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Synthesis and Characterization of Novel Isoform-Selective IP6K1 Inhibitors

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Abstract

Inositol hexakisphosphate kinases (IP6Ks) have been increasingly studied as therapeutically interesting enzymes. IP6K isoform specific knock-outs have been used to successfully explore inositol pyrophosphate physiology and related pathologies. A pan-IP6K inhibitor, *N*2-(m-trifluorobenzyl)-*N*6-(p-nitrobenzyl) purine (TNP), has been used to confirm phenotypes observed in genetic knock-out experiments; however, it suffers by having modest potency and poor solubility making it difficult to handle for *in vitro* applications in the absence of DMSO. Moreover, TNP's pan-IP6K inhibitory profile does not inform which IP6K isoform is responsible for which phenotypes. In this report we describe a series of purine-based isoform specific IP6K1 inhibitors. The lead compound was identified after multiple rounds of SAR and has been found to selectively inhibit IP6K1 over IP6K2 or IP6K3 using biochemical and biophysical approaches. It also boasts increased solubility and IP6K1 potency over TNP. These new compounds are useful tools for additional assay development and exploration of IP6K1 specific biology.

Keywords

Inositol hexakisphosphate kinase; Inositol pyrophosphate; Selective enzyme inhibitors; Structure activity relationships; Kinases

Inositol pyrophosphates (1PP-IP_5, 5PP-IP_5, 1,5PP-IP_4, Figure 1) are becoming recognized as important signaling molecules that may help regulate cellular metabolism and energy levels.[1–3] The intercellular pool of 5PP-IP_5 is formed from inositol hexakisphosphate (IP_6, phytic acid) by a family of three inositol hexakisphosphate kinases (IP6K1, IP6K2, and IP6K3). These kinases utilize ATP to form a high-energy pyrophosphate bond when they convert IP_6 to 5PP-IP_5. 5PP-IP_5 is thought to integrate with a number of cellular processes including signal transduction, vesicular trafficking, apoptosis, and metabolism.[4]

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

All three IP6K isoforms catalyze the same reaction; however, global genetic knock out experiments have uncovered unique phenotypes associated with each isoform. IP6K1 knock out mice show metabolic changes and resistance to diet-induced obesity and fatty liver.[5,6] IP6K2 knock out mice are sensitive to carcinogen-induced tumor formation,[7] and IP6K3 knock out mice are resistant to age-induced weight gain and have an increased lifespan.[8] IP6K1 (neuronal migration),[9] IP6K2 (Purkinje cell morphology and cerebellar synapse formation),[10] and IP6K3 (synapse formation)[11] have also been implicated with neuronal functions. Each isoform has differential cell-type expression,[8] sub-cellular localization,[12] and protein-protein interactions[13–16] that allow for the different phenotypes observed. Due to the importance of protein-protein interactions in maintaining normal cellular processes it is unclear if phenotypes observed in knock-out studies are fully driven by decreasing 5PP-IP_5 production or if the loss of protein-protein interactions and scaffolding have a significant effect.

Nearly a decade ago *N*2-(m-trifluorobenzyl)-*N*6-(p-nitrobenzyl) purine (TNP, compound **1**, Table 1) was discovered as a pan-IP6K inhibitor.[17] Since then TNP has been used in a number of studies to examine the effects of 5PP-IP_5 in various contexts (see[4] for a comprehensive review). In particular, Ghoshal et al. demonstrated that mice treated with TNP were resistant to high-fat diet induced weight gain while remaining sensitive to glucose and insulin.[18] While TNP has proved itself a useful tool, it can certainly be improved -- particularly in terms of potency, solubility, and isoform selectivity in order to better understand inositol pyrophosphate biology. TNP is reported to have low micromolar potency against the IP6K family,[17,19,20] however, it is sparingly soluble in aqueous buffers and is thus difficult to work with *in vitro* without precipitation and difficult to formulate for *in vivo* animal studies. Finally, TNP does not preferentially inhibit one IP6K isoform over the others. Therefore, it is not possible to use TNP to study the effects of one IP6K isoform in a context where the others are also present.

Puhl-Rubio and colleagues have identified a number of IP6K2 inhibitors after screening a library of kinase-focused compounds.[20] Gu et al. have also recently reported a series of flavonoids as inositol pyrophosphate kinase inhibitors.[21] However, we adopted a medicinal chemistry approach to identify IP6K1 specific inhibitors by systematically varying the *N*2 and *N*6 positions off of the purine core.

A simple two-step SNAr reaction depicted in Scheme 1 from 6-chloro-2-fluoro purine allows for rapid exploration of the 6- and 2- positions on the purine core. Initial efforts were spent exploring the *N6* position to determine if the nitrobenzyl moiety present on TNP was essential for compound potency (Table 1). The nitrobenzyl group is undesirable because it is capable of forming toxic metabolites *in vivo* and reduces compound solubility.[22] In this study, all compounds were tested in the Promega ADP-Glo Max assay optimized for IP6K performance as described previously.[23] Briefly, the assay is conducted with physiologically relevant concentrations of ATP (1 mM) that is consumed as the kinase reaction progresses over time. After a time, the reaction is stopped, the remaining ATP is enzymatically depleted, and the ADP produced is converted back to ATP with a recycling reaction. This regenerated ATP reacts with luciferin/luciferase to produce a bioluminescent signal proportional to IP6K activity. In this assay, TNP performance is inconsistent with previously

published work presumably due to the immense hydrophobic nature of the inhibitor causing it to precipitate out of the assay. Nitrobenzyl replacement with benzyl (**4)** or phenethyl (**5)** moieties caused a dramatic decrease in potency compared to the published value for TNP. A methoxyethyl (**6)** substitution produced a soluble compound capable of completely inhibiting IP6K1, albeit very weakly with a pIC_{50} of 3.93.

The *N*6-methoxyethyl substitution was initially retained for solubility while we attempted to regain potency at the 2-position (Table 2). Replacing the *m*CF_3-benzyl (**6)** with phenethyl (**7**) resulted in a modest increase in potency. Removing electron density (**8**) from the phenyl ring did not increase potency while adding electron density (**9**) increased potency from pIC_{50} 4.10 to 4.95. Large electron-rich aromatic rings (indole, **10**) at the 2-position further increase potency to pIC_{50} 5.08 while large hydrophobic aromatic rings are less active (napthyl, **11**). A smaller imidazole group (**12**) similarly abolishes potency.

Using compound **10** as a baseline, we set out to explore how essential the hydrogen bond donors in the molecule are by alkylating the NH groups with a methyl cap. We found that alkylating any of the exocyclic NHs abolished potency and 1-methyltrytamine dramatically reduced it (Supplementary Data Table 1). Likewise, purine *N*7 and *N*9 to carbon substitutions abolished potency. These data suggest that there is an intricate hydrogen bond network in the IP6K1 active site.

We next explored the chemical space surrounding the tryptamine (Table 3). Generally, substitutions at the 5 position on the indole are not tolerated (**13–16**). Substitutions at the 2 and 6 positions increase potency (**17, 18**). Increasing the linker length from 2 to 3 carbons (**19**) dramatically decreases potency and constraining free rotation of the tryptamine abolishes potency (data not shown).

After achieving low micromolar potency with compounds **17** and **18** by optimizing the *N*2 position off of the purine core, we returned to the *N*6 position to see if nitro replacements (relative to TNP) exist which are more potent than methoxyethylamine (Table 4). Benzoxadiazole (**20**), 4-cyanobenzyl (**22**), 4- and 5-methyl-2-pyridone (**21, 23**) derivatives were synthesized and tested. Compound **23** increased potency from pIC_{50} 5.08 to 5.76 over the parent compound **10**. This 5-methyl-2-pyridone substitution was then combined with the more potent *N*2–6-methoxytryptamine or *N*2–2-methyltryptamine derivatives to yield compounds **24** and **25**. *N*2–2-Methyltryptamine (**25**) is equipotent to unsubstituted tryptamine (**10**); however, *N*2–6-methoxytryptamine (**24**) is additive with 5-methyl-2-pyridone yielding for the first time a sub-micromolar IP6K1 inhibitor with a pIC_{50} of 6.13.

Compounds **1** (TNP), **10**, **17**, and **24** were selected for confirmation of binding to IP6K1 in an orthogonal biophysical thermal melt assay. In this assay purified IP6K1 was incubated over a temperature gradient in the presence of different concentrations of purine analogs. Compounds that bind tightly to IP6K1 will increase the protein melting temperature to a greater degree. Here, we observe good correlation between IC_{50} values obtained in the biochemical assay and an increase in melting temperature. As expected, compound **24** was able to increase the melting temperature to a greater degree than the other compounds at 11.1μM (Table 5). Moreover, compound **24** has a greater ΔT_{mMax} than the other compounds

tested, including TNP, indicating greater stabilization. Taken together with the biochemical data, these data suggest that compound **24** inhibits IP6K1 both enzymatically as well physically interacts with the enzyme in a meaningful way.

In order to examine IP6K isoform selectivity we tested compound **24** against purified IP6K2 and IP6K3 in the ADP-Glo Max assay (Table 6). We were gratified to find that compound **24** was ~25 and 50 fold more selective for IP6K1 over IP6K2 and IP6K3, respectfully, after converting observed IC_{50} values into K_i values with the Cheng-Prusoff equation.[24] These results suggest that there are distinct enough differences in the IP6K family to allow for subtype specific inhibitors to be developed.

Compound **24** was broadly profiled for off-target kinase inhibition in a diversity panel at Eurofins. This screen includes a number of kinases from different families and signaling pathways (see Supplementary Data Table 2). Compound **24** was tested at 10μM against 58 kinases and found to potently inhibit 4 kinases, 3 of which are part of the RAF/MAPK pathway in addition to casein kinase γ. The remaining 54 kinases were not significantly inhibited by compound **24**. Importantly, this panel included a number of metabolically important enzymes including GSK3β, AKT, mTOR, and PI3K. 5PP-IP_5 is thought to interact intimately with the PI3K/AKT/GSK3β pathway.[5] As such, it is important that tools that manipulate 5PP-IP_5 concentration do not inhibit these enzymes or the results may be confounded and difficult to interpret. These profiling results suggest that, surprisingly, the purine-based compound **24** is a relatively selective IP6K1 inhibitor against the rest of the kinome.

In summary, given the increasing body of evidence that IP6Ks and inositol pyrophosphates play an important role in cellular processes and pathologies, we became interested in developing IP6K specific inhibitors to serve as pharmacological compliments to the published genetic knock out models. These chemical tools will help deconvolute inositol pyrophosphate signaling and inform what each IP6K isoform is contributing to a particular phenotype. Our approach to systematically vary the *N*2 and *N*6 positions of TNP have uncovered compound **24** which boasts a number of improvements over TNP (Table 7). Primarily, compound **24** has sub-micromolar potency against IP6K1, it is 25 to 50-fold selective against IP6K2 and IP6K3, respectively, in addition to being inactive against 54/58 kinases tested in a diversity panel. Moreover, compound **24** eschews the nitrobenzyl moiety and possesses meaningful aqueous solubility compared to TNP, thus enabling *in vitro* experiments and *in vivo* formulations. We propose that compound **24** will be a useful tool to evaluate IP6K1-specific activity in physiological and pathological models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights:

- Structure-activity relationships based on a nonselective lead revealed compound **24** as a selective inhibitor of IP6K1.
- Compound **24** has good selectivity over closely related kinases IP6K2 and IP6K3 as well as a panel of other diverse kinases.
- Compound **24** is a useful tool to investigate the role of IP6K1 selective inhibitors.

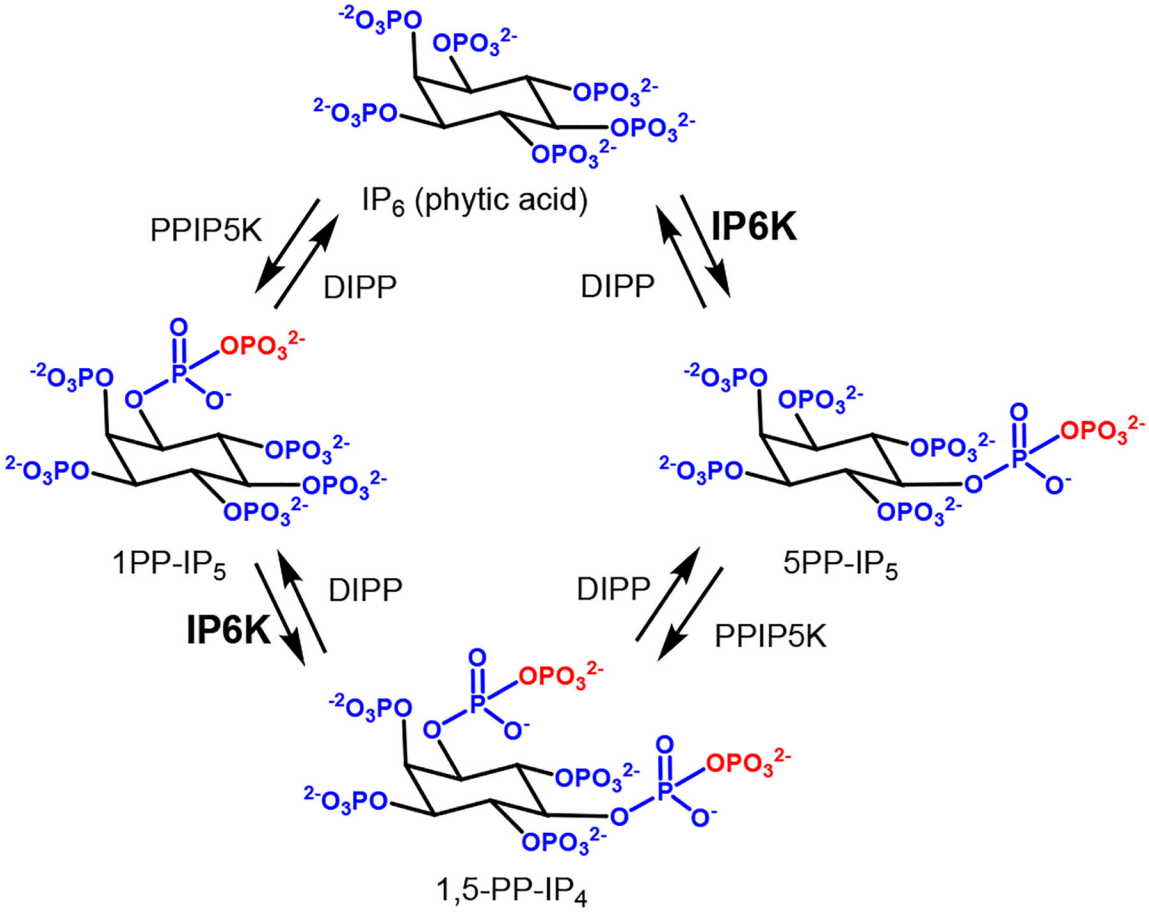
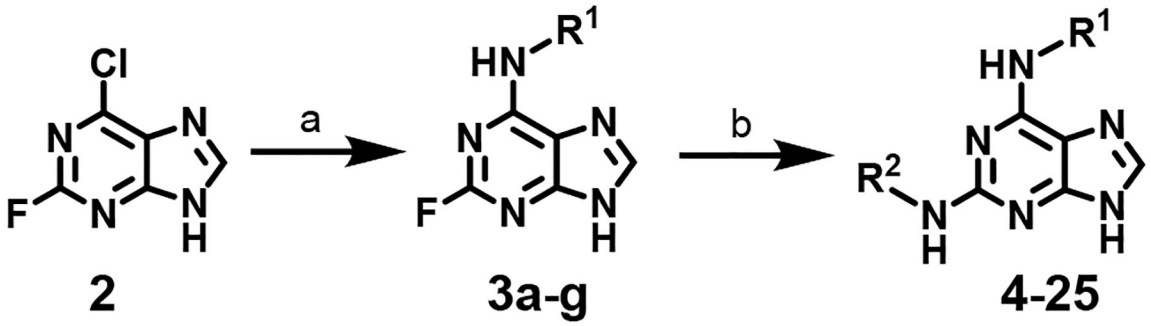


Figure 1.
Biosynthesis of inositol pyrophosphates

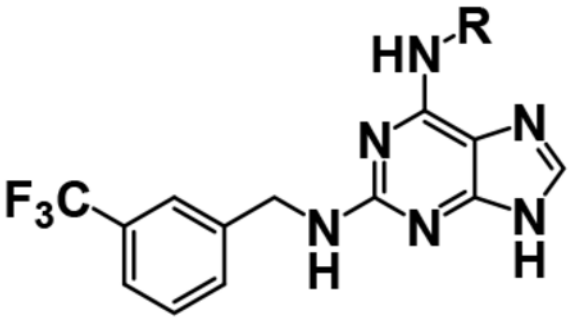


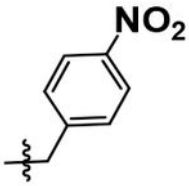
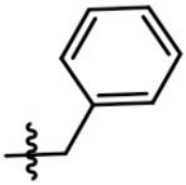
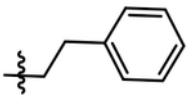
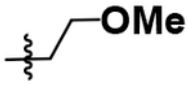
Scheme 1.

Preparation of *N*2- and *N*6- substituted purine analogs 2–25. Reagents and reaction conditions: (a) R^1-NH_2, DIPEA, nBuOH, 110°C (b) R^2-NH_2, DIPEA, DMSO, 110°C.

Table 1.

Inhibitory activities of the first iteration of purine analogs with variation at the *N*6-position.



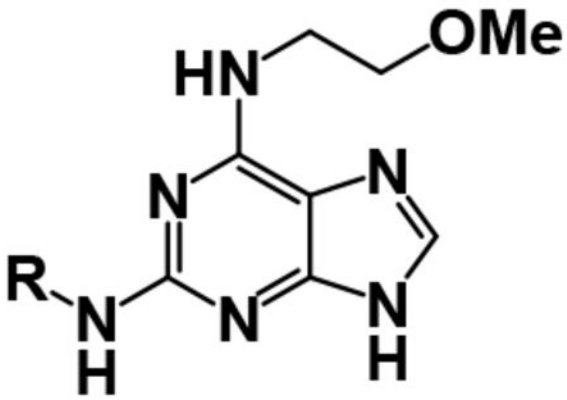
Compound	R	IP6K1 pIC_{50}
1, TNP		< 3.70 *
4		< 3.70
5		< 3.70
6		3.93 ± 0.10

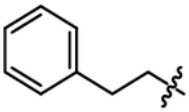
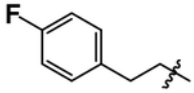
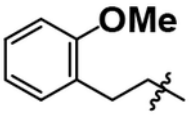
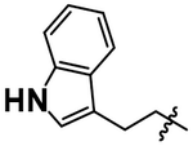
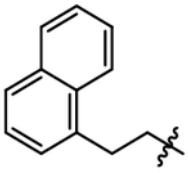
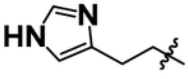
* Solubility issues preclude proper IC_{50} determination

Data represented as the mean ± SEM.

Table 2.

Inhibitory activities of the second iteration of purine analogs with variation at the *N*2-position.

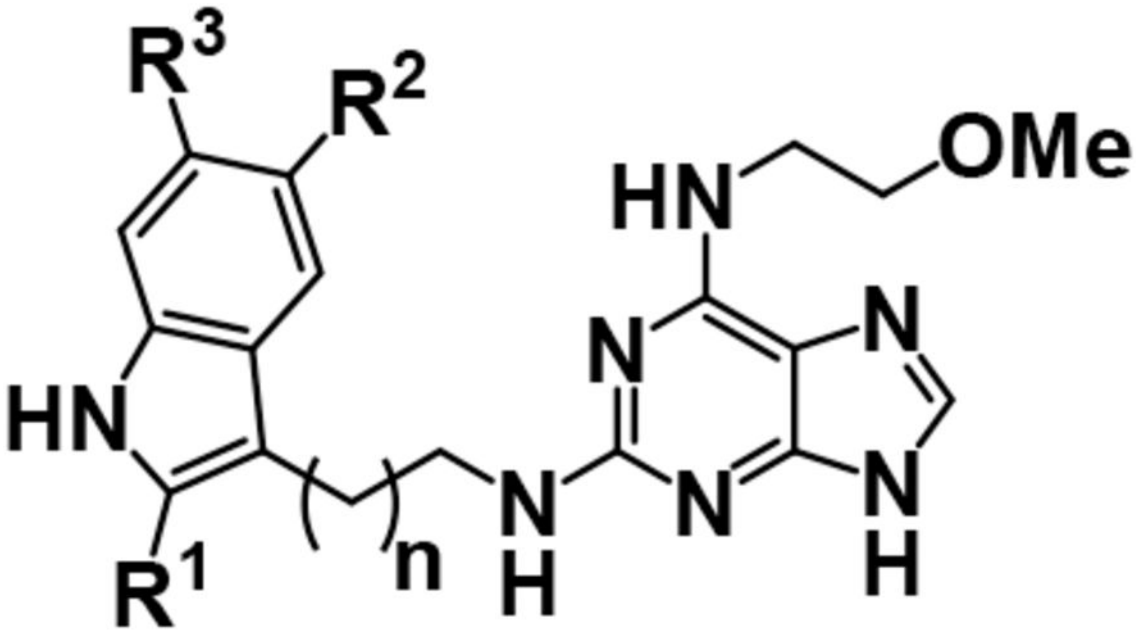


Compound	R	IP6K1 pIC_{50}
7		4.10 ± 0.12
8		4.05 ± 0.04
9		4.95 ± 0.19
10		5.08 ± 0.10
11		4.03 ± 0.09
12		< 3.70

Data represented as the mean ± SEM.

Table 3.

Inhibitory activities of the third iteration of purine analogs with exploration around an *N*2-tryptamine.

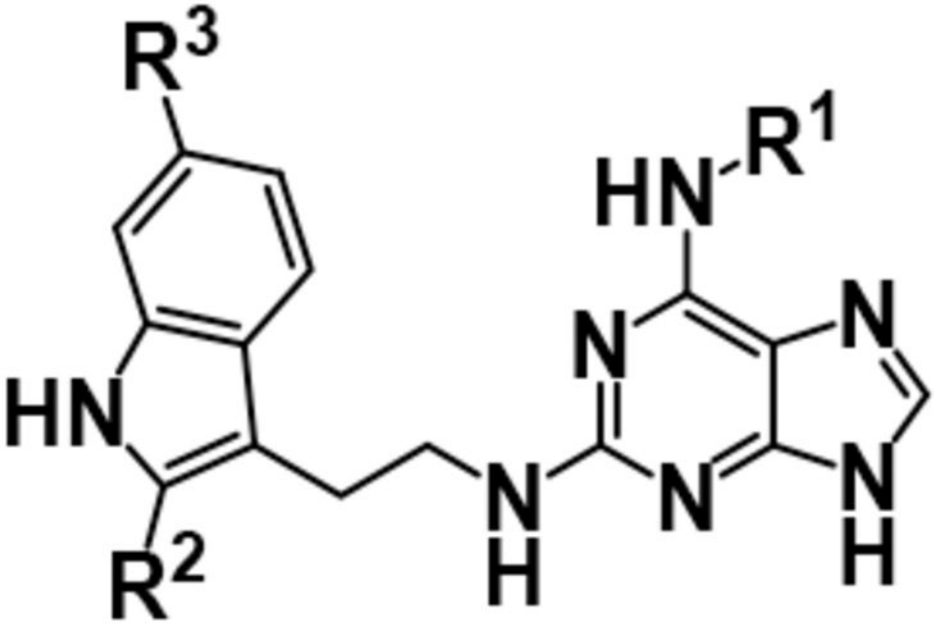


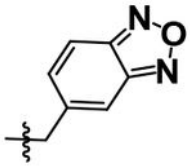
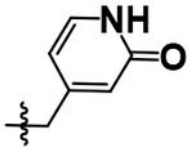
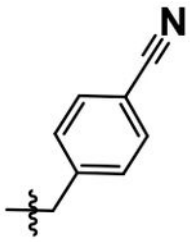
Compound	R^1	R^2	R^3	n	IP6K1 pIC_{50}
13	H	OMe	H	1	3.86 ± 0.08
14	H	OH	H	1	4.50 ± 0.14
15	H	Cl	H	1	4.57 ± 0.20
16	H	Me	H	1	3.91 ± 0.08
17	H	H	OMe	1	5.56 ± 0.09
18	Me	H	H	1	5.71 ± 0.14
19	H	H	H	2	4.16 ± 0.01

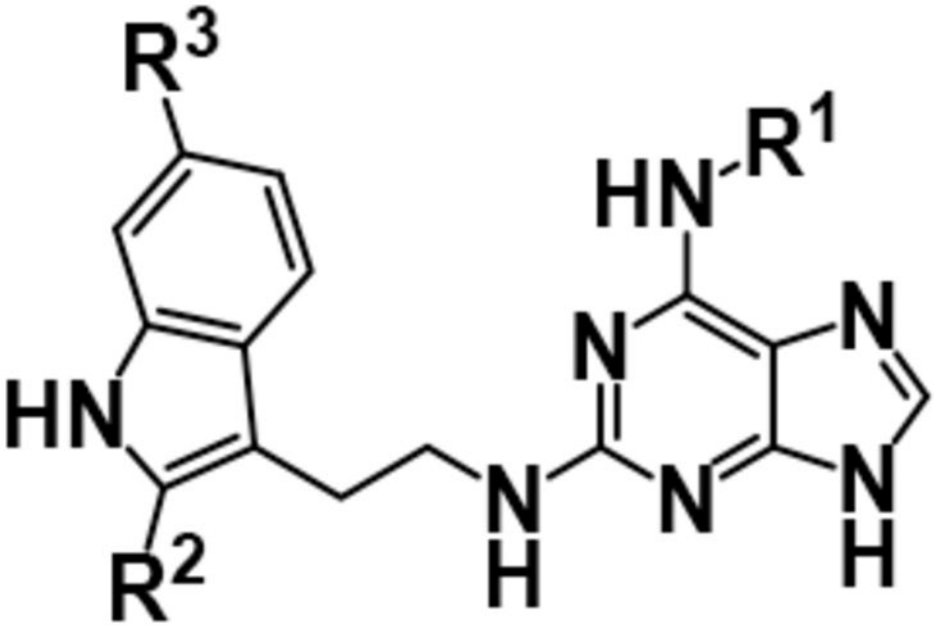
Data represented as the mean ± SEM.

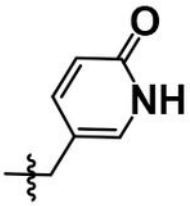
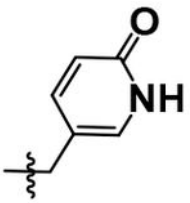
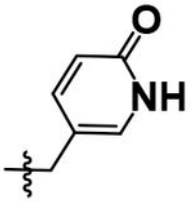
Table 4.

Inhibitory activities of the fourth iteration of TNP analogs with nitro replacements at *N*6.



Compound	R^1	R^2	R^3	IP6K1 pIC_{50}
20		H	H	3.82 ± 0.00
21		H	H	5.00 ± 0.11
22		H	H	4.33 ± 0.17



Compound	R^1	R^2	R^3	IP6K1 pIC_{50}
23		H	H	5.76 ± 0.14
24		H	OMe	6.13 ± 0.08
25		Me	H	5.52 ± 0.07

Data represented as the mean ± SEM.

Table 5.

Compound **24** thermally stabilizes IP6K1 to a greater degree than other purine analogs.

Compound	ΔT_m at 11.1μM (°C)	Δ_{TmMax} (°C)
24	5.5	6.6 ± 0.6
17	3.6	4.7 ± 0.6
10	2.4	4.3 ± 0.9
1, TNP	1.8	3.3 ± 0.6

Data represented as the mean ± SEM.

Table 6.

Selectivity of compound **24** against IP6K isoforms

	Compound 24		
	IC_{50} (μM)	**K_i (μM)**	**Selectivity (K_i/K_i IP6K1)**
IP6K1	0.75	0.20	-
IP6K2	20	4.88	24.4
IP6K3	15	9.62	48.1

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Table 7.

Comparison of compound **24** with TNP

Parameter	Compound 24	TNP, 1
Nitrobenzyl	Removed	Present
Solubility	51μg/mL (PBS pH 7.4)	Mostly insoluble in 2% DMSO
Potency	0.20μM K_i	0.24μM K_i (published value) *
IP6K selectivity	24.4 fold over K2 48.1 fold over K3	Not selective *
Microsome stability ($t_{1/2}$)	Rat 38 min	Mouse 9 min **
	Human 42 min	Human 32 min **

* published in Padmanabhan, et al. *J Biol Chem* 2009

** published in Ghoshal, et al. *Mol Metab*. 2016