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Towards pharmacological intervention in inositol pyrophosphate signalling

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Abstract

To help define the molecular basis of cellular signalling cascades, and their biological functions, there is considerable value in utilizing a high-quality chemical ‘probe’ that has a well-defined interaction with a specific cellular protein. Such reagents include inhibitors of protein kinases and small molecule kinases, as well as mimics or antagonists of intracellular signals. The purpose of this review is to consider recent progress and promising future directions for the development of novel molecules that can interrogate and manipulate the cellular actions of inositol pyrophosphates (PP-IPs) – a specialized, ‘energetic’ group of cell-signalling molecules in which multiple phosphate and diphosphate groups are crammed around a cyclohexane polyol scaffold.

Keywords

inositol phosphate; cell-signalling; drug; pharmacology; structure

Introduction – the multifunctionality of PP-InsPs

The inositol pyrophosphates (PP-IPs; Figure 1) comprise a unique class of cell-signalling molecules characterized by their functionally-significant and highly ‘energetic’ diphosphate groups [1,2]. As many as seven (‘InsP₇’) or eight (‘InsP₈’) phosphates are crammed around the six-carbon inositol ring; these molecules possess the most crowded 3D phosphate arrays seen in Nature [1]. The pathways of PP-InsP synthesis involve 5-kinases [inositol hexakisphosphate kinases (IP6Ks)] and 1-kinases [diphosphoinositol pentakisphosphate kinases (PPIP5Ks)]. Thus, two InsP₇ isomers are generated, distinguished by the presence of a diphosphate group at either the 5- or 1-position on the inositol ring; InsP₈ has both of these diphosphates (Figure 1).

Genetic studies indicate that PP-InsPs can regulate many different biological processes. The manipulation of IP6K expression has shown PP-InsPs to participate in maintenance of telomere length [3,4], vesicle trafficking [5,6], apoptosis [7,8], autophagy [9], repair of DNA repair by homologous recombination [10,11], transcription of glycolytic genes [12], haemostasis [13], phagocytic and bactericidal activities of neutrophils [14], craniofacial morphogenesis [15], epigenetic modifications to chromatin [16] and exocytic insulin

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secretion [17]. Genetic targeting of PPIP5K/Vip expression has contributed to demonstrations that PP-InsPs regulate phosphate homeostasis [18] and cellular morphogenesis [19] in yeasts, and stimulate IFN- β expression in mammalian cells during viral invasion [20].

Current evidence supports two different molecular mechanisms of action of PP-InsPs: association with specific ‘receptors’ [18,21–24] and the ability to non-enzymatically phosphorylate certain proteins [25,26] (since β -phosphates of PP-InsPs are transferred to a phosphoserine residue, the net result is actually protein pyrophosphorylation [25]). 5-InsP₇, 1-InsP₇ and InsP₈ each appear to be equally effective as phosphate donors [25]. This absence of isomer specificity, and the ever-increasing number of proteins identified as targets, could help account for PP-InsP multifunctionality. However, it has been noted that there is no *direct* evidence this covalent modification occurs *in vivo* [1,2]. There are as yet no antibody-based or proteomic methods that can address this issue [27]. An indirect, ‘back-phosphorylation’ assay for protein pyrophosphorylation has been developed [28], although such methodology may instead act as a bioassay for the degree of endogenous CK2-mediated protein phosphorylation [29].

There are other actions of PP-InsPs that *are* isomer-specific. For example, *Saccharomyces cerevisiae* express a cyclin kinase regulator-protein that is activated by 1-InsP₇, but not by 5-InsP₇ [18]. There is also selectivity in the inhibition by PP-InsPs of the binding of PtdIns(3,4,5)P₃ to pleckstrin-homology domains: 5-InsP₇ generally appears to be more potent than both 1-InsP₇ and InsP₈ [24]. The large number of PtdIns(3,4,5)P₃-binding proteins that 5-InsP₇ may regulate [23,24] could contribute to PP-InsP multifunctionality.

PP-InsP mimicry

Phosphates and diphosphates are typically targets of active cellular phosphatases, so PP-InsPs are particularly labile in cells and cell extracts. Therefore, there is interest in preparing biologically-stable PP-InsP analogues for functional studies, such as screening cell and tissue lysates for candidate receptors. This research topic has only developed recently – a comprehensive 2010 review of inositide analogues was unable to list any stable PP-InsP mimics [30]. In no small part, that situation reflected synthetic challenges imposed by the high charge density of PP-InsPs and the involvement of unstable phospho-anhydrides and phospho-esters. Characterization and purification of PP-InsP analogues has also been hindered by their lack of UV-absorbance and low level of optical rotation. All of these problems are at last being circumvented [31–34].

There has been a particular focus on replacing the metabolically labile diphosphates in PP-InsPs with α -phosphonoacetic acid esters (PA) or methylenebisphosphonates (PCP) [31–34]. One application for such molecules is for experiments performed *in vitro* that aim to distinguish between proposed alternative mechanisms of interaction of PP-InsPs with proteins: non-covalent binding compared with non-enzymatic transfer of the ‘energetic’ β -phosphates [25,26]. The latter phenomenon cannot be recapitulated by the metabolically stable analogues. Thus, phosphate transfer can be excluded as the mechanism by which 5-InsP₇ enhances CK2 activity (by attenuating inhibition of CK2 by NOPP140), since 5-PCP-

InsP₇ reproduces this effect [35]. Nevertheless, in circumstances in which a biological effect of a natural PP-InsP is not imitated by a PA- or PCP-analogue, the negative result should be interpreted cautiously; the bioisosteric properties are not perfect. Even in the case of the PCP-analogues, which are considered to be the superior mimics from a structural standpoint, the replacement of an oxygen atom with a methylene group leads to a reduction in acidity and higher p*K*_a values; at physiological pH, even a PCP analogue is expected to be less electronegative than the corresponding natural PP-InsP [36], which by itself may reduce affinity for a protein target. Thus, it is best to utilize the analogues in excess of the concentration of the natural PP-InsP that gives a biological effect. It has been noted that the p*K*_a of a difluoromethylene-bisphosphonate group is closer to that of a diphosphate, but the incorporation of this fluorinated group into a PP-InsP analogue is a considerable synthetic challenge [34,36].

An unexpected development to emerge from experiments with stable PP-InsP analogues has been the discovery that, adjacent to the PPIP5K catalytic pocket, there is a second ligand-binding site that resides on the surface of the protein [37]. The latter has been described as a 'substrate capture site.' As its name implies, it enhances 'capture' of substrate from the bulk phase, following which the ligand is transferred into the catalytic site – a 'catch-and-pass' catalytic mechanism [37]. The capture site was originally identified in a structural analysis [37]. In that study, either 5-PA-InsP₅ or another substrate analogue, 2,5-di-*O*-benzyl-inositol-1,3,4,6-tetrakisphosphate (di-Bn-InsP₄), were separately soaked into crystals of the PPIP5K2 kinase domain. It was the electron density maps of the two analogues that revealed that the kinase has two ligand-binding sites. In contrast, when a natural substrate, 5-InsP₇, was soaked into the crystals, its electron density was restricted to the catalytic site alone [37]. Wang et al. [37] have interpreted these observations as indicating that natural substrates only transiently occupy the capture site during the catalytic cycle. More recent structural work with a PA analogue of InsP₈ has shown that the kinase reaction product also binds to the capture site during its egress from the catalytic site [31].

The path to inhibitors of IP6Ks and PPIP5Ks

Selective, cell-permeant inhibitors of IP6Ks and PPIP5Ks – 'probes' [38] – could be a useful complement to genetic intervention in PP-InsP signalling processes; there is always the possibility that a particular phenotype that results from gene deletion is complicated by secondary genetic changes. One observation that is particularly pertinent to this topic is the altered degree of transcription of over 900 genes (2-fold change in expression), following the deletion of *vip1* [39]. This raises ample opportunity for off-target, secondary genetic changes to yield confounding data. The extent of this genetic penetration may reflect functional promiscuity, as a consequence of an inositol phosphate kinase having scaffolding roles separate from its catalytic roles (as is the case, for example, for the inositol pentakisphosphate kinase [40] and the inositol polyphosphate multikinase [41]). A genetic approach can distinguish between catalytic and scaffolding functions. For example, in yeast the wild-type Vip1 can be replaced with a version hosting a point mutation that renders it kinase-dead. However, even then, the degree of transcription of more than 250 genes is still modified [39]. One genetic complication of particular relevance is the reduction in *vip1* cells of the expression of Pho81 [39], an inhibitor of Pho80-Pho85 cyclin/cyclin-dependent

kinase activity [18]. This could complicate genetic research into the mechanism by which Vip1 regulates cyclin kinase activity during phosphate starvation [18]. In contrast, short-term application of a cell-permeant inhibitor of PP-InsP synthesis is an experimental approach that is not entangled by secondary genetic changes and also leaves scaffolding functions intact.

One of the features of PP-IPs that render them amenable to perturbation by defined reagents is the rapidity of their metabolic flux through futile cycles [42] (Figure 1). Indeed, the very demonstration of these fluxes arose from the first reported pharmacological intervention in PP-InsP turnover: the use of fluoride as a diphosphoinositol polyphosphate phosphohydrolase (DIPP) inhibitor, which rapidly (within minutes) brought about manyfold elevations in PP-InsP levels [42,43]. More recent work introduced a cell-permeant pan-IP6K inhibitor, *N*2-(*m*-(trifluoromethyl)benzyl) *N*6-(*p*-nitrobenzyl) purine (TNP) [44]. This and subsequent studies showed that within 2 h treatment of various cell types with 10–30 μ MTNP, levels of InsP₇ fell by 60–90% [15,22,44,45]. Naturally (Figure 1), IP6K inhibition does not just affect 5-InsP₇ levels; InsP₈ synthesis is also compromised [15]. Another collateral impact of TNP addition to intact cells is to elevate InsP₆ levels by as much as 40%; deletion of the *IP6K2* gene has a similar effect [15,22]. That outcome may in part reflect InsP₆ levels normally being curbed through their dephosphorylation by the InsP₆/ADP phosphotransferase activity of IP6Ks [45].

TNP has been used in several studies to explore biological functions of IP6Ks [15,22,45–49], so it seems appropriate to assess its specificity profile. In a counter-screen, TNP did not significantly affect a panel of 71 protein kinases [44], although they represent less than 15% of the human kinome. The catalytic site of the IP6K family is structurally related to that of two other groups of inositol phosphate kinases: the IPMKs and the IP3Ks [50]. Nevertheless, TNP is considered a competitive inhibitor of ATP binding and the IP6Ks have around 100-fold lower affinity for ATP than do IPMKs and IP3Ks [44]. Thus, the IC₅₀ for inhibition of IP3K by TNP (18 μ M) is almost 40-fold higher than the corresponding value for inhibition of IP6K (0.47 μ M). Yet, TNP was originally developed as a cell-permeant inhibitor of IP3K, which is accomplished by using treatment protocols similar to those now used to block IP6K activity [51]. For example, within a couple of minutes of the addition of 5–10 μ M TNP to HL60 cells, Ca²⁺ levels were reported to be slightly elevated [51]. Perhaps because intracellular stores of Zn²⁺ and Ca²⁺ are closely coupled [52], cells treated with 20 μ M TNP show increased cytosolic Zn²⁺ levels – a potential off-target cell-signalling response [53]. Furthermore, 20–40 μ M TNP relieves the inhibition by IP3 kinase of neurite outgrowth from PC12 cells [54]. In the same cells, as little as 15 min treatment with 40 μ M TNP enhances the degree of stimulation of extracellular signal-regulated kinase (ERK) phosphorylation by nerve growth factor [54]. A separate study with human microvascular endothelial cells described *inhibition* of receptor-dependent ERK phosphorylation after 4 h of TNP treatment [55]. Since IP6K does not appear to regulate ERK phosphorylation [22], the effects upon that kinase of TNP should be considered off-target. Thus, it would be wise not to consider TNP as a substitute for a molecular biology approach to studying IP6K biology.

Not only would it be useful to develop alternate IP6K inhibitors with an improved specificity profile, there could also be great utility in a drug that specifically targets an individual

isoform of IP6Ks, particularly as certain biological functions of these kinases are not redundant. For example, mice null for either *IP6K1* [56] or *IP6K2* [57] each exhibit specific phenotypes. Only the *IP6K1*^(-/-) mice show lower circulating levels of insulin, reduced male fertility and lower body weight. It also appears that the pro-apoptotic effects of IP6K are predominantly exerted by the type 2 isoform [7,8]. The N-terminal ‘glycine loop’ [58] in IP6Ks, that may contribute to binding the gamma phosphate of ATP [50], offers possibilities for development of an isoform-specific drug; residues on either side of this loop do vary a little between IP6K isoforms.

As for the PPIP5Ks, currently there are no known, cell-permeant inhibitors of this group of kinases; the development of such reagents is one of our laboratory’s goals. Lead inhibitors could be identified by high-throughput screening (HTS) of drug libraries. In general, small molecule kinases offer their catalytic pocket as a potential target, most likely to substrate analogues. In the case of the PPIP5Ks, the ‘druggability’ [59] of substrate analogues is doubtful; polyphosphates are highly charged and therefore inherently membrane-impermeant. It remains to be seen whether or not these problems can be overcome with cell-permeant, bioactivatable analogues [60,61] or drug encapsulation technologies [62]. However, the substrate capture site of PPIP5Ks (see above) may be a more viable drug target. Being on the surface of the kinase, it is much less sterically constrained. Additionally, the capture site can accept analogues that are more hydrophobic than natural substrate [75].

It can be a challenge to select an appropriate drug library for screening purposes that might include a suitable inhibitor; as has been noted [63], millions of molecules are commercially available and trillions more could be synthesized. To avoid HTS being a tedious, expensive and ultimately frustrating exercise, focused libraries have been prepared, such as those that are dominated by natural products [63]. Another approach has been to curate a library of approximately 5000 molecules that are either known or predicted to target nucleotide binding [64,65]. In addition, such molecules have been pre-selected to be membrane-permeant, by imposing admission criteria based on lipophilicity, hydrogen-bond formation and size [66]. This particular library has a proven track record, already generating useful reagents from previous screens of protein kinase and lipid kinase activities [65,67]. We aim to deploy this library in our own, future studies. In the case of the ATP-driven kinases that synthesize PP-InsPs, assays of changes in nucleotide levels represent a HTS-compatible assay. This is the approach that we are taking, but in an unusual manner that takes advantage of the ‘highenergy’ of InsP₈ hydrolysis, and some unique properties of PPIP5K: its $V_{\max}$ in the ‘reverse’ direction of ATP hydrolysis is significantly higher than the $V_{\max}$ in the forward direction [68]. These unusual kinetic characteristics in the ‘reverse-kinase’ direction can be exploited to increase the sensitivity of an HTS. As for preparing pure enzyme for these assays, full-length recombinant versions are difficult to express and purify in large quantities. Fortunately, the kinase activity of PPIP5Ks is self-contained in the N-terminally-located catalytic domain that is readily expressed in *Escherichia coli* [69–71].

Fragment-based screening offers another approach [72]. Fragments, being low-complexity molecules, interact with proteins weakly but in ideal conformations. They sample chemical space exponentially more effectively than drug-sized molecules, to the extent that a diverse set of 1000 fragments has been calculated to represent chemical space as effectively as

would 10 trillion diverse drug-sized molecules [72]. Chemists can then assimilate these different molecular components into more complex drug leads. Since the structure of the PPIP5K kinase domain is available [73], drug development can be assisted by computational methods such as virtual screening and rational design [74,75].

Conclusions

Beyond the use of chemical probes as research tools there is the holy grail: development of therapeutically-beneficial drugs that improve human health. The desire for new anti-inflammatory reagents, and more importantly, improved anti-cancer drugs, is what has driven pharmacological interest in another group of inositide-kinases, the PI3Ks [76]. Indeed, a disrupted PI3K signalling pathway has been associated with 30–50% of human cancers [76]. Even though the lipid products have many functions, over 30 small molecule inhibitors of PI3K activity have progressed into clinical trials for cancer treatment [76]. Furthermore, some of these drugs exhibit good specificity against particular PI3K isoforms [76].

PP-InsPs may play roles in the development of diabetes [17], inflammation [20] and cancer [77]; these are three human health conditions that could conceivably benefit from inhibitors of PP-InsP synthesis or their biological actions. It is too soon to hypothesize if the potential for therapeutic intervention might be thwarted by too many off-target effects of drug treatment due, in particular, to PP-InsP multifunctionality (see above).

There appears to be an opening to develop reagents that might specifically target a particular isoform of IP6K (see above). The preparation of isoform-specific inhibitors of the two PPIP5Ks is also a worthwhile endeavour, but more challenging: the kinase domains of PPIP5K1 and PPIP5K2 are 84% identical, with all non-conserved residues lying away from the ATP-binding site, the catalytic pocket, and even the substrate capture site. On the other hand, we can be sure there remains much to learn about PP-InsP function and metabolism; new opportunities for pharmacological intervention in PP-InsP signalling will surely reveal themselves during future studies.

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Abbreviations

CK2	casein kinase 2
DIPP	diphosphoinositol polyphosphate phosphohydrolase
ERK	extracellular signal-regulated kinase
HTS	high-throughput screening

IPMK	inositol polyphosphate multikinase
IP3K	inositol-1,4,5-trisphosphate 3-kinase
IP6K	inositol hexakisphosphate kinase
PA	α -phosphonoacetic acid esters
PCP	methylenebisphosphonates
PI3K	phosphoinositide 3-kinase
PP-IP	inositol pyrophosphate
PPIP5K	diphosphoinositol pentakisphosphate kinase
PtdIns(3,4,5)P₃	phosphatidylinositol-3,4,5-trisphosphate
TNP	<i>N</i> 2-(<i>m</i> -(trifluoromethyl)benzyl) <i>N</i> 6-(<i>p</i> -nitrobenzyl)purine.

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Figure 1. Synthesis and metabolism of the PP-InsPs

The figure describes the metabolic reactions that account for the turnover of the PP-InsPs in both yeasts and mammalian cells. IP6K (Asp1 in *Schizosaccharomyces pombe*, Vip1 in *Saccharomyces cerevisiae*; PPIP5K (Ksc1 in yeasts); DIPP (Ddp1 in yeasts).