 375.0456.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-fluorophenyl)-2-oxoacetohydrazonoyl cyanide (12). Compound **12** was prepared in 62% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 140-141 ^oC). HPLC purity 99.4% (t_R = 21.73 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.88 (s, 1H), 7.46 (td, J = 8.2, 6.5 Hz, 1H), 7.38 – 7.31 (m, 1H), 7.22 (d, J = 10.8 Hz, 1H), 7.03 (td, J = 8.4, 2.2 Hz, 1H), 6.67 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.9, 179.2, 162.6 (d, J = 242.3 Hz), 160.2, 131.2 (d, J = 8.85 Hz), 113.3, 112.9, 111.9 (d, J = 21.2 Hz), 110.8, 103.7,

103.6, 100.3, 32.4, 28.4. HRMS (ESI) calcd for $C_{16}H_{16}FN_4O_2$ 315.1252 $(M + H)^+$, found 315.1248.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-trifluoromethylphenyl)-2-oxoacetohydrazonoyl cyanide (13). Compound **13** was prepared in 33% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 147-148 ^oC). HPLC purity 96.0% (t_R = 21.80 min). ^1H NMR (600 MHz, DMSO-d_6) δ 7.74 (d, J = 8.0 Hz, 1H), 7.70 (s, 1H), 7.64 (t, J = 7.9 Hz, 1H), 7.51 (d, J = 7.6 Hz, 1H), 6.63 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.2, 179.7, 161.1, 131.1, 130.5 (q, J = 31.5 Hz), 125.5, 123.5, 122.4, 121.8, 113.7, 112.2, 100.6, 32.9, 28.9. HRMS (ESI) calcd for $C_{17}H_{16}F_3N_4O_2$ 365.1220 $(M + H)^+$, found 365.1230.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-nitrophenyl)-2-oxoacetohydrazonoyl cyanide (13). Compound **14** was prepared in 29% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 151-152 ^oC). HPLC purity 96.4% (t_R = 20.33 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.03 (s, 1H), 8.19 (s, 1H), 7.97 (d, 1H, J = 8.4 Hz), 7.85 (d, 1H, J = 7.8 Hz), 7.67 (t, 1H, J = 7.8 Hz), 6.62 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.5, 179.0, 171.9, 160.8, 148.5, 130.7, 124.6, 119.1, 113.4, 112.1, 111.2, 100.1, 32.4, 28.4. HRMS (ESI) calcd for $C_{16}H_{16}N_5O_4$ 342.1197 $(M + H)^+$, found 342.1207.

Ethyl 3-(2-(2-(5-*tert*-butylisoxazol-3-yl)-1-cyano-2-oxoethylidene)hydrazinyl)benzoate (15). Compound **15** was prepared in 74% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 155-156 ^oC). HPLC purity 98.8% (t_R = 21.53 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.92 (s, 1H), 8.16 (s, 1H), 7.76 (d, 1H, J = 7.8 Hz), 7.74 (d, 1H, J = 8.4 Hz), 7.57 (t, 1H, J = 8.4 Hz), 6.67 (s,

1H), 4.32 (q, 2H, *J* = 7.2 Hz), 1.37 (s, 9H), 1.34 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.1, 179.2, 165.1, 159.9, 142.4, 131.2, 129.8, 125.9, 121.0, 117.3, 113.2, 110.5, 100.1, 61.0, 32.5, 28.4, 14.1. HRMS (ESI) calcd for $C_{19}H_{21}N_4O_4$ 369.1557 $(M + H)^+$, found 369.1558.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-cyanophenyl)-2-oxoacetohydrazonoyl cyanide (16). Compound **16** was prepared in 58% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 181-182 ^{o}C). HPLC purity 99.3% (t_R = 19.87 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.75-7.77 (m, 1H), 7.69 (s, 1H), 7.61-7.63 (m, 2H), 6.69 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.3, 179.5, 160.3, 143.8, 131.0, 128.7, 122.0, 119.8, 118.4, 113.9, 112.4, 110.8, 100.5, 32.7, 28.6. HRMS (ESI) calcd for $C_{17}H_{16}N_5O_2$ 322.1299 $(M + H)^+$, found 322.1303.

***N*'-(3-Acetylphenyl)-2-(5-*tert*-butylisoxazol-3-yl)-2-oxoacetohydrazonoyl cyanide (17).** Compound **17** was prepared in 68% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 152-153 ^{o}C). HPLC purity 98.2% (t_R = 19.80 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.05 (s, 1H), 7.79 (d, 1H, *J* = 7.8 Hz), 7.72 (d, 1H, *J* = 8.4 Hz), 7.57 (t, 1H, *J* = 7.8 Hz), 6.66 (s, 1H), 2.57 (s, 3H), 1.37 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 197.4, 181.4, 179.6, 160.2, 142.7, 138.0, 130.1, 125.6, 121.3, 116.0, 113.3, 100.7, 100.3, 32.7, 28.6, 26.8. HRMS (ESI) calcd for $C_{18}H_{19}N_4O_3$ 339.1452 $(M + H)^+$, found 339.1459.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-methoxyphenyl)-2-oxoacetohydrazonoyl cyanide (18). Compound **18** was prepared in 61% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 125-126 ^{o}C). HPLC purity 95% (t_R = 21.73 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.32 (t, *J* = 8.1 Hz,

1H), 7.11 (m, 2H), 6.78 (dt, J = 9.0, 4.5 Hz, 1H), 6.65 (s, 1H), 3.77 (s, 3H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 181.0, 179.4, 160.1, 130.3, 112.3, 111.4, 110.7, 109.3, 102.5, 100.1, 55.1, 32.4, 28.4. HRMS (ESI) calcd for $C_{17}H_{19}N_4O_3$ 327.1452 $(M + H)^+$, found 327.1450.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3-(hydroxymethyl)phenyl)-2-oxoacetohydrazonoyl cyanide (19). Compound **19** was prepared in 63% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **7**. The title compound was obtained as a yellow solid (mp 147-148 ^{o}C). HPLC purity 99.6% (t_R = 17.86 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.81 (s, 1H), 8.28 (s, 1H), 7.46 (s, 1H), 7.35-7.40 (m, 1H), 7.13-7.18 (m, 1H), 6.65 (s, 1H), 4.49 (s, 2H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.9, 179.4, 160.0, 144.2, 142.0, 129.1, 123.6, 115.4, 114.5, 112.4, 110.7, 100.2, 62.5, 32.4, 28.4. HRMS (ESI) calcd for $C_{17}H_{19}N_4O_3$ 327.1452 $(M + H)^+$, found 327.1457.

2-(5-*tert*-Butylisoxazol-3-yl)-2-oxo-*N*'-m-tolylacetohydrazonoyl cyanide (20). Compound **20** was prepared in 50% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 131-132 ^{o}C). HPLC purity 99.0% (t_R = 21.29 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.73 (s, 1H), 7.31 (m, 2H), 7.28 (s, 1H), 7.04 (d, J = 6.0 Hz, 1H), 6.68 (s, 1H), 2.30 (s, 3H), 1.37 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.9, 179.4, 160.0, 142.0, 138.9, 129.3, 126.5, 117.0, 114.4, 112.3, 110.7, 100.3, 32.4, 28.4, 21.1. HRMS (ESI) calcd for $C_{17}H_{19}N_4O_2$ 311.1503 $(M + H)^+$, found 311.1514.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(2,5-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (21). Compound **21** was prepared in 62% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 193-194 ^{o}C). HPLC purity 97.1% (t_R = 23.69 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.45-7.54 (m, 1H),

7.34 (s, 1H), 7.13-7.22 (m, 1H), 6.56 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.4, 179.6, 162.1, 147.3, 132.8, 132.0, 125.7, 125.4, 118.7, 114.4, 113.7, 100.8, 31.2, 29.0. HRMS (ESI) calcd for $C_{16}H_{15}Cl_2N_4O_2$ 365.0567 $(M + H)^+$, found 365.0576.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3,5-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (22). Compound **22** was prepared in 41% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 191-192 ^{o}C). HPLC purity 99.0% (t_R = 23.20 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.42 (s, 2H), 7.39 (s, 1H), 6.70 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.6, 179.7, 160.5, 145.4, 135.3, 124.7, 115.9, 114.7, 110.9, 100.9, 33.0, 29.0. HRMS (ESI) calcd for $C_{16}H_{15}Cl_2N_4O_2$ 365.0567 $(M + H)^+$, found 365.0563.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(3,4-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (23). Compound **23** was prepared in 55% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 189-190 ^{o}C). HPLC purity 97.5% (t_R = 23.74 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.66 (d, J = 8.8 Hz, 1H), 7.56 (d, J = 2.2 Hz, 1H), 7.42 (dd, J = 8.7, 2.4 Hz, 1H), 6.62 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.1, 179.5, 161.2, 145.6, 132.3, 131.7, 127.3, 118.9, 118.8, 113.7, 112.3, 100.8, 33.0, 29.0. HRMS (ESI) calcd for $C_{16}H_{15}Cl_2N_4O_2$ 365.0567 $(M + H)^+$, found 365.0577.

2-(5-*tert*-Butylisoxazol-3-yl)-*N*'-(2,3-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (24). Compound **24** was prepared in 68% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 187-188 ^{o}C). HPLC purity 97.5% (t_R = 23.74 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.35-7.62 (m, 3H), 6.65 (s, 1H), 1.35 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.4, 179.2, 160.8, 133.0, 129.1,

127.4, 118.6, 100.5, 33.0, 29.0. HRMS (ESI) calcd for $C_{16}H_{15}Cl_2N_4O_2$ 365.0567 $(M + H)^+$, found 365.0568.

***N*'-(3,5-Bis(trifluoromethyl)phenyl)-2-(5-*tert*-butylisoxazol-3-yl)-2-oxoacetohydrazon -oyl cyanide (25).** Compound **25** was prepared in 43% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 138-139 ^oC). HPLC purity 96.4% (t_R = 22.96 min). ^1H NMR (600 MHz, DMSO-d_6) δ 7.91 (s, 2H), 7.76 (s, 1H), 6.58 (s, 1H), 1.33 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.6, 179.3, 161.1, 149.0, 131.3 (q, J = 32.6 Hz), 123.3 (q, J = 271.5 Hz), 118.2, 117.2, 114.1, 112.5, 100.2, 32.5, 28.5. HRMS (ESI) calcd for $C_{18}H_{15}F_6N_4O_2$ 433.1094 $(M + H)^+$, found 433.1098.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3-fluoro-5-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide (26). Compound **26** was prepared in 47% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 160-161 ^oC). HPLC purity 97.5% (t_R = 22.36 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.60 (s, 1H), 7.54 - 7.42 (m, 2H), 6.68 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.5, 179.7, 163.02 (d, J = 244.5 Hz), 160.6, 146.6, 132.2 (qd, J = 33.0 & 10.5 Hz), 125.4, 121.7, 114.7, 111.2, 110.1, 109.4, 108.5, 108.2, 100.7, 33.0, 28.8. HRMS (ESI) calcd for $C_{17}H_{15}F_4N_4O_2$ 383.1131 $(M + H)^+$, found 383.1124.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3,5-difluorophenyl)-2-oxoacetohydrazonoyl cyanide (27). Compound **27** was prepared in 50% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 183-184 ^oC). HPLC purity 96.1% (t_R = 21.56 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.21 – 6.98 (m, 3H), 6.69 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.6, 179.7, 163.5 (d, J = 243.0 Hz),

163.3 (d, J = 243. 8 Hz), 160.5, 145.5 (t, J = 12.0 Hz), 114.4, 110.8, 101.1, 100.9, 100.8, 100.5, 100.4, 33.0, 28.9. HRMS (ESI) calcd for $C_{16}H_{15}F_2N_4O_2$ 333.1163 $(M + H)^+$, found 333.1162.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3-chloro-5-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide (28). Compound **28** was prepared in 46% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 173-174 ^oC). HPLC purity 99.4% (t_R = 23.11 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.71 (s, 2H), 7.64 (s, 1H), 6.71 (s, 1H), 1.36 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.7, 179.8, 160.4, 145.1, 135.5, 132.2 (q, J = 32.3 Hz), 125.3, 121.6 (d, J = 3.8 Hz), 120.6, 115.1, 112.6 (d, J = 3.8 Hz), 110.9, 100.8, 33.0, 28.9. HRMS (ESI) calcd for $C_{17}H_{15}ClF_3N_4O_2$ 399.0836 $(M + H)^+$, found 399.0830.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3-chloro-5-fluorophenyl)-2-oxoacetohydrazonoyl cyanide (29). Compound **29** was prepared in 52% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 175-176 ^oC). HPLC purity 98.3% (t_R = 22.31 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.31 (s, 1H), 7.23 (s, 2H), 6.69 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 186.4, 184.5, 167.8 (d, J = 245.3 Hz), 165.2, 150.4, 140.1 (d, J = 12.8 Hz), 119.3, 118.1, 117.7, 117.3, 115.7, 108.4, 108.1, 105.6, 37.7, 33.7. HRMS (ESI) calcd for $C_{16}H_{15}ClFN_4O_2$ 349.0868 $(M + H)^+$, found 349.0862.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(4-chloro-3-fluorophenyl)-2-oxoacetohydrazonoyl cyanide (30). Compound **30** was prepared in 40% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 170-171 ^oC). HPLC purity 99.2% (t_R = 21.94 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.65 (t, J = 8.4 Hz, 1H), 7.36 (t, J = 9.0 Hz, 2H), 6.68 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-

d$_6$) δ 181.6, 179.6, 160.6, 158.0 (d, *J* = 244.4 Hz), 143.5, 132.0, 116.1, 115.9, 114.5, 114.1, 111.0, 105.7, 105.4, 100.8, 33.0, 28.9. HRMS (ESI) calcd for $C_{16}H_{15}ClFN_4O_2$ 349.0868 $(M + H)^+$, found 349.0860.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3-chloro-4-fluorophenyl)-2-oxoacetohydrazonoyl cyanide (31). Compound **31** was prepared in 40% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 173-174 ^{o}C). HPLC purity 97.4% (t_R = 21.70 min). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 7.55 (d, *J* = 5.9 Hz, 1H), 7.52 – 7.45 (m, 2H), 6.69 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ 181.6, 179.7, 160.6, 155.2 (d, *J* = 243.7 Hz), 140.3, 121.1, 120.8, 118.7, 118.4, 118.1, 117.9, 113.6, 111.1, 100.8, 33.0, 29.0. HRMS (ESI) calcd for $C_{16}H_{15}ClFN_4O_2$ 349.0868 $(M + H)^+$, found 349.0859.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(4-chloro-3-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide (32). Compound **32** was prepared in 42% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 175-176 ^{o}C). HPLC purity 99.8% (t_R = 22.58 min). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 7.91 (s, 1H), 7.79 (d, *J* = 8.6 Hz, 1H), 7.72 (d, *J* = 8.7 Hz, 1H), 6.67 (s, 1H), 1.35 (s, 9H). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) δ 181.7, 179.7, 160.5, 142.4, 133.3, 128.0 (q, *J* = 30.8 Hz), 126.7, 124.8, 122.3, 121.2, 116.3, 116.2, 114.5, 111.1, 100.6, 33.0, 28.9. HRMS (ESI) calcd for $C_{17}H_{15}ClF_3N_4O_2$ 399.0836 $(M + H)^+$, found 399.0829.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3,4-difluorophenyl)-2-oxoacetohydrazonoyl cyanide (33). Compound **33** was prepared in 45% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 185-186 ^{o}C). HPLC purity 98.2% (t_R = 21.12 min). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) δ 7.53 (dd, *J* = 18.7,

9.3 Hz, 1H), 7.45 – 7.29 (m, 2H), 6.68 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.6, 179.7, 160.6, 150.2 (dd, J = 13.8 & 244.3 Hz), 148.0 (dd, J = 12.7 & 167.7 Hz), 140.0 (d, J = 7.4 Hz), 118.9 (d, J = 18.5 Hz), 113.9, 113.5, 111.1, 106.4 (d, J = 21.9 Hz), 100.8, 33.0, 28.9. HRMS (ESI) calcd for $C_{16}H_{15}F_2N_4O_2$ 333.1163 $(M + H)^+$, found 333.1154.

2-(5-*tert*-Butylisoxazol-3-yl)-*N'*-(3-chloro-4-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide (34). Compound **34** was prepared in 35% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 176-177 ^{o}C). HPLC purity 98.9% (t_R = 22.61 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90 (d, J = 8.7 Hz, 1H), 7.63 (s, 1H), 7.56 (d, J = 8.8 Hz, 1H), 6.70 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.7, 179.7, 160.5, 147.7, 132.4, 129.8, 122.6 (q, J = 31.2 Hz), 119.2, 116.2, 115.4, 111.0, 100.9, 33.0, 29.0. HRMS (ESI) calcd for $C_{17}H_{15}ClF_3N_4O_2$ 399.0836 $(M + H)^+$, found 399.0828.

2-(5-*tert*-Butylisoxazol-3-yl)-2-oxo-*N*'-(3,4,5-trichlorophenyl)acetohydrazonoyl cyanide (35). Compound **35** was prepared in 52% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 208-209 ^{o}C). HPLC purity 98.6% (t_R = 13.22 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.50 (s, 2H), 6.55 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.6, 179.4, 161.8, 133.8, 124.9, 119.1, 113.6, 100.8, 32.9, 29.0. HRMS (ESI) calcd for $C_{16}H_{14}Cl_3N_4O_2$ 399.0177 $(M + H)^+$, found 399.0171.

2-(5-*tert*-Butylisoxazol-3-yl)-2-oxo-*N*'-(3,4,5-trifluorophenyl)acetohydrazonoyl cyanide (36). Compound **36** was prepared in 24% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 190-191 ^{o}C). HPLC purity 97.7% (t_R = 21.62 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.28

(d, J = 6.3 Hz, 1H), 7.26 (d, J = 6.3 Hz, 1H), 6.69 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 181.6, 179.5, 160.6, 152.8 (dd, J = 10.5, 5.2 Hz), 149.6 (dd, J = 10.4, 4.9 Hz), 139.7, 114.2, 111.1, 102.2, 101.8, 100.8, 33.0, 28.9. HRMS (ESI) calcd for $C_{17}H_{15}ClF_3N_4O_2$ 399.0836 $(M + H)^+$, found 399.0828.

2-(5-*tert*-Butylisoxazol-3-yl)-2-oxo-*N'*-(2,4,6-trichlorophenyl)acetohydrazonoyl cyanide (37). Compound **37** was prepared in 37% yield (two steps from **4a**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 185-186 ^{o}C). HPLC purity 98.9% (t_R = 12.77 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.55 (s, 2H), 6.39 (s, 1H), 1.27 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 179.3, 179.0, 162.7, 148.2, 128.9, 128.5, 128.2, 116.2, 113.5, 100.5, 32.7, 29.0. HRMS (ESI) calcd for $C_{16}H_{14}Cl_3N_4O_2$ 399.0177 $(M + H)^+$, found 399.0169.

***N'*-(3-Chlorophenyl)-2-(5-methylisoxazol-3-yl)-2-oxoacetohydrazonoyl cyanide (38)** Compound **38** was prepared in 42% yield (two steps from **4b**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 143-144 ^{o}C). HPLC purity 98.4% (t_R = 19.18 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.51 (s, 1H), 7.41-7.45 (m, 2H), 7.20-7.25 (m, 1H), 6.63 (s, 1H), 2.51 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 179.3, 170.3, 160.6, 144.4, 134.1, 131.3, 125.2, 116.8, 115.9, 113.3, 111.0, 103.0, 11.8. HRMS (ESI) calcd for $C_{13}H_{10}ClN_4O_2$ 289.0487 $(M + H)^+$, found 289.0492.

***N'*-(3-Chlorophenyl)-2-(5-cyclopropylisoxazol-3-yl)-2-oxoacetohydrazonoyl cyanide (39).** Compound **39** was prepared in 66% yield (two steps from **4c**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 169-170 ^{o}C). HPLC purity 99.6% (t_R = 10.02 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.51 (s, 1H), 7.45 (d, J = 4.6 Hz, 2H), 7.25 (s, 1H), 6.62 (s, 1H), 2.25 (m, 1H), 1.14 (m, 2H), 0.98 (m, 2H). ^{13}C

NMR (75 MHz, DMSO-d_6) δ 179.7, 176.1, 160.7, 144.0, 134.4, 131.7, 125.6, 116.9, 116.1, 113.8, 110.9, 100.2, 9.1, 8.0. HRMS (ESI) calcd for $C_{15}H_{12}ClN_4O_2$ 315.0643 $(M + H)^+$, found 315.0625.

***N'*-(3-Chlorophenyl)-2-(5-cyclohexylisoxazol-3-yl)-2-oxoacetohydrazonoyl cyanide (40).** Compound **40** was prepared in 58% yield (two steps from **4d**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 176-177 ^oC). HPLC purity 99.7% (t_R = 12.20 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.46 (t, J = 5.1 Hz, 3H), 7.25 (dt, J = 5.1, 2.5 Hz, 1H), 6.68 (s, 1H), 3.00 – 2.86 (m, 1H), 2.04 (d, J = 11.5 Hz, 2H), 1.83 –1.63 (m, 3H), 1.42 (dt, J = 25.3, 11.9 Hz, 4H), 1.31 – 1.17 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.9, 178.3, 160.5, 144.0, 134.5, 131.7, 125.6, 116.7, 116.2, 113.9, 110.9, 101.4, 35.8, 31.1, 25.7, 25.5.HRMS (ESI) calcd for $C_{18}H_{18}ClN_4O_2$ 357.1118 $(M + H)^+$, found 357.1128.

***N*'-(3-Chlorophenyl)-2-oxo-2-(5-phenylisoxazol-3-yl)acetohydrazonoyl cyanide (41).** Compound **41** was prepared in 55% yield (two steps from **4e**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 199-200 ^oC). HPLC purity 99.8 % (t_R = 21.88 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.98 (dd, J = 7.4, 2.1 Hz, 2H), 7.57 (m, 4H), 7.47 (m, 3H), 7.26 (d, J = 7.4 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.4, 170.1, 161.5, 144.1, 134.4, 131.7, 131.4, 129.8, 126.6, 126.3, 125.7, 117.1, 116.2, 113.8, 111.0, 102.0. HRMS (ESI) calcd for $C_{18}H_{12}ClN_4O_2$ 351.0649 $(M + H)^+$, found 351.0641.

***N*'-(3,5-Dichlorophenyl)-2-(5-methylisoxazol-3-yl)-2-oxoacetohydrazonoyl cyanide (42).** Compound **42** was prepared in 35% yield (two steps from **4b**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 185-186 ^oC). HPLC purity 98.4% (t_R = 20.79 min). ^1H NMR (600 MHz, DMSO-d_6) δ 7.45 (s, 2H), 7.36 (s, 1H), 6.63 (s, 1H), 2.50 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 179.1, 170.2, 160.8, 146.4,

134.9, 124.1, 116.0, 113.9, 111.2, 103.0, 11.8. HRMS (ESI) calcd for $C_{13}H_9Cl_2N_4O_2$ 323.0097 $(M + H)^+$, found 323.0103.

2-(5-Cyclopropylisoxazol-3-yl)-*N'*-(3,5-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (43). Compound **43** was prepared in 44% yield (two steps from **4c**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 209-210 ^oC). HPLC purity 98.7% (t_R = 11.05 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.45 (s, 2H), 7.39 (s, 1H), 6.61 (s, 1H), 2.30 – 2.19 (m, 1H), 1.14 (m, 2H), 1.03 – 0.92 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.5, 176.1, 160.8, 145.7, 135.3, 124.6, 116.1, 114.5, 111.1, 100.2, 9.2, 8.0. HRMS (ESI) calcd for $C_{15}H_{11}Cl_2N_4O_2$ 349.0259 $(M + H)^+$, found 349.0257.

2-(5-Cyclohexylisoxazol-3-yl)-*N'*-(3,5-dichlorophenyl)-2-oxoacetohydrazonoyl cyanide (44). Compound **44** was prepared in 48% yield (two steps from **4d**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 215-216 ^oC). HPLC purity 99.7% (t_R = 13.30 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.44 (d, J = 1.8 Hz, 2H), 7.39 (t, J = 2.0 Hz, 1H), 6.68 (s, 1H), 2.93 (t, J = 6.5 Hz, 1H), 2.04 (d, J = 11.5 Hz, 2H), 1.80 – 1.66 (m, 3H), 1.54 – 1.33 (m, 4H), 1.26 (t, J = 11.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.6, 178.3, 160.5, 145.7, 135.3, 124.6, 116.1, 114.7, 111.0, 101.3, 35.9, 31.2, 25.7, 25.5. HRMS (ESI) calcd for $C_{18}H_{17}Cl_2N_4O_2$ 391.0729 $(M + H)^+$, found 391.0724.

***N'*-(3,5-Dichlorophenyl)-2-oxo-2-(5-phenylisoxazol-3-yl)acetohydrazonoyl cyanide (45).** Compound **45** was prepared in 40% yield (two steps from **4e**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 213-214 ^oC). HPLC purity 97.7% (t_R = 23.17 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.96 (m, 2H), 7.57 (m, 3H), 7.47 (s, 3H), 7.36 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.0, 169.9, 161.8, 146.9,

135.2, 131.3, 129.8, 126.7, 126.3, 124.6, 116.5, 114.3, 111.7, 102.1. HRMS (ESI) calcd for $C_{18}H_{11}Cl_2N_4O_2$ 385.0259 $(M + H)^+$, found 385.0252.

2-(5-Cyclopropylisoxazol-3-yl)-2-oxo-*N'*-(3,4,5-trichlorophenyl)acetohydrazonoyl cyanide (46). Compound **46** was prepared in 36% yield (two steps from **4c**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 220-221 ^oC). HPLC purity 98.3% (t_R = 12.18 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.53 (s, 2H), 6.49 (s, 1H), 2.21 (dt, J = 8.3, 3.8 Hz, 1H), 1.11 (dt, J = 8.6, 3.2 Hz, 2H), 0.99 – 0.91 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.2, 175.2, 162.0, 133.8, 125.0, 119.0, 113.6, 100.1, 9.0, 8.0. HRMS (ESI) calcd for $C_{15}H_{10}N_4O_2$ 382.9869 $(M + H)^+$, found 382.9881.

2-(5-Cyclohexylisoxazol-3-yl)-2-oxo-*N'*-(3,4,5-trichlorophenyl)acetohydrazonoyl cyanide (47). Compound **47** was prepared in 35% yield (two steps from **4d**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 228-229 ^oC). HPLC purity 99.2% (t_R = 13.85 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.57 (s, 2H), 6.64 (s, 1H), 2.94 – 2.87 (m, 1H), 2.03 (d, J = 11.2 Hz, 2H), 1.79 – 1.64 (m, 3H), 1.54 – 1.36 (m, 4H), 1.29 – 1.17 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.5, 178.1, 160.7, 134.0, 125.7, 117.9, 114.7, 111.4, 101.4, 35.8, 31.2, 25.7, 25.5. HRMS (ESI) calcd for $C_{18}H_{16}ClN_4O_2$ 425.0335 $(M + H)^+$, found 425.0338.

***N'*-(3,4,5-Trichlorophenyl)-2-oxo-2-(5-phenylisoxazol-3-yl)acetohydrazonoyl cyanide (48).** Compound **48** was prepared in 51% yield (two steps from **4e**) by a procedure similar to that used to prepare compound **8**. The title compound was obtained as a yellow solid (mp 230-231 ^oC). HPLC purity 98.5% (t_R = 24.07 min). ^1H NMR (300 MHz, DMSO-d_6) δ 7.98 – 7.93 (m, 2H), 7.65 (s, 2H), 7.57 (m, 3H), 7.48 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 179.0, 170.0, 161.7,

144.2, 134.1, 131.4, 129.8, 126.7, 126.3, 125.8, 118.1, 114.6, 102.1. HRMS (ESI) calcd for $C_{18}H_{10}Cl_3N_4O_2$ 418.9864 $(M + H)^+$, found 418.9864.

***N*-(5-*tert*-Butyl-isoxazol-3-yl)-2-[(3-chlorophenyl)-hydrazono]-2-cyanoacetamide (51).** To a solution of 5-*tert*-butyl-isoxazol-3-ylamine **49a** (140 mg, 1.0 mmol) and cyanoacetic acid (85 mg, 1.0 mmol) in 10 mL of DCM was added DIPEA (258 mg, 2.0 mmol). EDCI (191 mg, 1.0 mmol) was added at 0 ^oC. The resulting mixture was stirred at r.t. for 16 h. The solution was diluted with DCM (50 mL), washed with 1 N HCl (aq.) (10 mL) and brine (10 mL). The organic layer was dried over anhydrous Na_2SO_4, and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to give the desired product **50a** as a white solid (163 mg, 90%). ^1H NMR (600 MHz, $CDCl_3$) δ 10.59 (s, 1H), 6.70 (s, 1H), 3.65 (s, 2H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 182.9, 159.8, 157.6, 113.4, 93.7, 33.4, 28.7, 26.9.

To a solution of 3-chloroaniline (25 mg, 0.2 mmol) in H_2O (1 mL cooled to -5°C) was added 0.2 mL of 1 N HCl (aq.). To the resulting acidic aniline solution, 1 mL solution of sodium nitrite (14 mg, 0.2 mmol) in H_2O was added dropwise to generate the aryldiazonium salt solution. To the aryldiazonium salt solution was added sodium acetate (33 mg, 0.4 mmol), followed by 1 mL solution of **50a** (29 mg, 0.14 mmol) in ethanol. The reaction mixture was stirred at 0 ^oC for 5 min, and then poured onto H_2O (2 mL) and extracted with ethyl acetate (20 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by short column chromatography on silica gel eluting with hexane/ethyl acetate (2/1) to provide the desired product **51** (32 mg, 66%) as a yellow solid (mp 238-240 ^oC). HPLC purity 96.7% (t_R = 20.97 min). ^1H NMR (600 MHz, DMSO-d_6) δ 12.04 (s, 1H), 11.22 (s, 1H), 7.98 (s, 1H), 7.69 (d, 1H, J = 9.0 Hz), 7.40 (t, 1H, J = 7.8 Hz), 7.17 (d, 1H, J = 7.8 Hz), 6.63 (s, 1H),

1.32 (s, 9H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 180.2, 159.5, 157.2, 143.0, 133.6, 130.4, 123.8, 115.8, 115.0, 110.6, 107.9, 93.6, 32.2, 28.1. HRMS (ESI) calcd for $C_{16}H_{17}ClN_5O_2$ 346.1065 $(M + H)^+$, found 346.1074.

2-[(3-Chlorophenyl)-hydrazono]-2-cyano-*N*-(5-methyl-isoxazol-3-yl)acetamide (52). Compound **52** was prepared in 53% yield (two steps) by a procedure similar to that used to prepare compound **51**. The title compound was obtained as a yellow solid (mp 241-243 ^{o}C). HPLC purity 98.5% (t_R = 18.55 min). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.03 (s, 1H), 11.19 (s, 1H), 7.98 (s, 1H), 7.69 (d, 1H, J = 8.4 Hz), 7.39 (t, 1H, J = 7.8 Hz), 7.18 (d, 1H, J = 6.6 Hz), 6.67 (s, 1H), 2.43 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 169.6, 159.8, 157.9, 143.4, 133.9, 130.7, 124.1, 116.1, 115.3, 110.9, 108.3, 97.0, 12.2. HRMS (ESI) calcd for $C_{13}H_{11}ClN_5O_2$ 304.0596 $(M + H)^+$, found 304.0606.

***N*-(5-(*tert*-Butyl)isoxazol-3-yl)-3-chlorobenzamide (53).** To a solution of 3-chlorobenzoic acid (35 mg, 0.25 mmol) in 5 mL DCM, $(COCl)_2$ (212 μL, 2.5 mmol) was added at 0 ^{o}C, and the mixture was allowed to rt for 1 h. The solution was concentrated and then dissolved in 5 mL of DCM. 5-(*tert*-Butyl)isoxazol-3-amine **49a** (78 mg, 0.5 mmol) and Et_3N (151 mg, 1.5 mmol) were added at 0 ^{o}C, then the mixture was stirred at rt overnight. The solution was diluted by DCM and washed with 1 N $NaHSO_4$, saturated $NaHCO_3$ and brine (5 mL each). The organic layer was concentrated and purified by PTLC to obtain **53** (25 mg, 33%) as a white solid (mp 145-146 ^{o}C). HPLC purity 96.5% (t_R = 20.08 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.74 (s, 1H), 8.05 (t, J = 1.9 Hz, 1H), 7.96 (ddd, J = 7.7, 1.7, 1.1 Hz, 1H), 7.59 (ddd, J = 8.0, 2.1, 1.1 Hz, 1H), 7.47 (t, J = 7.8 Hz, 1H), 6.91 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.8, 164.6, 135.2, 134.9, 132.4, 129.9, 128.2, 126.0, 94.0, 33.1, 28.7. HRMS (ESI) calcd for $C_{14}H_{16}ClN_2O_2$ 279.0900 $(M + H)^+$, found 279.0894.

1-(5-*tert*-Butylisoxazol-3-yl)-3-(3-chlorophenyl)urea (54). A mixture of 5-(*tert*-butyl)isoxazol-3-amine **49a** (30 mg, 0.21 mmol) and 1-chloro-3-isocyanatobenzene (33 mg, 0.21 mmol) in 5 mL DCM was stirred at rt overnight. The mixture was concentrated under vacuum. The residue was purified with silica gel column (Hexane/EtOAc=10/1 to 5/1) to obtain **54** (10 mg, 17%) as a white solid (mp 114-116 ^{o}C). HPLC purity 98.0% (t_R = 19.99 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.08 (s, 1H), 9.24 (s, 1H), 7.75 (t, J = 2.0 Hz, 1H), 7.55 (d, J = 8.0 Hz, 1H), 7.36 (t, J = 8.0 Hz, 1H), 7.29 (s, 1H), 5.81 (s, 1H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 181.6, 177.7, 158.4, 139.3, 134.4, 129.8, 126.9, 125.1, 123.1, 91.3, 33.0, 28.6. HRMS (ESI) calcd for $C_{14}H_{17}ClN_3O_2$ 294.1009 $(M + H)^+$, found 294.0992.

5-*tert*-Butyl-*N*-(3-chlorophenyl)isoxazole-3-carboxamide (55). 4a (1000 mg, 5.1 mmol) and LiOH (638 mg, 15.3 mmol) were dissolved in 30 mL MeOH and 10 mL H_2O, respectively. Then the solution was stirred at rt for 1 h. In an ice-cooled bath, 1 N Na_2SO_4 (10 mL) was added, and the mixture was extracted by ethyl acetate (20 mL × 3). The organic layer was separated and washed with brine (10 mL). After drying over anhydrous Na_2SO_4, the solution was concentrated to give 5-*tert*-butylisoxazole-3-carboxylic acid as a light yellow oil (818 mg, 95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.64 (s, 1H), 6.42 (s, 1H), 1.37 (s, 9H).

To a solution of 5-*tert*-butylisoxazole-3-carboxylic acid (70 mg, 0.41 mmol) and 3-chloroaniline (53 mg, 0.41 mmol) in 5 mL of DCM, HBTU (395 mg, 1.23 mmol) and DIPEA (271 mg, 2.1 mmol) were added. The mixture was stirred at rt for 18 h. The mixture was washed with 1 N Na_2SO_4, saturated $NaHCO_3$ and brine (5 mL each). After drying over anhydrous Na_2SO_4, the solution was concentrated and purified with silica gel column (Hexane/EtOAc=10/1 to 7/1) to obtain **55** (54 mg, 51%) as a colorless oil. HPLC purity 99.6% (t_R = 21.11 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.58 (s, 1H), 7.81 (t, J = 2.0 Hz, 1H), 7.50 (ddd, J = 8.1, 2.1, 1.0 Hz,

1H), 7.30 (d, *J* = 7.8 Hz, 1H), 7.16 (ddd, *J* = 8.0, 2.0, 1.0 Hz, 1H), 6.52 (s, 1H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.8, 158.3, 157.1, 138.1, 134.8, 130.1, 124.9, 120.1, 118.0, 98.4, 33.1, 28.8. HRMS (ESI) calcd for $C_{14}H_{16}ClN_2O_2$ 279.0900 $(M + H)^+$, found 279.0895.

(*E*)-5-(*tert*-Butyl)-*N*'-(3-chlorobenzylidene)isoxazole-3-carbohydrazide (56). Ethyl 5-(*tert*-butyl)isoxazole-3-carboxylate **4a** (100 mg, 0.5 mmol) and NH_2NH_2 (76 mg, 1.5 mmol) were dissolved in 5 mL EtOH and refluxed for 2 h. 3-Chlorobenzaldehyde was then added and the solution was allowed to stir at rt overnight. Then the solution was concentrated and extracted with DCM (10 mL × 3). After drying over anhydrous Na_2SO_4, the solution was concentrated and purified with silica gel column (Hexane/EtOAc = 20/1 to 10/1) to obtain **56** (89 mg, 61% for two steps) as a white solid (mp 157-159 ^{o}C). HPLC purity 99.5% (t_R = 19.74 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.88 (s, 1H), 8.23 (s, 1H), 7.84 (d, *J* = 1.7 Hz, 1H), 7.70 – 7.60 (m, 1H), 7.45 – 7.32 (m, 2H), 6.58 (s, 1H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 183.6, 157.4, 155.5, 147.6, 135.1, 135.0, 130.8, 130.0, 127.5, 126.1, 98.8, 33.1, 28.8. HRMS (ESI) calcd for $C_{15}H_{17}ClN_3O_2$ 306.1009 $(M + H)^+$, found 306.1005.

3-(5-*tert*-Butylisoxazol-3-yl)-4-((3-chlorophenyl)diazenyl)-1*H*-pyrazol-5-amine (57). To the solution of **1** (90 mg, 0.27 mmol) in EtOH (4 mL) was added hydrazine hydrate (15 µL, 0.36 mmol). The reaction mixture was refluxed for 6 h, the solvent was evaporated and the crude product was crystallized from dichloromethane to yield **57** as a yellow solid (mp 230-232 ^{o}C) in 64% yield (60 mg). HPLC purity 99.2% (t_R = 21.18 min). ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.45 (s, 1H), 7.83 – 7.79 (m, 1H), 7.74 (d, *J* = 8.0 Hz, 1H), 7.51 (t, *J* = 7.9 Hz, 1H), 7.40 (m, 3H), 6.63 (s, 1H), 1.38 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 180.6, 156.6, 154.8, 141.3, 140.5, 134.2, 131.2, 128.0, 121.9, 120.6, 120.6, 99.0, 32.8, 29.0. HRMS (ESI) calcd for $C_{16}H_{18}ClN_6O$ 345.1231 $(M + H)^+$, found 345.1229.

Molecular Docking Protocol. The substituted 2-(isoxazol-3-yl)-2-oxo-*N*'-phenyl-acetohydrazonoyl cyanide analogues were docked with EPAC2 using the X-ray structure of EPAC2 (PDB code: 3CF6) and AutoDock 4.0. Water molecules and the ligand (Sp-cAMPS) within the crystal structure were removed and polar hydrogens were added using AutoDockTools. In the structures of the analogues, all bonds were treated as rotatable except for the aromatic, amide, cyano and double bonds, whereas the protein was treated as rigid. Docking runs were carried out using the standard parameters of the program for interactive growing and subsequent scoring, except for the parameters for setting grid box dimensions and center. For all of the docking studies, a grid box size of 60 × 60 × 60 Å, centered at coordinates 4.995 (x), -14.957 (y), and -60.351 (z) of the PDB structure. The binding affinities of the **47** output structures ranged from -10.61 to -8.64 kcal/mol.

Determination of Solubility. Solubility in water or ethanol for **25**, **28**, **31**, **35**, **36**, and **47** was determined by HPLC analysis according to a previously published protocol.[68-70] First, 1-2 mg of **25**, **28**, **31**, **35**, **36**, and **47** were weighed and added to 1 mL of water, respectively. Then, 10-15 mg of **25**, **28**, **31**, **35**, **36**, and **47** were weighed and added to 0.1-0.6 mL of ethanol, respectively. The suspensions were shaken at 25 °C for 24 h and then centrifuged, and the supernatants were filtered. Aliquots (5 μL) of the supernatants were injected into the HPLC system equipped with a C18 reverse-phase column under the same condition which was described in the general Experimental Section. One-point calibration[71] was done by injecting 5 μL aliquots of the corresponding buffer solutions of **25**, **28**, **31**, **35**, **36**, or **47** with known concentrations.

Biological Evaluation Methods. Protein Expression and Purification. Recombinant full-length human EPAC1, mouse EPAC2 and C-terminal truncated Rap1B(1-167) were purified as previously described.[72, 73]

8-NBD-cAMP Competition Assay. The 8-NBD-cAMP competition assay was performed in 96-well microplates from Corning Costar (Cambridge, MA, USA) as previously described [36]. Reaction mix containing 50 nM EPAC2 and 60 nM 8-NBD-cAMP in 20 mM Tris-HCl pH 7.5 buffer, with 150 mM NaCl, 1 mM EDTA and 1 mM DDT, was dispensed into 96-well plate. Test compounds were added to the reaction mix at different doses. The fluorescence intensity signals from the 8-NBD probe before and after the addition of test compounds were measured using a SpectaMaxM2 microplate reader (Molecular Devices, Silicon Valley, CA, USA) with excitation/emission wavelengths set at 470/540 nm. Lastly, fluorescence intensity was again monitored after the addition of cAMP at a saturated final concentration of 300 μM. Data were presented by nominalizing the observed fluorescence intensity (F) with the initial florescence signal (F_0) before the addition of compound and final florescence signal after cAMP addition (F_{cAMP}) using equation: $Relative\ fluorescence = (F - F_{cAMP})/(F_0 - F_{cAMP}) \times 100$.

***In Vitro* Guanine Nucleotide Exchange Factor (GEF) Activity Assay of EPAC1.** In vitro EPAC1 GEF activity was acquired as previously described.[39] Briefly, the assay was performed using 500 nM Rap1b-BODIPY-GDP and 200 nM EPAC1 in buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 50 mM GDP and the indicated concentrations of test compounds at room temperature using half-area 96-well plates (Corning Costar 3915). The exchange reaction was monitored using a Spectramax M2 Plate Reader (Molecular Devices) with the excitation/emission wavelengths set at 485/515 nm. The reaction rate constant (k_{obs}) was determined by globally fitting the experimental data to a single

exponential equation. Quantification was processed by nominalizing the observed k_{obs} in the presence of inhibitor with the rate constant in the presence of 20 μM cAMP (no inhibitor) (k_{cAMP}) and the rate constant without cAMP or inhibitor (k_0) using equation: *Relative GEF activity* = $(k_{obs} - k_0)/(k_{cAMP} - k_0) \times 100$.

ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

EPAC, exchange proteins directly activated by cAMP; SAR, structure-activity relationship; cAMP, cyclic adenosine monophosphate; 8-NBD-cAMP, 8-(2-[7-nitro-4-benzofurazanyl]aminoethylthio)adenosine-3',5'-cyclic monophosphate; GDP, guanosine diphosphate; PKA, protein kinase A; FRET, fluorescence resonance energy transfer; GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; Rap, Ras-related protein; HTS, high-throughput screening; 007-AM, 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate acetoxymethyl ester; TLC, thin layer chromatography; UV, ultraviolet; TMS, tetramethylsilane; HRMS, high-resolution mass spectrometry; HPLC, high-performance liquid chromatography; DCM, dichloromethane; EtOAc, ethyl acetate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; DDT, dichlorodiphenyltrichloroethane; ADP, adenosine diphosphate; NADH, nicotinamide adenine dinucleotide; PEP, phospho(enol)pyruvate; H89, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride; CBD, cAMP binding domain; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum

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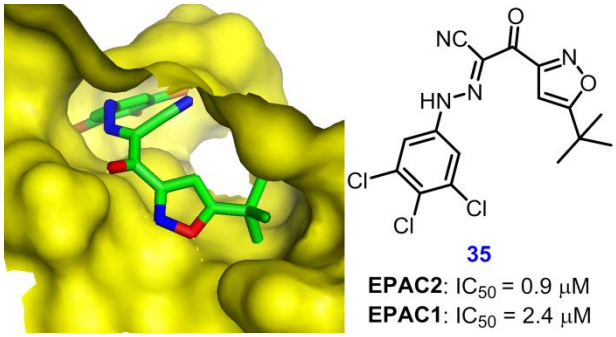
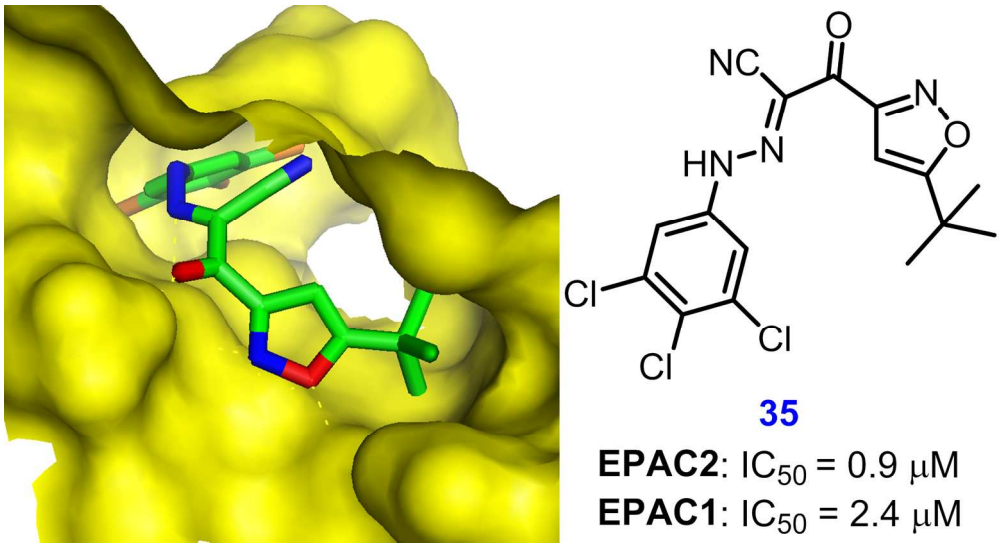
Table of Contents (TOC) Graphic





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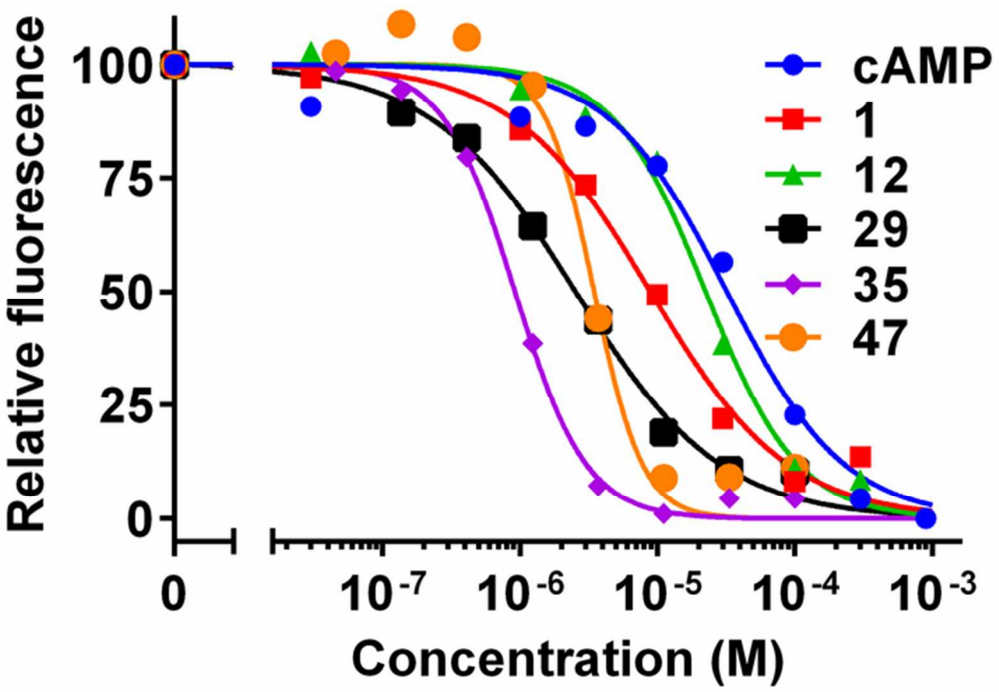

Figure 3
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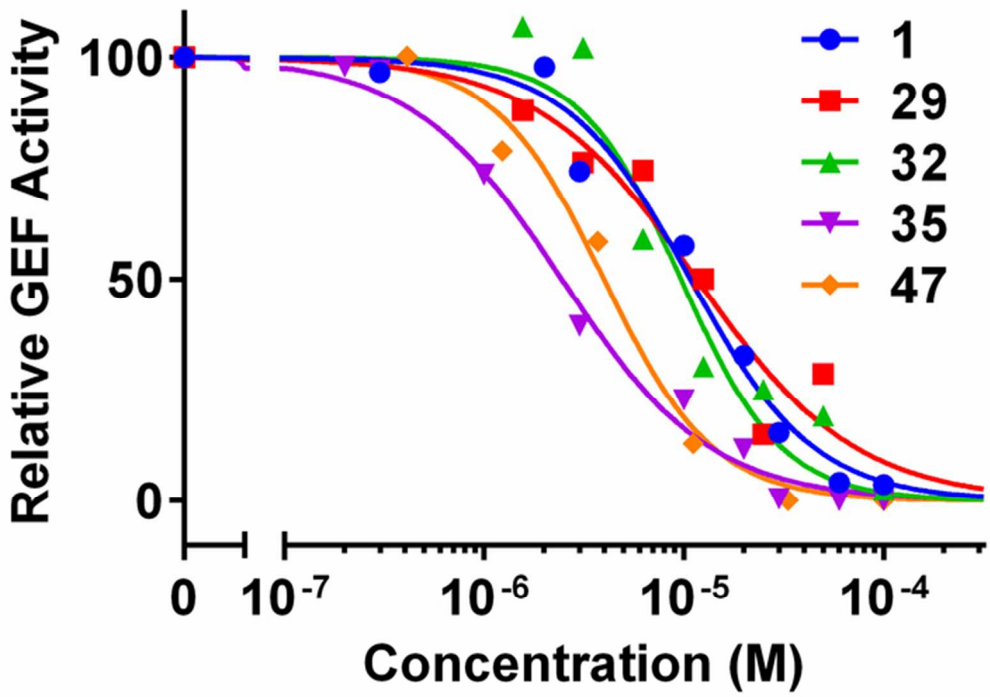

Figure 4
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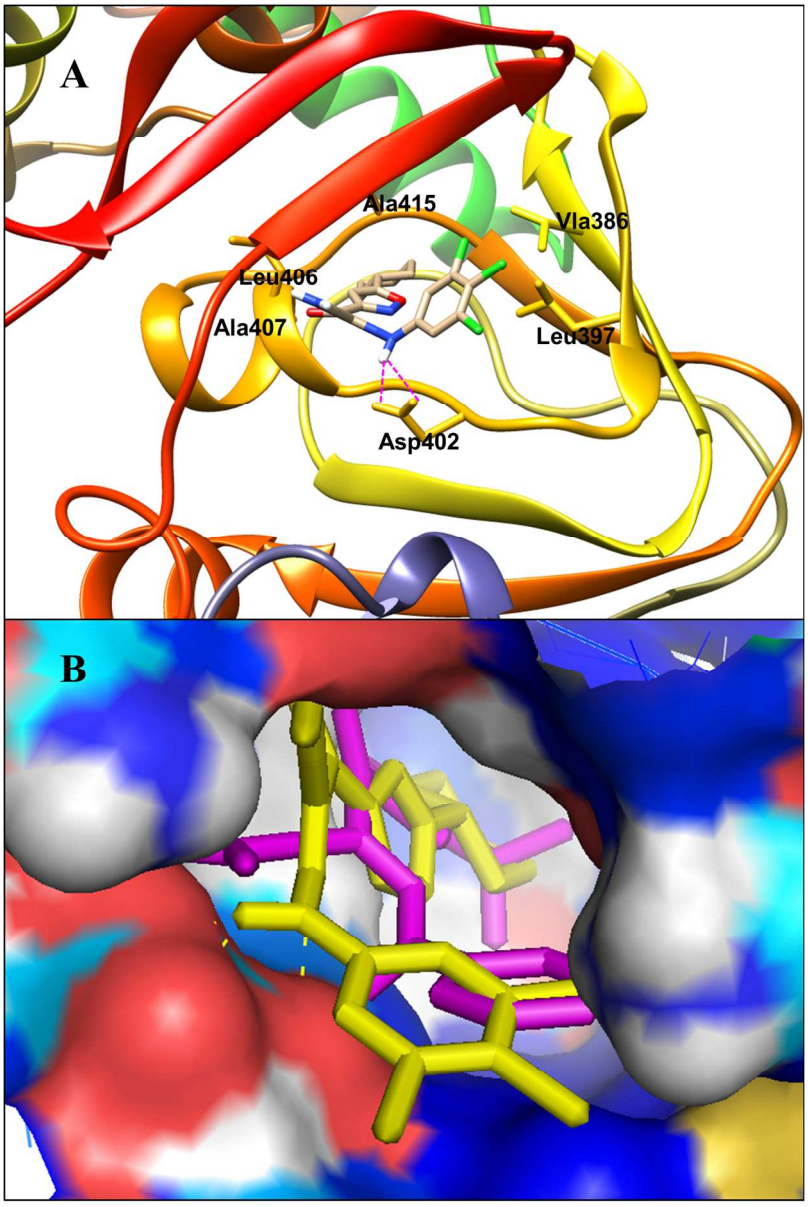

Figure 5
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