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Optimization of the anti-cancer activity of the phosphatidylinositol-3 kinase pathway inhibitor PITENIN-1: switching thiourea with 1,2,3-triazole†

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We previously reported encouraging *in vitro* and *in vivo* anti-cancer activity of *N*-((3-chloro-2-hydroxy-5-nitrophenyl)carbamothioyl)benzamide (termed PITENIN-1). In the current work, we describe the structure–activity relationship study of the PIT-1 series, based on the replacement of a central thiourea unit with 1,2,3-triazole, which leads to increased liver microsomal stability, drug likeness and toxicity towards cancer cells.

Overactivation of PI3K signaling is a common feature of many types of human cancer.[1] Increased activation of a variety of PI3K effectors, through their binding to the PI3K product, lipid PIP3, provides multiple growth, survival, migration and metabolic advantages to cancer cells. Not surprisingly, small molecule targeting of the PI3K pathway, including direct inhibition of PI3K isoforms as well as its multiple effectors (Akt, mTOR, PDK1, *etc.*) has attracted major attention. While many approaches focused on targeting enzymatic activities in the PI3K network, we and others have recently described a new approach aimed at targeting a universal central step in PI3K signal transduction, *i.e.* binding of PIP3 to PH domains of effector proteins.[2] In particular, we have developed two new classes of small molecule antagonists of PIP3, termed PIT-1 [*N*-((3-chloro-2-hydroxy-5-nitrophenyl)carbamothioyl)benzamide] and PIT-2 [(*Z*)-5-(2-benzyl-5-hydroxy-4-nitrobenzylidene)-2-thioxothiazolidin-4-one] (Fig. 1).[3,4] These two structurally dissimilar molecules displayed very similar activities in cancer cells, including induction of apoptosis and metabolic stress and inhibition of cell migration and invasion. Furthermore, both PIT-1 and PIT-2 displayed synergistic toxicity with TRAIL in human glioblastoma U87MG cells. These activities have been linked to the inhibition of Akt signaling and actin remodeling by ARF6, two pathways regulated by PI3K.[3,4] These *in vitro* activities of PITs translated into the significant inhibition of tumor growth and lung metastasis formation in 4T1 and B16-F10 syngeneic xenograft models by the dimethyl analog of PIT-1.[3,4]

Despite the promising initial results, PITs displayed obvious limitations, including high micromolar activity as well as multiple non-drug-like features. In particular, nitrophenyl and thiourea moieties of PIT-1 represent potential toxicity concerns and metabolic liabilities. Initial analysis of the PIT-1 series revealed surprisingly specific SAR for a micromolar compound suggesting several changes to the molecule, leading to some increase in activity and changes in targeting different PH domains.[3,4] In particular, the addition of two methyl groups to the phenyl ring in PIT-1 (DM-PIT-1, Fig. 1) resulted in some increase in activity and improved incorporation into long-circulating PEG–PE micelles for *in vivo* delivery.[3,5] Furthermore, changes to the nitrophenyl ring were identified. However, these results did not address the main limitations of the PIT-1 series, which was the target of our current work.

In the processes of refining the initially identified structural scaffold of the PIT-1, our first concern was to replace the susceptible thiourea unit with a stable bioisostere.[6] The 1,2,3-triazole structural motif has attracted our attention in this regard considering the fact that the triazole is a safe

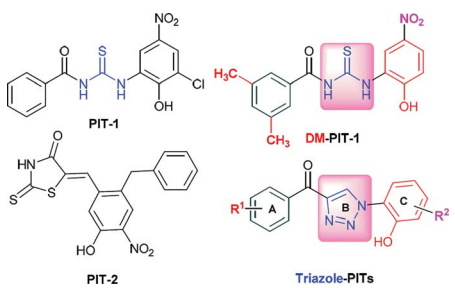

Fig. 1 Structures of the two classes (PIT-1 and PIT-2) of inhibitors of the phosphatidylinositol-3 kinase (PI3K) signaling pathway, termed PITENINs (PITs) and the newly designed 2nd generation triazole-PITs.

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[b]*Department of Developmental, Molecular and Chemical Biology, Tufts University, 136 Harrison Avenue, Boston, MA 02111, USA. E-mail: alexei.degterev@tufts.edu*

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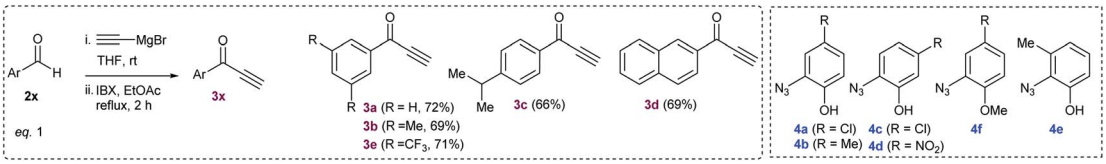
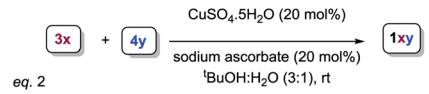
† Electronic supplementary information (ESI) available: Synthesis and characterization data and spectra of all the new compounds. See DOI: 10.1039/c4md00109e



Scheme 1 Synthesis of alkynones **3a–e** (eqn (1)) and their Cu-catalyzed [3 + 2]-cycloaddition (eqn (2)) with selected 2-azidophenols **4a–e**.

Table 1 The synthesis of and screening results with 1,4-disubstituted 1,2,3-triazoles[a]



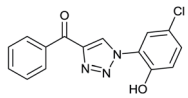
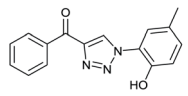
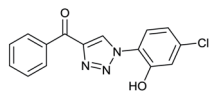
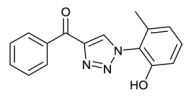
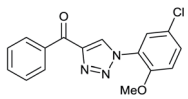
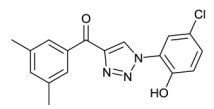
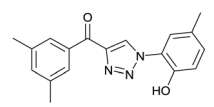
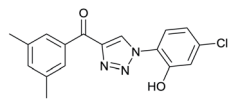
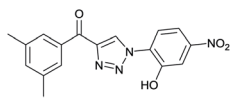
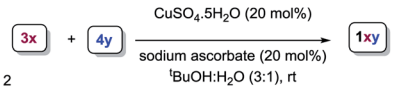
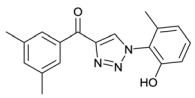
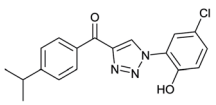
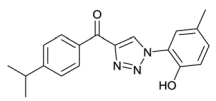
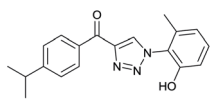
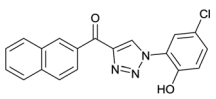
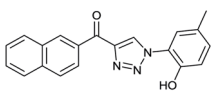
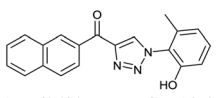
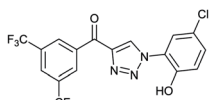
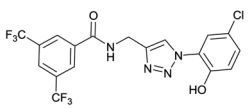
S. No	Triazole (yield, code)	EC_{50} (μmol) Alone	With TRIAL[b]
1	 **1aa** (87%, YK-NCL-176)	103.6	51.6
2	 **1ab** (91%, YK-NCL-184)	>100	>100
3	 **1ac** (87%, YK-NCL-191)	48.9	42.3
4	 **1ae** (84%, YK-NCL-185)	8.1	8.0
5	 **1af** (82%, YK-NCL-178)	>100	>100
6	 **1ba** (83%, YK-NCL-190)	57.8	30.6
7	 **1bb** (85%, YK-NCL-193)	>100	>100
8	 **1bc** (91%, YK-NCL-183)	>100	64.4
9	 **1bd** (86%, YK-NCL-194)	89.1	70.5

Table 1 (*Contd.*)

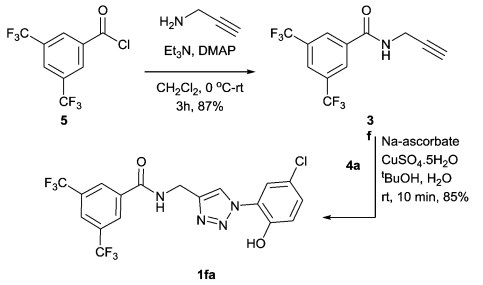


S. No	Triazole (yield, code)	EC_{50} (μmol) Alone	With TRIAL[b]
10	 **1be** (91%, YK-NCL-192)	104.0	98.4
11	 **1ca** (86%, YK-NCL-195)	64.1	41.6
12	 **1cb** (83%, YK-NCL-197)	>100	>100
13	 **1ce** (82%, YK-NCL-196)	>100	>100
14	 **1da** (87%, YK-NCL-186)	>100	>100
15	 **1db** (91%, YK-NCL-188)	>100	>100
16	 **1de** (84%, YK-NCL-187)	85.2	74.9
17	 **1ea** (82%, YK-NCL-240)	11.99	3.15
18	 **1fa** (85%, YK-NCL-234)	16.2	14.0

[a] Experiments were performed in A2780 cells as described in the ESI.
[b] TRIAL – tumor necrosis factor-related apoptosis-inducing ligand.

bioequivalent surrogate for the amide bond and that this concept has found its application in the area of anticancer agents as well as in developing non-nucleoside reverse transcriptase inhibitors.[7,8] As shown in Fig. 1, triazole-PITs have been designed as the structural mimics of DM-PIT-1 and as potential second-generation PITenins (PITs), in anticipation of better antitumor activity.

Scheme 1 reveals the salient features of the synthesis of the newly designed triazole PITENINS. The alkynone precursors **3a–e** were prepared by following a two-step sequence consisting of the addition of ethynylmagnesium chloride to corresponding aldehydes **2a–e** and subsequent IBX oxidation. The azidophenols **4a–f** were synthesized from the corresponding aminophenols by following the standard azidation procedures and are used immediately. The copper catalyzed [3 + 2] cycloaddition reaction of alkynones **3** with azidophenols **4** was carried out under established click reaction conditions (20 mol% $CuSO_4$, 20 mol% Na-ascorbate in *tert*-BuOH–water, at rt) to obtain the requisite 1,2,3-triazole PITENINS.[9] Table 1 summarizes the details of the compounds synthesized. All the new compounds have been characterized completely with the help of spectral and analytical data.



Scheme 2 Synthesis of triazole **1fa**.

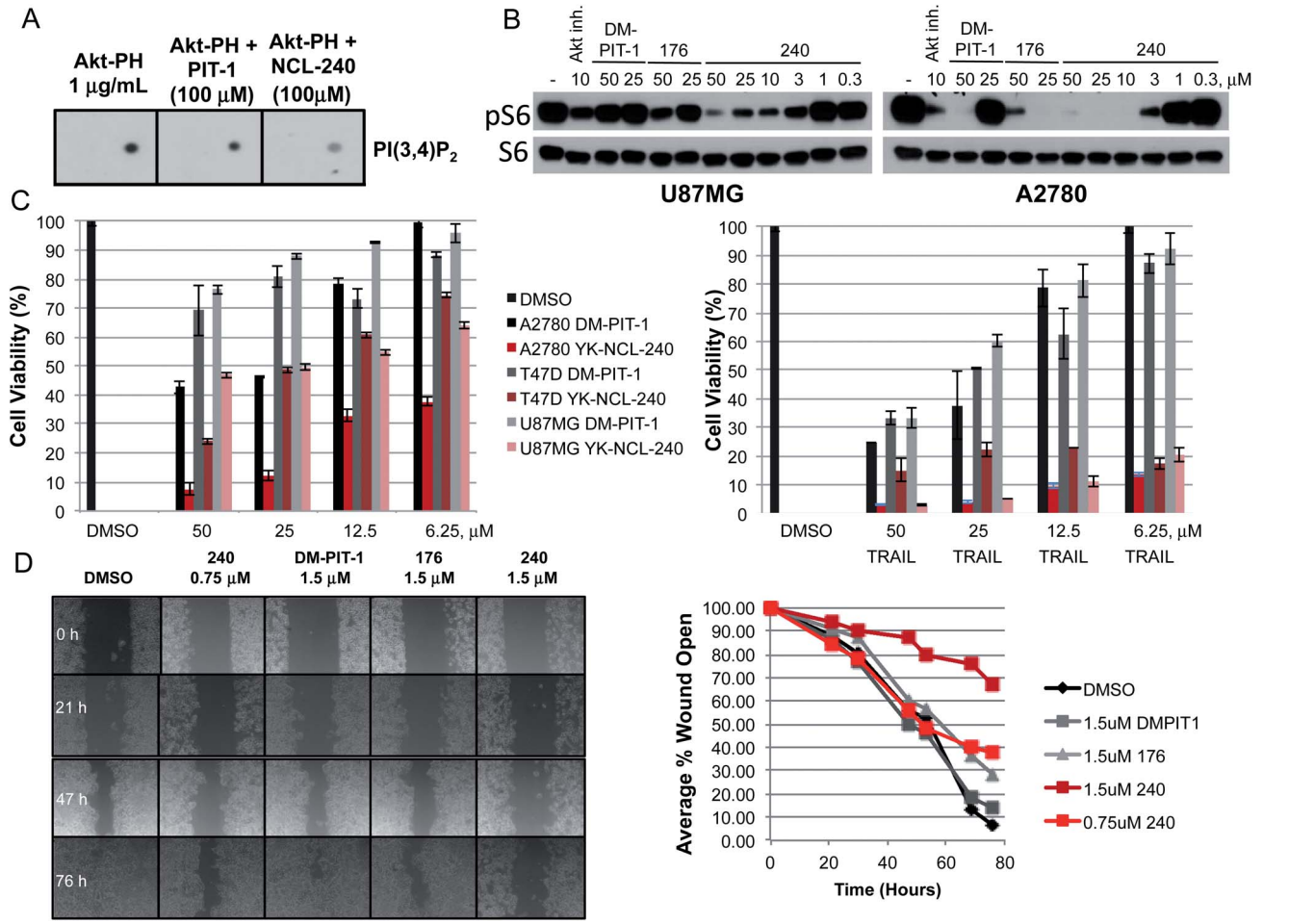


Fig. 2 Increased activity of **1ea**. (A) Increased inhibition of PI(3,4)P2/Akt-PH domain binding by **1ea**. Lipid overlay experiment was performed as described in ref. 3. (B) Inhibition of S6 phosphorylation by **1ea**. U87MG or A2780 cells were treated with indicated concentrations of inhibitors for 7 h, followed by Western blotting using phospho-S6 and total S6 antibodies. (C) Increased cytotoxicity of **1ea** compared to DM-PIT-1 in multiple cell types. Cells were treated with indicated concentrations of inhibitors alone or in combination with 1 μg mL^{-1} TRAIL for 24 h. Cell viability was determined using the CellTiter-Glo assay. (D) **1ea** efficiently blocks the migration of A2780 cells. Wound healing assay was performed as previously described.[4] Cell monolayers were treated with indicated concentrations of inhibitors, followed by a scratch wound. The size of the cell free area was photographed at indicated periods of time. Quantification of the open wound area is shown on the right.

The initial screening of **1aa** against growth of A2780 human ovarian cancer cells revealed that **1aa** has comparable activity with the corresponding PIT-1 analogue.[10] In addition, the corresponding methyl ether **1af** was completely inactive consistent with the importance of the free –OH group in the original thiourea series.[3] In this course of SAR studies, we have installed the dimethyl groups and isopropyl groups on the ring A along with variable functional groups on the ring C. However, this approach was not effective. Replacing the aryl ring A with a naphthyl unit has been also examined, albeit with no improvement in the activity. In contrast, we found that the addition of the $-CF_3$ group at the C-3 and C-5 positions on ring A in combination with a chloro group at the C-4 position of the ring C (**1ea**) showed excellent improvement (20 times higher than that of the DM-PIT-1) in anti-tumor activity.

In order to examine whether the addition of an extra hydrogen bond donor in between the triazole and ring A will have any effect, the compound **1fa** was synthesized (Scheme 2). The compound **1fa** has shown good activity, which, however is lower than that of **1ea**.

Having examined a wide range of analogs, compound **1ea** was selected for further progress in the direction of examining its pharmacological properties. The mode of activity of **1ea** has been studied by the lipid overlay assay, measuring the binding of the AktPH domain to PI(3,4)P2 spotted on the nitrocellulose membrane, as previously described.[3] Compound **1ea** displayed substantially higher activity compared to PIT-1 (Fig. 2A). We further examined the inhibition of PI3K/Akt signaling in human glioblastoma U87MG and ovarian carcinoma A-2780 cells. Interestingly, we observed very robust inhibition of the TORC1/p70S6K/S6 pathway downstream from Akt, which correlated with the cytotoxicity of the compounds and suggested that the increased activity of **1ea** translated into more specific inhibition of a particular pathway downstream from PI3K/Akt (Fig. 2B). Other targets of the compound **1ea** in the PI3K/Akt signaling pathway remain to be fully elucidated in the future.

Subsequently, the cytotoxicity of new PIT analogs in three different cancer cell lines (U87MG, A2780 and T47D) has revealed that **1ea** displayed the highest activity in all cases (Fig. 2C). In addition to activation of cell death, PITs displayed several additional important properties. First, we found that PIT-1 analogs reverse the resistance of cancer cells to anti-cancer cytokine TRAIL. This led to synergistic cytotoxicity of PIT-1 and TRAIL when applied in combination.[3] This useful property was retained with **1ea** (Fig. 2C and Table 1). Second, PIT-1 analogs not only increased the cell death, but also suppressed the cell migration and invasion through the attenuation of actin cytoskeleton remodeling.[4] Consistently, **1ea** displayed significantly increased activity in the cell migration assay (Fig. 2D).

Finally, one of the major goals of our study was to improve the pharmacological properties of PIT-1/DM-PIT-1. DM-PIT-1 displayed $T_{1/2} = 1.8$ min (CL = 1262 μL min^{-1} mg^{-1}) in mouse liver microsomal stability assay *in vitro*. Compound **1ea** displayed $T_{1/2} = 119$ min (CL = 19.4 μL min^{-1} mg^{-1}) in the same assay. Pharmacokinetic analysis following i.v. injection of 1 mg kg^{-1} of the drug showed reasonable $T_{1/2} = 3.22$ h with CL of 930 mL h^{-1} kg^{-1} and bioavailability of 85.1% following i.p. administration. Furthermore, **1ea**, unlike DM-PIT-1, complied with 4 out of 5 Lipinski rules, with the exception of liphophilicity (calculated log $P = 5.3$). Overall, our SAR analysis describes a new analog of PIT-1 with significantly improved anti-cancer activity and pharmacological properties, which may present a promising molecule for further analysis in mouse xenograft models.

Conclusions

In summary, a structure–activity relationship (SAR) study of the *N*-((3-chloro-2-hydroxy-5-nitrophenyl)carbamothioyl)benzamide (PIT-1) series revealed that increased liver microsomal stability and toxicity towards cancer cells could be achieved by replacing the central thiourea unit with the 1,2,3-triazole heterocycle unit. Secondly, the nitro group of the nitrophenyl moiety can be replaced with chlorine, removing another potential liability. Finally, the addition of two trifluoromethyl moieties to the second phenyl ring of the molecule further increased the activity, but also resulted in increased lipophilicity. Overall, a derivative incorporating all of the modifications (*i.e.* **1ea**) displayed good stability *in vitro* and *in vivo* and significantly increased the toxicity against a number of cancer cell lines alone and also in combination with TRAIL. Interestingly, compound **1ea** displayed particularly robust activity in inhibiting the mammalian Target Of Rapamycin Complex 1 (TORC1) signaling downstream from Akt. The mechanism of this inhibition is currently under investigation.

Acknowledgements

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