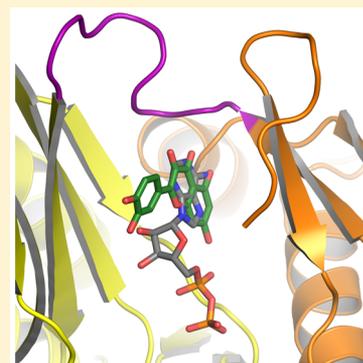


Inhibition of Inositol Polyphosphate Kinases by Quercetin and Related Flavonoids: A Structure–Activity Analysis

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Supporting Information

ABSTRACT: Dietary flavonoids inhibit certain protein kinases and phospholipid kinases by competing for their ATP-binding sites. These nucleotide pockets have structural elements that are well-conserved in two human small-molecule kinases, inositol hexakisphosphate kinase (IP6K) and inositol polyphosphate multikinase (IPMK), which synthesize multifunctional inositol phosphate cell signals. Herein, we demonstrate that both kinases are inhibited by quercetin and 16 related flavonoids; IP6K is the preferred target. Relative inhibitory activities were rationalized by X-ray analysis of kinase/flavonoid crystal structures; this detailed structure–activity analysis revealed hydrophobic and polar ligand/protein interactions, the degree of flexibility of key amino acid side chains, and the importance of water molecules. The seven most potent IP6K inhibitors were incubated with intact HCT116 cells at concentrations of 2.5 μM ; diosmetin was the most selective and effective IP6K inhibitor (>70% reduction in activity). Our data can instruct on pharmacophore properties to assist the future development of inositol phosphate kinase inhibitors. Finally, we propose that dietary flavonoids may inhibit IP6K activity in cells that line the gastrointestinal tract.



INTRODUCTION

All eukaryotes express a number of inositol phosphate (InsP) kinases (Figure 1). These enzymes synthesize a range of diffusible messenger molecules which contain various combinations of phosphate groups, all attached to the inositol ring.^{1–3} Valuable information concerning InsP function and metabolism has been derived from the creation of a number of InsP kinase knockouts, both in model organisms^{3,4} and in human cell lines.^{3,5} However, all physiological actions of InsP kinases are not simply related to their catalytic activities; in addition, these proteins exhibit a variety of functionally significant protein–protein interactions.^{2,3} These are phenomena that complicate the use of genetic procedures to explore InsP biology; the phenotype that arises from a “knock-down” of kinase expression may not simply reflect a loss of InsP synthesis but also the elimination of its scaffolding activities. Thus, there is growing need to develop cell-permeable inhibitors of the individual InsP kinases; such compounds could be very insightful in specifically deciphering the catalytic roles of these signaling enzymes^{6–9} and may also lead to the development of therapeutically beneficial inhibitors.³ For example, human inositol hexakisphosphate kinases (hIP6Ks;

Figure 1) synthesize the inositol pyrophosphate, InsP₇,¹⁰ a multifunctional molecule that receives particular attention, both for its ability to pyrophosphorylate proteins¹¹ and for its roles in bioenergetic homeostasis, cancer, and aging.^{3,12} A close relative of the IP6Ks is inositol polyphosphate multikinase (IPMK; Figure 1), a key enzyme in the pathway of InsP₆ synthesis. IPMK is a transcriptional regulator, and its phosphorylation of InsP₃ to InsP₅ produces a coactivator of the Wnt/ β -catenin signaling pathway.^{13,14} Furthermore, there is growing interest in the substantial contributions that pathogenic fungal and protozoan InsP kinases make to the fitness and virulence of these organisms. It has become a viable proposition to develop drugs that can specifically target microbial InsP kinases, due to their limited homology with human orthologs.^{15,16}

Despite the enormous potential value of InsP kinase inhibitors, work to produce such molecules is in its infancy. One such compound, 2-[3,5-dimethyl-1-(4-nitrophenyl)-1H-pyrazol-4-yl]-5,8-dinitro-1H-benzo[*de*]isoquinoline-1,3(2H)-

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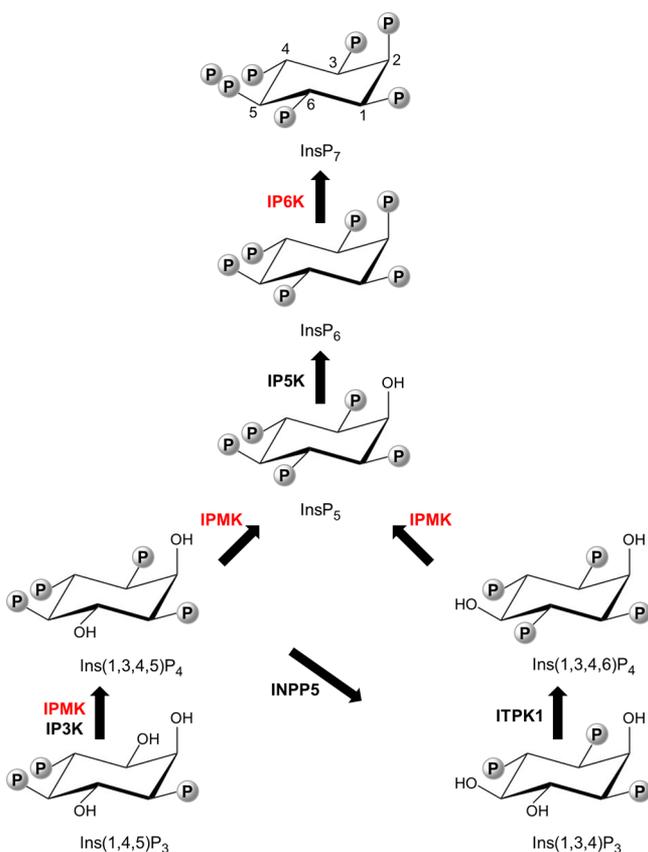


Figure 1. Roles of IPMK and IP6K in the mammalian InsP metabolic pathway. “Ins” refers to inositol; the subscripts denote the total number of phosphates (“P”), and the numbers in parentheses describe the positions of the phosphate groups around the inositol ring. Arrows depict metabolic steps, catalyzed by the following enzymes: IP3K, inositol trisphosphate 3-kinase; IPMK, inositol polyphosphate multi-kinase; INPP5, inositol polyphosphate 5-phosphatase; ITPK1, inositol trisphosphate 6-kinase/inositol tetrakisphosphate 1-kinase; IP5K, inositol pentakisphosphate 2-kinase; IP6K, inositol hexakisphosphate kinase. The enzymes that are the focus of this study (IPMK and IP6K) are highlighted in red font.

dione (BIP-4), inhibits IP3Ks (Figure 1).^{9,17} However, the target specificity profile of BIP-4 is unknown, while its polar nitro groups impede cellular uptake and are potentially toxic *in vivo*.^{9,17} Only one other InsP kinase inhibitor is in current use: *N*2-(*m*-(trifluoromethyl)benzyl)-*N*6-(*p*-nitrobenzyl)purine (TNP), which is a competitive inhibitor of the ATP-binding site of inositol hexakisphosphate (InsP₆) kinases (IP6Ks).⁶ Unfortunately, TNP also has substantial limitations, including a growing list of off-target effects: inhibition of cytochrome P450,¹⁸ inhibition of CaMK1,⁷ initiation of cellular Ca²⁺ fluxes,¹⁹ and perturbation of the activity of the ERK protein kinase.^{20,21} An additional restriction on the utility of TNP is that it inhibits all three IP6K isoforms.³ The development of isoform-specific IP6K inhibitors is an important future goal.

A new direction for the development of inhibitors of human IPMK (hIPMK) and InsP6Ks (hIP6K) has recently emerged from structural work which has demonstrated that these kinases exhibit substantial conservation of the protein kinase fold and in particular the ATP-binding site.^{7,22–24} Thus, prior development of protein kinase inhibitors that block nucleotide binding has established a knowledge base that could be applied productively to the design of new inhibitors that compete for

the ATP-binding site of InsPKs.⁷ To implement this approach through rational design, we need as much insight as possible into the pharmacophore space that is available. To this end, the flavonoids are one well-known group of competitive inhibitors of ATP-binding by protein kinases.^{25–27} Flavonoids (Figure 2) are low-molecular weight, aromatic compounds that are components of fruits, vegetables, and other plant components. It is possible that inhibition of protein kinases by flavonoids contributes to their reported ability to reduce the risks of cardiovascular disease, diabetes, and cancer.^{26–28} Flavonoids have other targets; they inhibit the structurally similar phosphatidylinositol 3-kinase- γ (PI3K γ),^{25,28,29} and they also interact with unrelated, non-kinase targets, such as mammalian F₁-ATPase,³⁰ xanthine oxidase,³¹ serine protease,³² and telomeric DNA.³³ This promiscuity limits the value of the flavonoid core structure *per se* as the basis for a specific kinase inhibitor, but it is still recognized that valuable pharmacophore information can be obtained from a structure–activity analysis of the interactions of flavonoids with the ATP-binding pocket of a particular kinase.²⁷

In the current study, our goal has been to assemble a logically derived, analogue series of flavonoids that are based on **1** (Figure 2) and to test their effects upon the catalytic activities of hIP6K2 and hIPMK. We have supported this work with orthogonal assays. We also sought to rationalize the inhibitory properties of our selection of flavonoids through the generation of X-ray crystallographic data. Flavonoids also have the advantage of penetrating across the plasma membrane,³⁴ which has allowed us to investigate if their inhibition of InsP kinases can be recapitulated in intact cells. Our rigorous structure–activity analysis has allowed us to derive pharmacophore insights for future development of non-flavonoid inhibitors that can be made specific to a particular kinase target. Finally, our data also suggest previously unsuspected biological functionality for dietary flavonoids, as inhibitors of InsP kinases.

RESULTS AND DISCUSSION

Structure–Activity Analysis of the Inhibition of hIP6K2 by Flavonoids. The ATP-binding sites of hIP6Ks and hIPMK are similar to those of protein kinases,²² which are inhibited by flavonoids.^{26,27} Thus, a goal for this study was to perform a structure–activity analysis to investigate if the flavonoid core structure can provide new chemical information to apply to the development of novel inhibitors of InsP kinases. We began this work by investigating if **2** is an inhibitor of hIP6K2. As in our earlier study of hIP6K2 activity,⁷ we used a time-resolved fluorescence resonance energy transfer (TR-FRET) assay in 384-well microplate format, using as substrates 10 μ M InsP₆ and 10 μ M ATP. It should be noted that these assays all contained 0.01% Brij-35. The use of detergent prevents false-positive inhibition through colloidal aggregation of flavonoids into “pan assay interference compounds” (PAINS).^{35–37}

We discovered that **2** inhibits hIP6K2 activity with an IC₅₀ value of 0.7 μ M (Table 1). We followed up this observation by examining the effects upon hIP6K2 of a range of flavonoids (Figure 2) in order to determine the structural determinants for inhibition of kinase activity.

We found that addition of a 5'-OH group to **2** (yielding **1**) had no impact upon the value of the IC₅₀ (Table 1). This observation that **1** is an inhibitor of hIP6K2 confirms a prior report;⁸ nevertheless, in the latter study IP6K was also

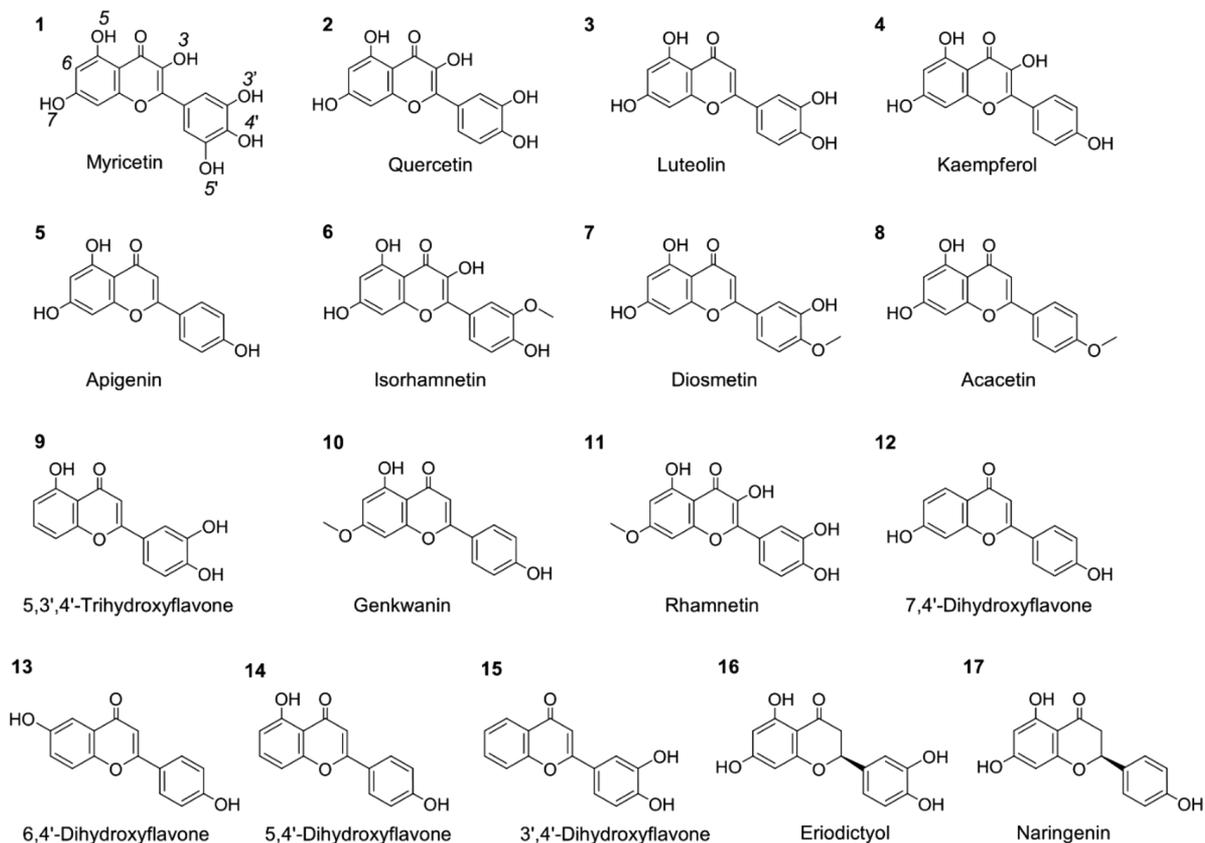


Figure 2. Chemical structures of the flavonoids used in this study.

Table 1. IC₅₀ Data for Inhibition of hIPMK and hIP6K2 by Various Flavonoids^a

compd	common name	IP6K2 IC ₅₀ (μM)	n	IPMK IC ₅₀ (μM)	n	IC ₅₀ ratio IP6K/IPMK
1	myricetin	0.7 ± 0.11	9	1.1 ± 0.2	7	1.6
2	quercetin	0.7 ± 0.13	9	2.3 ± 0.4	6	3.3
3	luteolin	1.3 ± 0.3	6	5.5 ± 1.2	6	4.2
4	kaempferol	2.5 ± 1.0	7	4.4 ± 1.4	4	1.8
5	apigenin	7.1 ± 2	7	29 ± 8	8	4.1
6	isorhamnetin	0.5 ± 0.06	5	2.2 ± 0.6	3	4.4
7	diosmetin	0.9 ± 0.18	9	7.2 ± 0.2	3	8
8	acacetin	5.7 ± 2	7	>30	6	>5.3
9	5,3',4'-trihydroxyflavone	8.0 ± 1.3	5	>30	7	>3.8
10	genkwanin	24 ± 8	4	>30	7	>1.3
11	rhamnetin	1.8 ± 0.2	8	14 ± 1	3	>7.8
12	7,4'-dihydroxyflavone	18.3 ± 3.6	6	>30	7	>1.6
13	6,4'-dihydroxyflavone	12.9 ± 3.2	4	>30	8	>2.3
14	5,4'-dihydroxyflavone	>30	5	>30	7	
15	3',4'-dihydroxyflavone	11.4 ± 1.7	6	>30	5	>2.6
16	eriodictyol	25.3 ± 7.5	4	>30	4	>1.2
17	naringenin	>30	5	>30	5	

^aThe two enzymes were assayed in vitro as described under [Experimental Procedures](#), using compound concentrations of up to 100 μM. Data shown are the mean ± standard errors. In all cases where the IC₅₀ is designated as >30 μM, a combination of weak inhibition and poor curve fitting together prevented an accurate designation of IC₅₀ values.

incubated with 2, and no inhibitory effect was observed, contrary to one of the findings in the current study (Table 1). Removal of the 3-OH group from 2 (yielding 3) results in a small (1.9-fold) decrease in inhibitory activity. A larger increase in the IC₅₀ value (3.6-fold) is observed for removal of the 3'-OH (compare 4 with 2). The degree of the impact upon the IC₅₀ value of removing from 2 both the 3- and the 3'-OH groups (yielding 5) is approximately additive, compared to

loss of either group alone: a 10-fold higher IC₅₀ value for 5, compared to 2 (Table 1). We also found that the 7-OH group has an important role: for example, in a comparison of 9 with 3, loss of the 7-OH results in a 6-fold increase in the IC₅₀ value (Table 1).

In some cases, O-methylation was better tolerated than loss of the equivalent OH group. For example, 3'-O-methylation of 2 (as in 6) did not significantly alter the degree of inhibition of

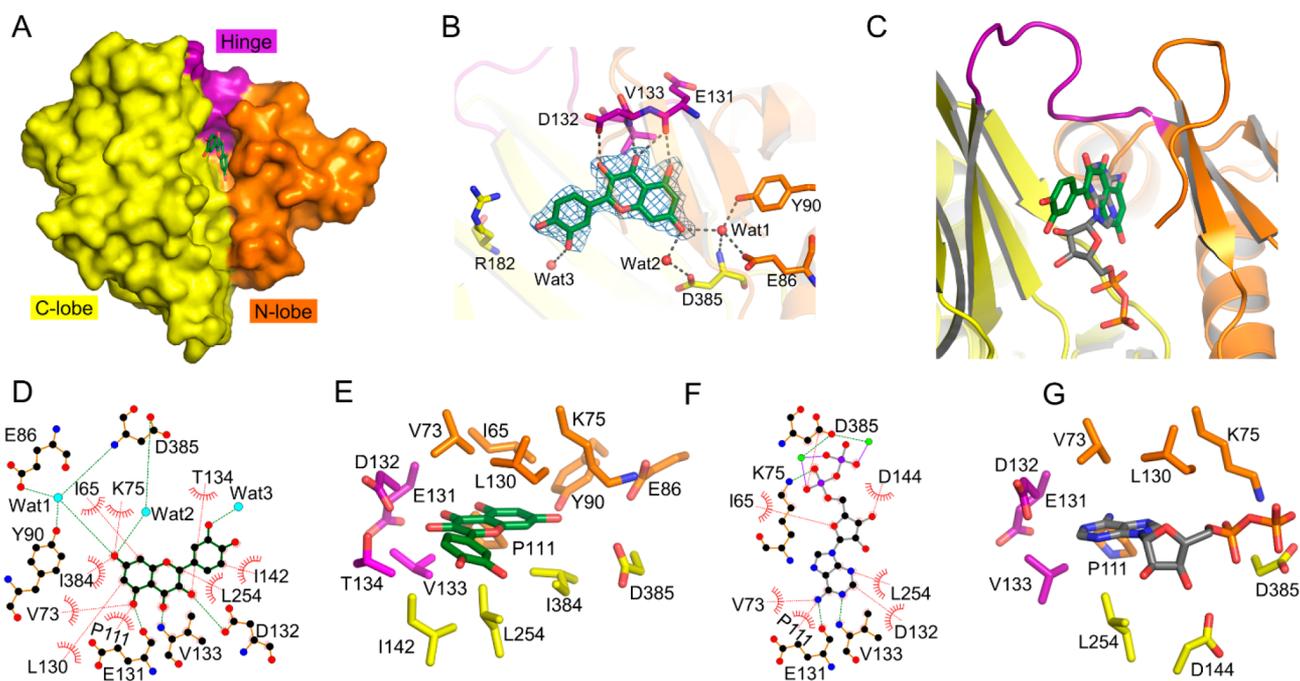


Figure 3. Structure of the hIPMK/2 crystal complex. (A) Surface representation of quercetin binding. The C- and N-lobes are depicted in yellow and orange, respectively; the hinge region (E131 to K139²³) is colored purple, and **2** is shown as a dark green stick model, with the oxygen atoms illustrated in red. All color coding is retained for structural elements shown in the other panels. (B) Polar interactions of **2** with hIPMK. $2F_o - F_c$ electron density maps (skyblue mesh) are contoured at 1.0σ . Hydrogen bonds are shown in broken gray lines. Water molecules that directly interact with **2** are depicted as red spheres. (C) Ribbon plot of hIPMK showing superimposition of bound **2** (this study, PDB code 6M89) and ADP (from ref 23, PDB code 5W2H; dark gray stick model; nitrogen atoms are shown in blue). (D–G) Ligplots and relative spatial positioning of all residues that make either van der Waals or polar interactions with either **2** (D, E) or ADP (F, G).

hIP6K2 (Table 1). O-methylation can also be tolerated by the OH group at the 4' position (similar IC_{50} data were obtained for **7** compared to **3** and for **8** compared to **5**; Table 1). On the other hand, the conversion of the 7-OH to a 7-O-methyl group does slightly impact inhibition of hIP6K2: **10** exhibits a 3-fold higher IC_{50} than **5**, and **11** shows a 2.6-fold higher IC_{50} than **2** (Table 1).

It is also significant that flavonoids with only two OH groups and no O-methyl groups (**12**, **13**, **14**, and **15**) are all relatively weak inhibitors of hIP6K2 (Table 1). In fact, we are unable to ascertain with accuracy the exact IC_{50} value against hIP6K2 for **14** (and also **17**; see below). This is an inherent technical issue, due in part to the relatively weak inhibition. Another likely contributory factor is the poor solubility of certain flavonoids at higher concentrations. For example, **14** and **17** are among the most hydrophobic of the flavonoids that we tested.

Finally, inhibitory activity against hIP6K2 is attenuated by reduction of the double bond between C2 and C3, and the concomitant disruption to the planarity of the chromen-4-one and phenyl rings. This is evident from a comparison of **16** with **3** (the latter has a 19-fold lower IC_{50} value against hIP6K2; Table 1). Consistent with the latter conclusion, **17** is at least 4-fold weaker an inhibitor than is **5** (Table 1).

Comparative Effects of Flavonoids upon the Inhibition of hIP6K2 and hIPMK. IPMK is the InsP kinase that is most closely related to the IP6Ks, both structurally,^{22–24} and in an evolutionary context.³⁸ Thus, we selected IPMK to investigate to what extent the flavonoid scaffold might give insight into the principle of developing target specificity against an InsP kinase. In these experiments we used 10 μ M ATP and 10 μ M InsP₃ as substrates (Table 1).

Each of the flavonoids that we have examined was less potent against hIPMK than against hIP6K2, albeit to various degrees (Table 1). This is useful information, as it underscores that it is plausible to develop inhibitors that are more selective for IP6Ks. Furthermore, we identified some modifications to the flavonoid core structure that somewhat increase the relative degree of inhibition of hIP6K2 compared to that for hIPMK (see Table 1): O-methylation of the 4'-OH of **3**, yielding **7**, elicited a 1.9-fold improvement in selectivity for hIP6K2, and O-methylation of the 7-OH of **2**, yielding **11**, increased selectivity 2.4-fold (Table 1). Conversely, the addition of a 5'-OH group to **2** (i.e., conversion to **1**) resulted in a 2-fold decrease in selectivity against hIP6K2 vs hIPMK (Table 1).

Finally, as is the case with hIP6K2, disruption to the planarity of the chromen-4-one and phenyl rings also impacts the degree of inhibition of hIPMK. For example, compare **16** with **3** (>5.5-fold loss of activity; Table 1).

Structural Rationalization of Quercetin-Mediated Inhibition of hIPMK. We next performed structural studies to rationalize the molecular recognition processes that underlie the inhibition of hIPMK by **2**, which we successfully soaked into crystals of apo-hIPMK (Figure 3A,B). The electron density of **2** assumes a crescent-like cross section within the nucleotide-binding pocket, with the larger chromen-4-one group penetrating deeper, leaving the smaller phenyl group closest to the entrance (Figure 3A–C). By comparing this new structure of the hIPMK/**2** complex with that of hIPMK/ADP,²³ we observed that the chromen-4-one group is coplanar with the adenine group of ADP (Figure 2C). This direct demonstration of competition by **2** for the nucleotide binding site provides a logical explanation for its inhibition of an InsP

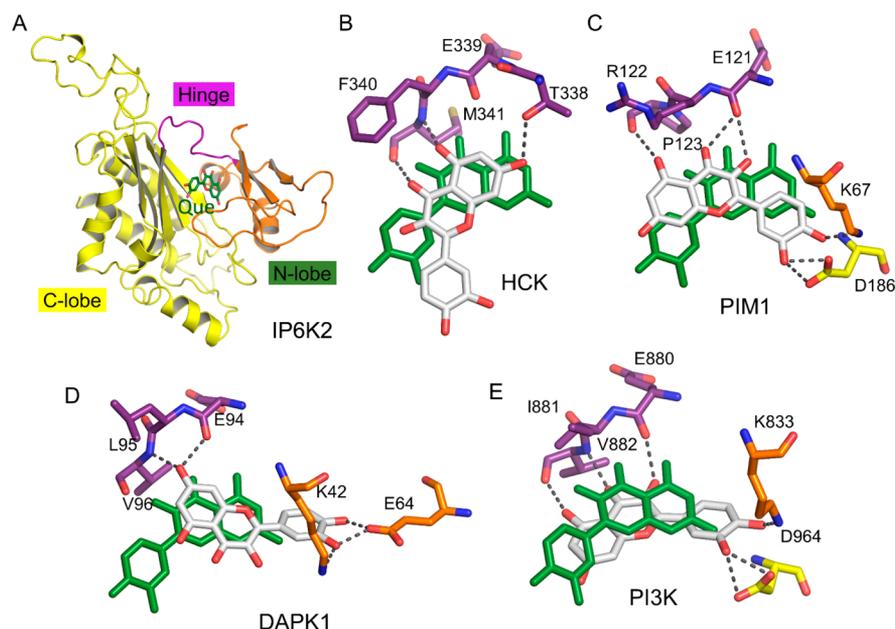


Figure 4. Variations in the relative spatial positioning of **2** in various kinases. (A) Ribbon plot of a homology model of hIP6K2²⁴ into which **2** is docked; color coding is as in Figure 3. (B–E) By use of the conserved nucleotide binding residues such as hinge region, metal binding residues, and invariant lysine, the pose of **2** in the hIPMK crystal complex (green stick model) is superimposed upon the configuration of **2** (light gray stick model; oxygen atoms are shown in red; broken dark gray lines depict polar contacts) for the following kinase/**2** crystal complexes: (B) HCK (PDB code 2HCK); (C) PIM1 (PDB code 2O3P); (D) DAPK1 (PDB code 5AUW); (E) PI3K (PDB code 1E8W).

kinase, thereby countering concern that this activity of the flavonoid might involve experimental artifacts (i.e., PAINS³⁵).

Moreover, the orientation of **2** within the nucleotide-binding pocket of hIPMK allows the flavonoid's ketone group to be positioned similarly to that of the N1 of ATP so that the inhibitor can act as a surrogate by accepting a hydrogen bond from the hinge region of the protein (Figure 3B–G). This situation is typical of the orientation of flavonoids within protein kinases and PI3Ks.²⁵ We also ascertained that the ribose group of ADP is displaced at approximately 120° from the phenyl group of **2**. There is no overlap of **2** with the diphosphate group of ADP.

We identify several polar contacts of **2** with hIPMK that can account for the inhibitory activity of this particular ligand. The 7-OH of **2** lies at the interface of the C- and N-lobes (Figure 3C), where it makes a hydrogen bond with two adjacent water molecules, which we have labeled as Wat1 and Wat2 (Figure 3B). A third water molecule (Wat3; Figure 3B) is associated with this hIPMK/**2** complex through a single hydrogen bond with the 3'-OH group. The identification of water molecules that contribute to ligand binding and target selectivity can be a significant step in inhibitor development;³⁹ such information is missing from the previously published nucleotide-bound structure.²³

Wat1 is particularly well-coordinated, forming four hydrogen bonds with E86 and Y90 from the N-lobe, D385 from the C-lobe, and the 7-OH of **2** (Figure 3B). Wat2 is also involved in ligand binding, as it makes hydrogen bonds with D385 and the 7-OH of **2** (Figure 3B). In the latter structure, the conformation of D385 (Figure 3B,F) is similar to that we previously observed in the inactive apoenzyme (PDB code SW2G).²³ This is an alternative rotamer, compared to its orientation in the nucleotide-bound state (Figure 3G), in which D385 is a member of an ion-paired, catalytic triad of

residues that activates Mg and interacts with the α and β phosphates of ATP.^{22,23}

Elsewhere in **2**, the 3- and 5-OH groups, as well as the 4-keto group, all make hydrogen bonds with the polypeptide backbone at the N-terminus of the hinge region (Figure 3B). Interestingly (see below), we report that the 4'-OH group of **2** is 4.1–4.2 Å distant from the R182 side chain, just outside the 4 Å distance that is typically considered the maximum possible range of a hydrogen bond.⁴⁰

In addition to the polar contacts between hIPMK and **2** (Figure 3B,D), the latter also has van der Waals interactions (Figure 3D) with residues in the N-lobe (I65, V73, K75, P111 and L130), the hinge region (T134), and the C-lobe (I142, L254, I384). We also draw attention to a number of amino acids in hIPMK that interact with **2** but not with ADP: I65, E86, Y90, T134, I142, and I384 (Figure 3D). These data also provide more information on the pharmacophore space than is evident from our previously published analysis of the nucleotide/hIPMK crystal complex.²³

Comparative Analysis of the Relative Spatial Orientation of **2 in Crystal Complexes with Various Kinases.** The rational design of an inhibitor that might be specific for one particular kinase requires the identification of molecular recognition processes that are unique to that target. This approach cannot yet be deployed to assist development of inhibitors that can select between hIPMK and hIP6Ks, since no experimentally determined structures for the latter are available. So instead we have docked **2** into a previously constructed homology model of hIP6K2²⁴ (Figure 4A). This model predicts that **2** adopts comparable orientations in hIP6K2 and hIPMK (Figure 3A,C; Figure 4A).

Data in the literature show the structures of **2** in complex with PI3K γ ²⁵ and some protein kinases.^{41,42} Among these, the orientation of **2** in the HCK/**2** complex⁴² is closest to the pose of **2** in the hIPMK/**2** complex (Figures 3A and 4B). In both

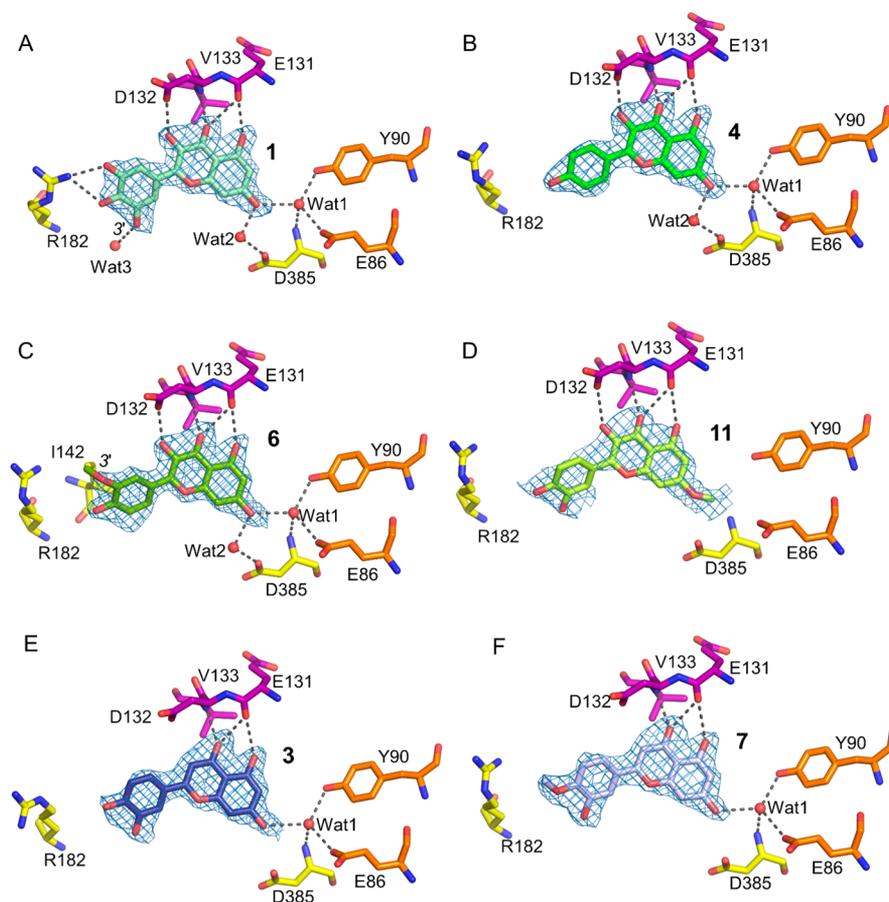


Figure 5. Crystal structure of hIPMK in complex with various flavonoids. (A)–(F) depict the data obtained from X-ray crystallographic analysis of selected flavonoids soaked into the crystal structure of hIPMK. A $2F_o - F_c$ map of each bound compound is shown (sky-blue mesh), contoured at 1σ . Broken lines depict hydrogen-bond interactions with associated water molecules and key residues. To highlight a rotamer switch, the groups attached to the 3'-carbon on the phenyl ring are annotated for compounds **1** and **6** (panels A and C, respectively).

cases, the 5-OH, 7-OH, and the keto group all make hydrogen bonds with the respective hinge regions. Despite these similarities, the relative position of **2** in HCK is rotated approximately 50° relative to its position in hIPMK (Figure 3B). There are other kinases that show more dramatic differences in the orientation of **2** in the ATP-binding pocket, as compared to that for hIPMK. For example, relative to the pose of **2** in hIPMK, there is a transposition of the relative positions of the phenyl ring and the chromen-4-one group in crystal complexes of **2** with either PIM1, DAPK1, and PI3K γ such that it is the phenyl ring that now makes polar contacts with the N-lobe (Figure 4C–E). Moreover, compared to the orientation of **2** in hIPMK, the DAPK1/**2** crystal complex shows that the chromen-4-one group has flipped 180° such that its keto group is now distal to the hinge region (Figure 4D).

These various similarities and differences in inhibitor orientation, as well as the accompanying alternate polar and hydrophobic interactions, together offer new directions for the design of an inhibitor that may show specificity for an InsP kinase over protein kinases and PI3Ks.

Structural Insights into Inhibition of hIPMK by Flavonoids. The rationalization of the inhibitory profiles of various flavonoids upon one particular InsP kinase (as in Table 1) can be confounded if the different ligands adopt alternative configurations within one protein's nucleotide-binding site, as is the case for both PI3K γ ²⁵ and some protein kinases.⁴¹ Thus,

in addition to compound **2** (Figure 3B and see above), we soaked into crystals of hIPMK the other six most potent inhibitors of this kinase (Figure 5A–F; Table 1). Such an approach acts as an orthogonal validation of the inhibitory data. Additionally, a thorough structure–activity analysis “is the most important criterion” for excluding experimental artifacts (i.e., PAINS³⁵).

One of three water molecules in the hIPMK/**2** complex, Wat3, is associated through a single hydrogen bond to the 3'-OH (Figure 3B). Removal of the latter OH group (in **4**) is associated with loss of Wat3 (Figure 5B) and a 2-fold reduction in inhibitory activity (Table 1). Wat3 is also missing from the hIPMK/**6** structure (Figures 2A and 5C), but interestingly, there is a rotamer switch in the ligand's phenyl ring such that the 3'-O-methyl group provides a gain of function 3.4 Å hydrophobic interaction with I142 (Figure 5C). We are not aware of any previous descriptions of this particular rotamer switch. This phenomenon may explain why **6** and **2** are equipotent inhibitors of hIPMK (Table 1), even though Wat3 is missing from the hIPMK/**6** complex. Moreover, the 3'-O-methyl group in **6**, by increasing compound hydrophobicity, is also expected to increase bioavailability by improving passive uptake across the cell membrane.

In the other hIPMK/ligand structural complexes (Figure 5), the orientations of the core structure of the various inhibitors show only subtle variations. These data give significant insight into several determinants of kinase inhibition. For example, we

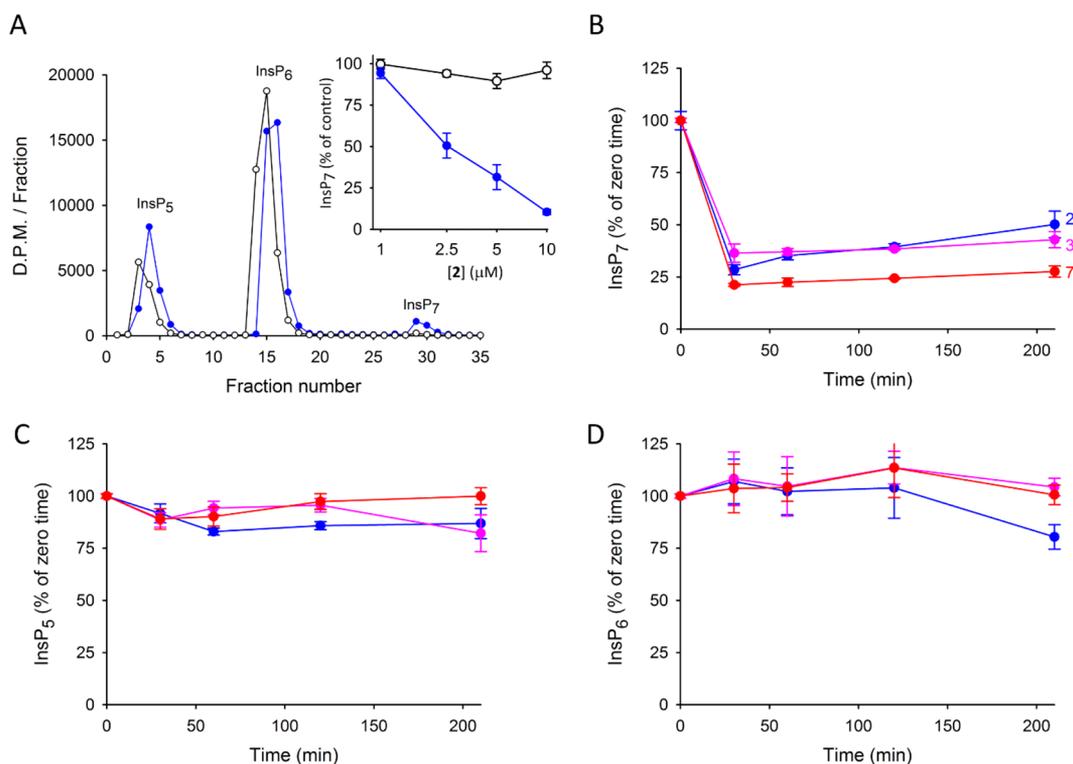


Figure 6. HPLC analysis of the time-dependent effects of selected flavonoids (**2**, **3**, and **7**) upon InsP₅, InsP₆, and InsP₇ in HCT116 cells. (A) Representative HPLC analysis of cell extracts prepared from [³H]inositol-labeled HCT116 cells incubated for 3.5 h with either DMSO vehicle (open circles) or 10 μM **2** (blue circles). The total DPM for each peak is the following: control, InsP₅ = 14 677, InsP₆ = 36 327, InsP₇ = 2160; treated with **2**, InsP₅ = 10 989, InsP₆ = 39 521, InsP₇ = 195. The inset shows the relative levels of InsP₇ in cells treated for 3.5 h with either the indicated concentration of **2** (blue circles) or corresponding DMSO vehicle controls (open circles). (B–D) HCT116 cells were treated with 2.5 μM of either **2** (data colored blue), **3** (pink), or **7** (red) for the indicated times. Levels of the following InsPs were assayed by HPLC: (B) InsP₇, (C) InsP₅, (D) InsP₆. Data are the mean ± standard errors (*n* = 3–4).

obtained structural complexes of hIPMK with **3** and **7** (Figure 5E,F). The latter lacks the 3-OH that in **1**, **2**, **4**, **6**, and **11** forms a hydrogen bond interaction with the hinge region (Figures 3B and 5A–D). These results may at least partly explain why the 3-OH makes about a 2-fold contribution to the degree of inhibition (i.e., compare the hIPMK IC₅₀ data for **2** and **3** in Table 1). Additional structural consequences appear to arise from the absence of the 3-OH in **3** and **7**: there is a change in the atomic-level pivot point at which these flavonoids interact with the hinge region, which may cause distal conformational changes that account for the loss of Wat2 (Figures 3B and 5E,F). These are further observations that help define the pharmacophore space. In any case, **3** and **7** exhibit IC₅₀ values against hIPMK that are 2.4- to 3-fold higher than that for **2** (Table 1).

We found that the addition of the 5'-OH to **2** (which yields **1**) combines with the 4'-OH to form a pincer of hydrogen bonds that brings R182 closer to the phenyl ring (Figure 5A). This can explain why **1** is an approximately 2-fold stronger inhibitor of hIPMK than is **2** (Table 1). In addition, these data reveal further information about the pharmacophore space by showing that the side chain of R182 has flexibility in this structure. This contrasts with the side chains in the hinge region, which appear more rigid, since their orientations are unaffected by changes in the nature of the ligand (Figure 5).

The hIPMK/**11** crystal complex, in which the 7-OH group of **2** is methylated, provides another example of loss of Wat3 (Figure 5D). However, the accompanying loss of Wat1 and Wat2 would seem to have more structurally significant

consequences, since this eliminates a significant hydrogen bond network (Figures 3B and 5D); the latter effect probably makes the largest contribution to the 6-fold higher IC₅₀ value for hIPMK inhibition by **11**, compared to that for **2** (Table 1). Note also that D385 remains in its catalytically inactive configuration (see above) in all of the hIPMK/flavonoidcrystal complexes (Figure 5).

Effects of Flavonoids upon Levels of Inositol Phosphates in Intact Cells. Each of the flavonoids that we have examined in this study is inherently less effective at inhibiting hIPMK than hIP6K2 (Table 1). In addition, IP6Ks have a much lower affinity for ATP than do IPMKs,^{10,43} rendering the latter less susceptible to ATP-competing inhibitors at physiological (1–5 mM) levels of this nucleotide. Thus (see Figure 1), we hypothesized that the addition of flavonoids to intact cells would have a greater impact upon IP6K activity (i.e., synthesis of InsP₇¹⁰) than IPMK activity (synthesis of InsP₅⁴⁴). To interrogate this idea in a physiologically relevant context, we used HCT116 colonic epithelial cells because they are a highly appropriate model of the human body's frontline exposure to significant levels of dietary flavonoids.⁴⁵ Indeed, there is considerable interest in the apparent protection from colon cancer afforded by these natural compounds.⁴⁶ Our treatment of HCT116 cells with the unconjugated flavonoids is also biologically relevant because conjugation hinders their passive diffusion into cells that line the gastrointestinal tract.^{34,45} Furthermore, the HCT116 model is especially pertinent to the current study in that the type 2 isoform of hIP6K that we have studied *in vitro* (Table

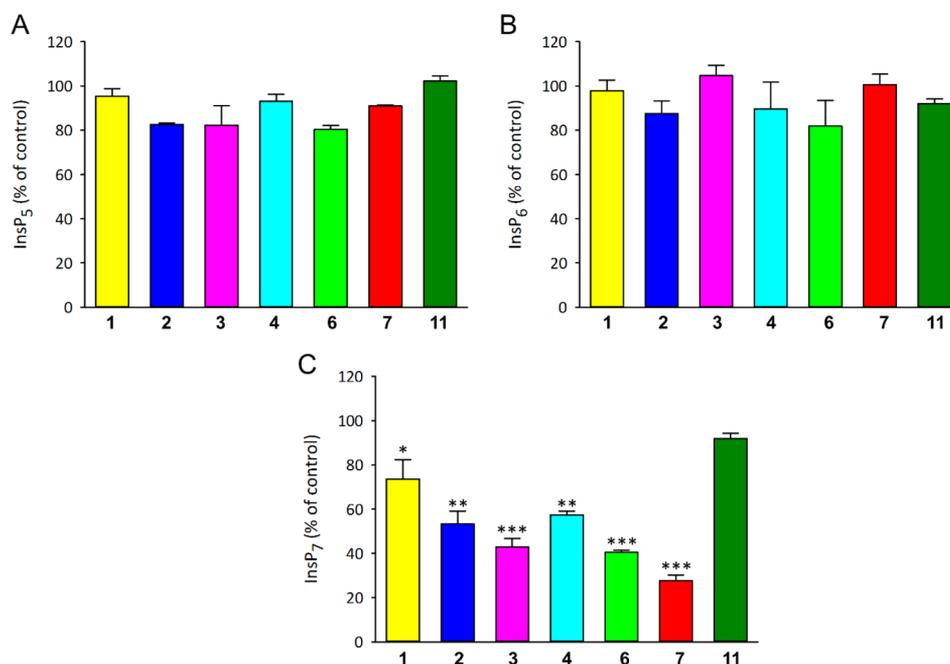


Figure 7. Effects of flavonoids upon the InsP profile in HCT116 cells. (A–C) Levels of the indicated InsPs in HCT116 cells were determined by HPLC as described in the legend to Figure 6A, following 3.5 h treatment with 2.5 μ M of the indicated flavonoids: (A) InsP₅, (B) InsP₆, (C) InsP₇. Data are the mean \pm standard errors ($n = 3$ –4). Data for 2, 3, and 7 are included from Figure 5 for comparative purposes (using the same color-coding): *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.001$; all versus controls.

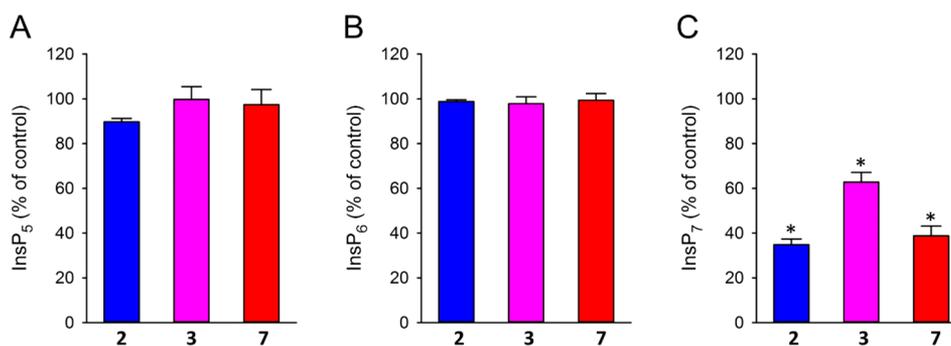


Figure 8. Effects of flavonoids upon the InsP profile in HEK293 cells. (A–C) Levels of the indicated InsPs in HCT116 cells were determined by HPLC as described in the legend to Figure 6A, following 3.5 h treatment with 2.5 μ M of the indicated flavonoids: (A) InsP₅, (B) InsP₆, (C) InsP₇. Data are the mean \pm standard errors ($n = 3$): *, $p < 0.05$ versus corresponding control values.

1) is responsible for approximately 90% of all InsP₇ synthesis in this cell type.⁴⁷ Finally, InsP kinases are located in cell cytoplasm and therefore not susceptible to possible nonspecific effects of flavonoids resulting from their partition into membrane bilayers.⁴⁸

We performed Carbopac-HPLC analysis of [³H]-prelabeled HCT116 cells to record levels of InsP₅, InsP₆, and InsP₇ (open circles in Figure 6A); we initially treated cells with 2. The concentrations of 2 in serum seem unlikely to surpass 1 μ M levels, even after dietary supplementation,⁴⁹ although cells that line the small intestine and colon are exposed to higher levels.^{45,50} We incubated cells with 1–10 μ M 2 for 3.5 h, which caused dose-dependent reductions in InsP₇ levels (Figure 6A), indicative of inhibition of cellular hIP6K2 activity. A dose of 10 μ M 2 reduced InsP₇ levels by 90% (Figure 6A); InsP₆ levels were not affected, while InsP₅ levels were reduced by approximately 25% (Figure 6A), consistent with 2 also being a weak inhibitor of cellular IPMK (Table 1), which synthesizes InsP₅ from InsP₃.^{23,44} These data are consistent with our

hypothesis (see above) that in intact cells the flavonoids used in our study will preferentially inhibit IP6K activity over IPMK activity.

We next obtained time course data, using 2.5 μ M levels of 2, which are sufficient to reduce InsP₇ levels by 50–70% (Figure 6B), without significantly impacting cellular synthesis of either InsP₅ or InsP₆ (Figure 6C,D). The diminution in cellular InsP₇ content was greatest after 30 min of treatment; a slight restoration of InsP₇ levels was observed thereafter, perhaps due to some cellular metabolism of 2.⁴⁵ We also performed a time-course analysis for the effects of 3 and 7 upon intact cells, again at concentrations of 2.5 μ M. We found that 7 produced the most sustained inhibition of InsP₇ synthesis (Figure 6B). Moreover, 7 had no effect upon InsP₅ (Figure 6C) and also did not significantly alter InsP₆ levels (Figure 6D). The degree of inhibition of InsP₇ synthesis by 7 was slightly stronger and more sustained than that of either 2 or 3 (Figure 6B). These demonstrations that flavonoids inhibit IP6K activity in intact

cells serve as an orthogonal validation of the data obtained in vitro with recombinant enzyme (Table 1).

We also determined cellular levels of InsP₅, InsP₆, and InsP₇ after 3.5 h treatment with 2.5 μ M concentrations of **1**, **4**, **6**, and **11**, which together with **2**, **3**, and **7** comprise our seven strongest IP6K inhibitors (Table 1). Levels of InsP₅ and InsP₆ were not significantly affected (Figure 7A,B), and all but **11** inhibited InsP₇ synthesis to varying degrees levels (Figure 7C).

The inability of **11** to reduce InsP₇ in HCT116 cells (Figure 7C) may reflect it being the weakest of the IP6K inhibitors that we used in this experiment (see Table 1). In addition, **11** might be more rapidly inactivated by its metabolism and/or less efficiently accumulated from the culture medium. As for **1**, which only slightly altered InsP₇ levels, its greater degree of hydroxylation (i.e., lower hydrophobicity) may hinder its permeation through the cell membrane. Nevertheless, it is clear from our data (Table 1; Figure 7) that a range of flavonoids are effective inhibitors of IP6K activity in intact HCT116 cells. Among these flavonoids, **7** emerged as the most effective inhibitor of InsP₇ levels.

It is significant that the concentration of **2** that we used in these experiments (2.5 μ M) is equivalent to that recorded in the human small intestine and colon,^{45,50} reflecting it being one of the most abundant flavonoids in the human diet;⁴⁶ dietary supplementation would raise the concentration further. Thus, we conclude that IP6K in cells that line the gastrointestinal tract should be considered as a biologically relevant target of dietary flavonoids.

We have also analyzed the effects of **2**, **3**, and **7** in HEK293 cells (Figure 8A,B,C). We found that all three flavonoids reduced InsP₇ levels, indicating IP6K activity was inhibited in these cells; levels of InsP₅ and InsP₆ were not affected.

Effects of Flavonoids upon AKT Activity in Intact Cells. Flavonoids such as **2** have been shown to inhibit PI3K activity in vitro²⁵, and that effect is proposed to underlie the inhibition of the downstream protein kinase AKT that has been observed in some cell types following their treatment with flavonoids.^{28,29,51,52} However, the alternative possibility that AKT may in some cases be *activated* by flavonoids is raised by our demonstration that this group of molecules inhibit IP6K activity in intact cells (Figures 7 and 8), since the InsP₇ product of IP6K activity is an inhibitor of AKT.^{3,18,53} Indeed, in some cell types, inhibition of IP6K activity by TNP is accompanied by activation of AKT.^{3,18,53}

We used two different cell types to study the net effect of these potentially competing influences of flavonoids upon AKT activity. In HCT116 cells, we did not observe any statistically significant effect upon AKT activity following treatment with 2.5 μ M concentrations of either **2**, **3**, or **7** (Figure 9A), which is sufficient to strongly inhibit cellular IP6K activity (Figures 7 and 8). In control experiments, inhibition of IP6K by TNP also did not affect AKT activity in HCT116 cells (Figure 9A). On the other hand, AKT activity increased following TNP treatment of 3T3-L1 preadipocytes (Figure 9B); similar data were previously reported following TNP addition to adipocytes.¹⁸ We also found AKT activity to be increased upon treatment of 3T3-L1 cells with 2.5 μ M concentrations of either **2**, **3**, or **7** (Figure 9B). Our data (Figure 9A,B) indicate that the nature of the impact of flavonoids upon AKT signaling pathways will be determined by multiple inputs that vary depending upon the cell type.

In conclusion, our detailed structure–activity data, which include new crystal structures of hIPMK in complex with

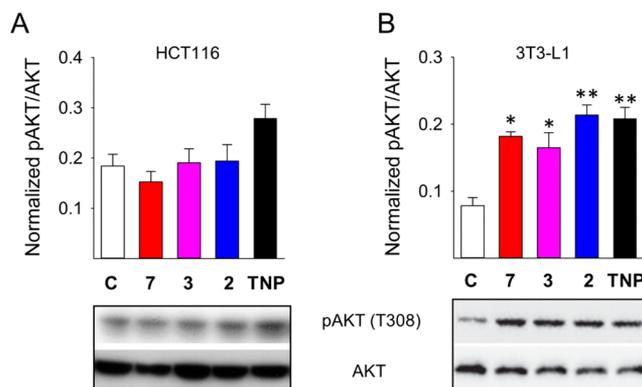


Figure 9. Effects of flavonoids and TNP upon AKT activity in HCT116 cells and 3T3-L1 fibroblasts. Western analysis of AKT activity (T308 phosphorylation) and total AKT in (A) HCT116 cells and (B) 3T3-L1 preadipocytes, in each case following cell treatment for 3 h with either DMSO vehicle control (C), TNP, or 2.5 μ M of either **2**, **3**, or **7**. The upper panels depict the mean \pm standard errors (A, $n = 5$; B, $n = 3$) as a ratio of p-AKT (phosphorylated AKT; T308) versus total AKT. The lower panels provide representative Western blots. Color-coding facilitates comparisons with the data in Figures 6 and 7: *, $p < 0.01$; **, $p < 0.001$; vs control.

various flavonoids, together improve insight into the pharmacophore space within the ATP-binding pocket of InsPKs, which may accelerate the design of InsPK inhibitors. Finally, our data suggest that dietary flavonoids may inhibit IP6K activity in cells that line the gastrointestinal tract, which opens up a new area of focus for those studying the human health significance of flavonoids as dietary supplements.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. Recombinant hIP6K2 and hIPMK were prepared as previously described.^{23,24} The purity of these proteins was estimated to be >90% as judged by SDS–PAGE. The purified proteins were concentrated to between 1 and 2 mg/mL and stored at -80 °C.

Compound IC₅₀ Determination for Inhibition of hIP6K2 and hIPMK. ATP-driven kinase activity was measured by detecting ADP formation from substrate phosphorylation using the Adapta Universal kinase assay. Dilution plates of selected flavonoids (Figure 1) were made using a Freedom EVO liquid handler (Tecan) (3-fold dilutions, 10 points, at 100 \times), using DMSO as diluent into 384-well plates. Compounds were subsequently dispensed using a Mosquito (TTP Labtech) to deliver 50 nL into assay plates to give a 10-point dose–response with the following concentrations (all μ M): 0.005, 0.015, 0.046, 0.14, 0.41, 1.23, 3.7, 11.1, 33.3, 100. The final DMSO concentration was 1%.

The Adapta kinase reaction buffer was used to prepare all additions; it contained 50 mM HEPES, pH 7.5, 0.01% Brij-35, 10 mM MgCl₂, and 1 mM EGTA. A Multidrop Combi reagent dispenser (ThermoFisher) was used to dispense 2.5 μ L of (2 \times) kinase to the assay plates (to final concentrations of 400 nM for hIP6K2 and 12 nM for hIPMK). After a 15 min equilibration period at room temperature, 2.5 μ L of (2 \times) ATP/substrate mix was added to final concentrations of either 10 μ M ATP and 10 μ M InsP₆ (IP6K assays) or 10 μ M ATP and 10 μ M InsP₃ (for IPMK assays). Reaction conditions were selected such that displacement of the ADP tracer from the antibody was 70–80% (according to the vendor's guidelines). The enzymatic reaction was performed for 30 min and the amount of ADP produced was detected by adding 2.5 μ L of detection solution Adapta Eu-anti-ADP antibody, Alexa Fluor 647 ADP tracer, and EDTA, for a final concentration of 2 nM, 12 nM, and 10 mM, respectively (using the provided TR-FRET dilution buffer). After an additional 30 min equilibration period, the plate was read on an EnVision (PerkinElmer)

plate reader (excitation = 320 nm, emission = 665 and 615 nm). The HTRF signal was calculated as a ratio of the signals from the 665 nm (acceptor) and 615 nm (donor) channels. For each plate, percent inhibition was calculated relative to the interquartile mean of 16 wells each of positive controls (DMSO alone) and negative controls (no enzyme added). ADP titration curves were also run routinely to ensure that the enzyme assays produced approximately 20% ATP turnover. IC₅₀ values were generally calculated by applying a four-parameter curve fit using ScreenAble Solutions.

Structural Studies. Crystals of apo-hIPMK were prepared as described previously.²³ Complex crystals were produced by soaking apo crystals into a mixture of 2–10 mM compounds with 35% (w/v) PEG 400, 0.1 M Li₂SO₄, 100 mM HEPES (pH 7.5) at 25 °C for 3 days. Diffraction data were collected using APS beamlines 22-ID and 22-BM. All data were processed with the program HKL2000.⁵⁴ The crystal structures were determined by using rigid body and direct Fourier synthesis and refined with the equivalent and expanded test sets by using programs in the CCP4 package.^{55,56} The molecular graphics representations were prepared with the program PyMol (Schrödinger, LLC). Atomic coordinates and structure factors for hIPMK/ligand complexes have been deposited with the Protein Data Bank with the following accession codes (with ligands in parentheses): 6M88 (1), 6M89 (2), 6M8A (3), 6M8B (4), 6M8C (6), 6M8D (7), 6M8E (11). All structural refinement data are shown in Table 1 in Supporting Information. The structure of hIP6K2 was modeled as previously described,²⁴ and 2 was docked into the structure by superimposing the hIPMK/2 complex structure on the calculated hIP6K2 model.

Cell culture and Assay of Intracellular Inositol Phosphates. The 3T3-L1 fibroblasts were cultured in 6-well plates in DMEM + 10% fetal bovine serum. The culture conditions for HEK293 cells and HCT116 cells, and conditions for [³H]inositol-labeling, are as described previously⁵⁷ except that the concentration of [³H]inositol was increased to 20 μCi/mL. Added flavonoids were dissolved in DMSO; the final concentration of DMSO in the cell cultures (and DMSO vehicle controls) was 0.1%. Cells were PCA-quenched, and [³H]InsPs were extracted and assayed by CarboPac HPLC, as previously described.⁵⁷ Statistical significance of each sample, versus DMSO control, was evaluated by one-way ANOVA (Dunnett's method).

AKT Activity Assays. We studied AKT activity in either 2-day, postconfluent 3T3-L1 preadipocytes or 1-day post confluent HCT116 cells. These cells were treated for 3 h with either DMSO vehicle control or 2.5 μM of the indicated flavonoid (in DMSO) or 1 μM TNP (for 3T3-L1 cells) or 10 μM TNP (for HCT116 cells), also in DMSO. In each case, the final DMSO concentration was 0.1%. Thereafter, each well of cells was washed with ice-cold PBS and lysed in 200 μL of ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and a protease-phosphatase inhibitor tablet (Pierce; no. A32961). The lysate was cleared by centrifugation (16000g for 20 min; 4 °C), and then protein concentrations were assayed with a BCA kit. A total of 20 μg of protein per sample was resolved by SDS-PAGE gel, transferred to either a nitrocellulose or PVDF membrane, and probed with antibodies for both phospho-AKT (T308; Cell Signaling no. 13038; dilution, 1:1000) and total AKT (either Santa Cruz, sc-81434 or Cell Signaling no. 9272; dilution, 1:1000). Band intensities were quantified using the ImageJ software. The intensity of each phospho-AKT band was calculated relative to total AKT, and those resultant ratios were then normalized to the sum of all ratios obtained from a single gel.⁵⁸ Statistical significance of each sample, versus DMSO control, was evaluated by one-way ANOVA (Dunnett's method).

Other Reagents. Compounds 1–8, 11 were purchased from Cayman Chemical, and 9, 10, 12–17 were obtained from Indofine Chemical. [³H]Inositol was purchased from American Radiolabeled Chemicals. InsP₃ was purchased from CellSignals. InsP₆ was procured from Calbiochem. The Adapta Universal kinase assay kits were obtained from Thermo Fisher.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.8b01593.

Supplementary Table 1 of data collection and structure refinement statistics; supplementary Table 2 of molecular formula strings and some data (PDF)

Molecular formula strings and some data (CSV)

Accession Codes

The accession codes for hIPMK/ligand crystal complexes have been deposited with the PDB as follows (ligands in parentheses): 6M88 (1, myricetin), 6M89 (2, quercetin), 6M8A (3, luteolin), 6M8B (4, kaempferol), 6M8C (6, isorhamnetin), 6M8D (7, diosmetin), 6M8E (11, rhamnetin).

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DMSO, dimethylsulfoxide; hIP6K, human inositol hexakisphosphate kinase; hIPMK, human inositol polyphosphate multikinase; InsP, inositol phosphate; InsP₃, inositol 1,4,5-trisphosphate; InsP₅, inositol 1,3,4,5,6-pentakisphosphate; InsP₆, inositol hexakisphosphate; InsP₇, 5-diphosphoinositol 1,2,3,4,6-pentakisphosphate; PBS, phosphate buffered saline; TNP, N₂-(*m*-(trifluoromethyl)benzyl)- N₆-(*p*-nitrobenzyl)-purine; TR-FRET, time-resolved fluorescence resonance energy transfer

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