Multiple Elements Jointly Determine Inhibitor Selectivity of Cyclic Nucleotide Phosphodiesterases 4 and 7*

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Phosphodiesterase (PDE) inhibitors have been widely studied as therapeutics for treatment of human diseases. However, the mechanism by which each PDE family recognizes selectively a category of inhibitors remains a puzzle. Here we report the crystal structure of PDE7A1 catalytic domain in complex with non-selective inhibitor 3-isobutyl-1-methylxanthine and kinetic analysis on the mutants of PDE7A1 and PDE4D2. Our studies suggest at least three elements play critical roles in inhibitor selectivity: 1) the conformation and position of an invariant glutamine, 2) the natures of scaffolding residues, and 3) residues that alter shape and size of the binding pocket. Kinetic analysis shows that single PDE7 to PDE4 mutations increase the sensitivity of PDE7 to PDE4 inhibitors but are not sufficient to render the engineered enzymes comparable with the wild types. The triple S373Y/S377T/I412S mutation of PDE7A1 produces a PDE4-like enzyme, implying that multiple elements must work together to determine inhibitor selectivity.

Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and cGMP that are the second messengers modulating cellular signaling in many biological and metabolic processes (1, 2). The human genome encodes 21 genes of PDE, which are categorized into 11 families. Alternative mRNA splicing of the 21 genes generates over 60 isoforms of PDE in various human tissues (3–5). Molecules of PDEs contain a conserved catalytic domain of about 300 amino acids but exhibit different substrate preferences. Thus, PDE4, 7, and 8 prefer to hydrolyze cAMP, whereas PDE5, 6, and 9 are cGMP-specific. PDE1, 2, 3, 10, and 11 act on both nucleotides (5).

Family-selective inhibitors of PDEs have been widely studied as cardiotonics, vasodilators, smooth muscle relaxants, antidepressants, antithrombotics, antiasthma therapeutics, and agents for improving cognitive functions such as learning and memory (6–12). For example, PDE5 inhibitors sildenafil (Viagra™), vardenafil (Levitra™), and tadalafil (Cialis™) have been used for the treatment of male erectile dysfunction (9), and PDE4 inhibitors have shown potential for the treatment of asthma and chronic obstructive pulmonary disease (8, 12, 13).

PDE7 is abundantly expressed in immune cells and has been implicated in T lymphocyte activation (14–18), although it has also been reported that PDE7A knock-out mice show no deficiency in T cell proliferation (19). Like PDE4, PDE7 prefers to hydrolyze cAMP with a $K_m$ value of 0.03–0.2 μM (20–24). However, PDE7 is not sensitive to PDE4 inhibitors (Fig. 1) and thus was originally named as a “rolipram-insensitive PDE family”. To understand the inhibitor selectivity of the PDE7 and PDE4 families, we have determined the crystal structure of the PDE7A1 catalytic domain in complex with 3-isobutyl-1-methylxanthine (IBMX) and performed mutagenesis on PDE7A1 and PDE4D2. Our results show that several key residues must work together to switch the inhibitor selectivity between PDE4 and PDE7 and thus provide a valuable insight into selectivity of PDE inhibitors.

EXPERIMENTAL PROCEDURES

Subcloning, Protein Expression, and Purification—The cDNA of PDE7A1 was purchased from American Type Culture Collection (BE783968) and subcloned into vector pET32a following the standard protocols. A pair of oligonucleotide primers of GCCCTGATCTCAAAT-TCCCTAAAC and CGCGGCTGAGTTATGATAACCGATTTTC was synthesized for amplification of the PDE7A1 coding region of amino acids 130–482 by PCR. The amplified PDE7A1 DNA and the expression vector pET32a were separately digested by the restriction enzymes BamHI and Xhol, purified from agarose gel, and then ligated by T4 DNA ligase. The plasmid pET32-PDE7A1-(130–482) was transformed into Escherichia coli strain BL21 (codonplus) for overexpression. The E. coli cell carrying pET-PDE7A1 was grown in 2×YT medium at 37 °C to absorbance $A_{600}$ = 1.0, and then 0.1 mM isopropyl β-d-thiogalactopyranoside was added for further growth at 15 °C for 12–16 h.

The catalytic domain of PDE7A1 was purified with three chromatographic columns of nickel-nitriilotriacetic acid (Qiagen), Q-Sepharose (Amersham Biosciences), and Sephacyr S300 (Amersham Biosciences). The nickel-nitriilotriacetic acid affinity column was washed with 15 mM imidazole and eluted with a buffer of 20 mM Tris base, pH 8.0, 50 mM NaCl, 150 mM imidazole, and 1 mM β-mercaptoethanol. After removal of the His tag by thrombin cleavage, the PDE7A1 catalytic domain was loaded into a Q-Sepharose column and eluted with 20 mM Tris base, pH 7.5, 200 mM NaCl, 1 mM β-ME, 1 mM MgCl₂, and 10% glycerol. The PDE7A1 was finally purified by passing through a Sephacyr S300 column in a buffer of 20 mM Tris base, pH 7.5, 1 mM β-ME, and 1 mM MgCl₂. A typical batch of purification yielded about 5 mg of PDE7A1 from 4 liters of cell culture. Purified protein showed a single band in SDS-PAGE and was found to be more than 95% pure.

The cDNA clone of full-length PDE4D2 was purchased from American Type Culture Collection (BF693794) and subcloned into pET15b. The wild type full-length PDE4D2 (1–507) was overexpressed and purified by the same protocols for the PDE4D2 catalytic domain (25). SDS-PAGE showed a purity >95% of the recombinant PDE4D2 (Fig. 2).
The mutants of PDE4D2-(1–507) and PDE7A1-(130–482) were produced with a QuikChange™ site-directed mutagenesis kit and verified by DNA sequencing. All mutants were overexpressed in BL21 (codon- plus) and purified using the same procedure as that for the wild types. The expression levels of the PDE7A1 mutants are comparable with that of the wild type PDE7A1. A typical batch of purification of PDE7A1 mutant produced about 2 mg from 2 liters of cell culture. The purified PDE7A1 mutants are estimated to have purity >95% as shown by the SDS gel (Fig. 2).

**Crystallization and Structure Determination**—Crystals of PDE7A1-IBMX were grown by vapor diffusion. The catalytic domain of 5 mg/ml SDS gel (Fig. 2). PDE7A1 mutants are estimated to have purity >95% as shown by the SDS gel (Fig. 2).

Crystallographic Data Collection and Structure Refinement—X-ray diffraction data were collected on beamline X29 of National Synchrotron Light Source. The PDE7A1-IBMX crystal in liquid nitrogen. Diffraction data were collected on beamline X29 of National Synchrotron Light Source. The PDE7A1-IBMX crystal has the space group P321 with cell dimensions of a = 115.8 and c = 64.3 Å. The diffraction data were processed by the program HKL (26). The raw diffraction images were automatically indexed and integrated by the subroutine Denzpack of HKL, and then all the images were scaled and reduced by subroutine Scalepack of HKL. The statistics on the data process are shown in Table I. The structure of PDE7A1-IBMX was solved by the molecular replacement program AMoRe (27), using the catalytic domain of PDE4D2 as the initial model (25). The structure determination by AMoRe includes two steps: rotation function and translation search. The rotation function of AMoRe produces a peak with correlation coefficient of 0.12 for 3795 reflections at 4.8 Å resolution, which is 6 times the remaining solutions. A further search of the peak by the translation function of AMoRe yielded a correlation coefficient of 0.73 and R factor of 0.31 for 3795 reflections at 4.8 Å resolution. The phases from the molecular replacement were improved by the density modification package of program CCP4 (Collaborative Computational Project Number 4) (28). The atomic model output from AMoRe and the electron density maps from CCP4 were displayed in a Silicon graphic system by program O (29). The amino acid differences between PDE4 and PDE7 and residue positions were built by O for the best fit to the electron density maps. The structure was refined by the program CNS (Crystallography and NMR System) (30).

**Assay of Phosphodiesterase Activities**—The enzymatic activities of the catalytic domain of PDE7A1-(130–482) and full-length PDE4D2-(1–507) were assayed by incubating the enzymes with 100 µl of reaction mixture of 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 50 mM dithiothreitol, and 1 mM EDTA (pH 8.5) at 37°C. The reaction was terminated by addition of 200 µl of 0.2 M Na2CO3. The reaction mixture was incubated at 37°C for 5 min. The absorbance at 405 nm was measured using a microplate reader.

**Kinetic Properties**—The catalytic domain of PDE7A1 has a Kcat value of 0.2 ± 0.03 µM and a kcat value of 1.3 ± 0.1 s⁻¹ for cAMP, in contrast to a Km of 0.2 ± 0.03 µM and a kcat value of 0.7 ± 0.1 s⁻¹ for cGMP. The ratio of the specificity constants (kcat/Km) is 50 times that of PDE4D2. The kcat values of our PDE7A1 catalytic domain is ~5 times that of PDE7A1 refolded from the inclusion bodies (24) and about half that of PDE4D2, suggesting that the purified PDE7A1 catalytic domain has a native-like folding. It is interesting to note that both PDE4D2 and PDE7A1 have similar kcat values for cAMP and cGMP. However, the Kcat value for PDE7A1 is ~500-fold bigger than those for PDE4D2, thus implicating Kcat as the dominant factor of the substrate specificity.
Overall Structure of PDE7—The catalytic domain of PDE7A1 (residues 130–482) contains 17 α-helices (Fig. 3) and has the same topology of folding as do other PDEs (31–40). Two divalent metals were tentatively interpreted as zinc and magnesium for the purpose of structure refinement. Residues 130–138 and 458–482 were not traceable in the structure because of the lack of electron density. The N-terminal fragments of the catalytic domains in the PDE families (130–157 in PDE7A1) do not have comparable amino acid sequences and vary in their secondary structure elements. Among them, PDE4B, PDE4D, and PDE1B (25, 31, 38) containing helices H1 and H2 have a similar folding; PDE3B (40) shows only a comparable helix H2; and PDE5 and PDE9 have different secondary and tertiary structures for their N-terminal residues (36, 37). The catalytic domain of PDE7A1 resembles PDE4 mostly, and their entire catalytic domains are comparable, as shown by an average difference of 0.93 Å for the superposition of Ca atoms of PDE7A1 residues 139–455 on the equivalents of PDE4D2.
FIG. 4. The inhibitor binding. A, stereoview of electron density for IBMX. The $(2F_o - F_c)$ map was calculated from the structure with omission of IBMX and contoured at 1σ. B, IBMX (golden sticks-balls) binding to the active site of PDE7A1 catalytic domain. The hydrogen bonds are represented by dotted lines. C, superposition of rolipram binding residues of PDE4D2 (cyan sticks and blue labels) over those of PDE7A1 (green sticks and red labels). The cyclopentanyl ring of rolipram will clash with the side chain of Ile-412 if Gln-413 forms hydrogen bonds with the oxygen atoms of phenylmethoxy of rolipram. D, surface presentation of the subpocket for the cyclopentanyl group of rolipram in PDE4D2. E, surface presentation for the same binding pocket in PDE7A1. It is clear that the size and shape of the subpocket for the cyclopentanyl group are different.
Structure and Kinetics of PDE7

However, minor differences are observed between PDE7A1 and PDE4D2. First, PDE7A1 has two deletions around residues 297 and 341 (Fig. 3C). Second, two α helices (a type of helix involving 3 residues/turn) in PDE4D2 appear as α-helices in PDE7A1 (Fig. 3C, Hε and H11) and a 310 helix in PDE4D2 (residues 98–102) becomes a coil in PDE7A1. The most significant change is associated with the N-terminal portion of helix H11 (residues 304–310 of PDE7A1), which shows an average positional movement of 2.8 Å, ~3 times the overall average of 0.93 Å for the whole catalytic domain. This structural change appears to be statistically meaningful, but the biological significance is not clear. The positional change of helix H11 may either reflect intrinsic conformational variation between PDE4 and PDE7 or may be due to the tetramer formation in the PDE4D2 crystal, because H11 contributes a small portion of the interface of the PDE4D2 tetramer but is not involved in lattice packing in PDE7A1.

**IBMX Binds to a Subpocket Common for All PDE Inhibitors**—IBMX (Fig. 1) is a non-selective inhibitor for most PDEs and shows IC50 of 2–9 μM for PDE7A and PDE7B (18, 22, 23). The catalytic domain of PDE7A1 has an IC50 of 8.1 ± 0.4 μM for IBMX, thus implying that the binding determinants are located within the catalytic domain of PDE7. This argument is supported by the crystal structure of PDE7A1, in which IBMX occupies the bottom of the deep pocket and is not exposed at the surface of the catalytic domain. The xanthine ring of IBMX stacks against Phe-416 (Fig. 4), and its oxygen O6 forms a hydrogen bond with Gln-413. It also contacts PDE7A1 residues Tyr-211, Val-380, and Phe-384 via van der Waals interactions. Two water molecules bound to O2 and N7 of xanthine, respectively. The isopropyl group of IBMX interacts with residues Phe-384, Ile-412, and Phe-416. The above interactions of IBMX with PDE7A1 are similar to those in other PDEs (36, 37). For an accurate comparison, IBMX binding in the families of PDE4, 5, and 9 was re-examined. IBMX shows good electron density and temperature factors in PDE5 and PDE9 so that both orientation and conformation in these crystals are uniquely defined (36, 37). However, the B-factor, a thermal movement index, for IBMX in PDE4D2 crystal is significantly higher than the average B-factor for all the atoms of the protein (36), indicating a low occupancy or high thermal flexibility of IBMX in PDE4D2. Therefore, we re-adjusted the orientation of IBMX in PDE4D2 (new Protein Data Bank entry code 1ZKN) for the best comparison. Overall, IBMX shows conservation of stacking against phenylalanine and a hydrogen bond to the invariant glutamine in all the PDE families. However, IBMX shows significant positional shifts among PDE4, 5, 7, and 9. For example, IBMX in PDE7A1 has an average positional shift of ~1.3 Å from that in PDE4D2. This may reflect that the large pockets in PDEs allow orientational variation of IBMX, as long as the stacking and the hydrogen bond are conserved. Based on the crystal structures of PDE4, 5, and 9 in complex with IBMX, we proposed that the hydrogen bond with the invariant glutamine and stacking against the phenylalanine are two key components for common binding of all PDE inhibitors (36, 37). This argument is supported by later studies on the structures of PDE4 and PDE5 in complex with various inhibitors (39). However, it remains a puzzle how the conserved catalytic domains of the PDE families selectively recognize their individual inhibitors.

**Inhibition of PDE4D2-(1-507) and PDE7A1-(130-482) by PDE4 inhibitors**

| Rolipram (IC50) | RO-20–1724 (IC50) | Zardaverine (IC50) | Etazolate (IC50) |
|----------------|------------------|-------------------|-----------------|
| PDE4D2 Wild type | 0.55 ± 0.05     | 2.9 ± 0.1        | 1.95 ± 0.15     | 1.1 ± 0.1    |
| PDE4D2 Y329S    | 7.5 ± 1.0       | 16 ± 1           | 75 ± 5          | 2.3 ± 0.2   |
| PDE 7A1 Wild type | 129 ± 10       | 245 ± 5          | 75 ± 8          | 18 ± 1     |
| PDE 7A1 S373Y   | 43 ± 3          | 48 ± 2           | 5.5 ± 0.1       | 6.3 ± 0.8   |
| PDE 7A1 S377T   | 54 ± 6          | 28 ± 2           | 53 ± 7          | 10 ± 1     |
| PDE 7A1 I412S   | 53 ± 9          | 115 ± 5          | 110 ± 10        | 16 ± 1     |
| PDE 7A1 S373Y/I412S | 25 ± 1    | ND               | ND              | ND         |
| PDE 7A1 S373Y/S377T | 23 ± 3 | ND               | ND              | ND         |
| PDE 7A1 S377T/I412S | 32 ± 4 | ND               | ND              | ND         |
| PDE 7A1 S373Y/S3777/I412S | 3.6 ± 0.4 | 5.5 ± 0.5       | 2.3 ± 0.2       | 5.2 ± 0.8   |

Errors were calculated from two or three repeated measurements. ND, not determined.

In summary, the PDE7 structure suggests the inhibitor sensitivity can be explained on the basis of the crystal structures of PDE4D2-rolipram and PDE7A1-IBMX. The structure of PDE4D2-rolipram showed that the rolipram binding in PDE4 is stabilized by two hydrogen bonds with the invariant glutamine Gln-369, whose conformation is fixed by the hydrogen bond to Tyr-329 (25, 32, 39). In PDE7A1, the invariant glutamine Gln-413 does not form a hydrogen bond with the Tyr-329 equivalent, Ser-373, but with Ser-377. The structural superposition between the catalytic domains of PDE7A1 and PDE4D2 shows that Gln-413 in PDE7A1 exhibits the same side chain conformation as that of Gln-369 in PDE4D2 with an average positional shift of 1.1 Å (Fig. 4C). This positional change is slightly higher than the average difference of 0.93 Å between PDE7A1 and PDE4D2 but is about twice those of other active site residues (e.g. 0.56 Å for Ser-373/ Tyr-329). Thus, the conformation and positioning of the glutamine side chain and the scaffolding residues must be the factors for recognition of inhibitors. On the other hand, Ile-412 of PDE7A1, which neighbors on Gln-413, has a positional shift of 1.6 Å from its corresponding partner Ser-368 in PDE4D2, almost twice the overall average difference. The positional change and variation of amino acid type make a different size and shape of the binding pocket in PDE7A1 from PDE4D2 (Fig. 4, D and E), so that Ile-412 may be the third factor for inhibitor selectivity. In fact, modeling of rolipram into PDE7A1 on the basis of the structural superposition shows that the side chain of Ile-412 is located less than 1 Å from the cyclopentanyl group of rolipram (Fig. 4C), indicating that it sterically hinders the binding of rolipram. In summary, the PDE7 structure suggests that at least three factors play roles in inhibitor selectivity: 1) the conformation and positioning of the invariant glutamine (Gln-413 in PDE7A1), 2) residues scaffolding the glutamine such as Tyr-329 in PDE4D2 and Ser-377 in PDE7A1, and 3) residues affecting the shape and size of the binding pockets such as Ile-412 in PDE7A1. Multiple Determinants for Inhibitor Selectivity—To further understand the inhibitor selectivity in PDE4 and PDE7, kinetic...
analysis was carried out on the mutations on residues Ser-373, Ser-377, and Ile-412 of PDE7A1 and Tyr-329 of PDE4D2. Single mutations of the PDE7A1 residues to the equivalents of PDE4D2 showed decrease of the IC_{50} values by 2–3-fold for rolipram (Table II, Fig. 5). Because the changes of IC_{50} values are at least 8-fold that of the experimental errors (Table II), the sensitivity increase of the mutants to rolipram inhibition is biochemically significant. The double PDE7/4 mutations reduced the IC_{50} values for rolipram by 4–6-fold (Fig. 5, Table II). The most dramatic change is the S373Y/S377T/I412S triple mutation that showed IC_{50} of 3.2 μM for (R,S)-rolipram, k_{cat} of 2.7 s^{-1}, and K_{m} of 2.2 μM for cAMP. All three kinetic parameters are very close to 0.55, 3.9, and 1.5 for the wild type PDE4D2, respectively.

Consistently, the PDE7A1 mutants showed increases of sensitivity to the other PDE4 inhibitors (Table II). RO-20–1724 had IC_{50} of 245 μM for the wild type PDE7A1, but the IC_{50} values of the single mutants were reduced by 2–9-fold. The triple mutation exhibited IC_{50} of 5.5 μM, only ~2 times higher than 2.9 μM for the wild type PDE4D2. The dual PDE3/4 inhibitor zardaverine showed IC_{50} values of 75 μM for wild type PDE7A1, 1.0 μM for wild type PDE4D2, and 2.3 μM for the triple mutant of PDE7A1 (Table II). The insensitivity of zardaverine to the I412S mutant of PDE7A1 probably is due to the small fluoride group.

To see whether introduction of a PDE7 residue to PDE4 could make a PDE7-like enzyme, we examined the Y329S mutant of PDE4D2. This single mutation of PDE4D2 resulted in 2–75-fold loss in sensitivity to the PDE4 inhibitors (Fig. 6, Table II), confirming the importance of the tyrosine residue as the scaffold and also cross-verifying the effects observed in the PDE7A1 mutants. In short, the kinetic data suggest that the 3 residues must work together to significantly change inhibitor selectivity.

**DISCUSSION**

Extensive structural studies have shed light on the substrate specificity and inhibitor selectivity of PDEs (25, 31–40). The structures of PDE4 in complex with rolipram (25, 32, 39) showed that an invariant glutamine (Glu-369 in PDE4D2) was carried out on the mutations on residues Ser-373, Ser-377, and Ile-412 of PDE7A1 and Tyr-329 of PDE4D2. Single mutations of the PDE7A1 residues to the equivalents of PDE4D2 showed decrease of the IC_{50} values by 2–3-fold for rolipram (Table II, Fig. 5). Because the changes of IC_{50} values are at least 8-fold that of the experimental errors (Table II), the sensitivity increase of the mutants to rolipram inhibition is biochemically significant. The double PDE7/4 mutations reduced the IC_{50} values for rolipram by 4–6-fold (Fig. 5, Table II). The most dramatic change is the S373Y/S377T/I412S triple mutation that showed IC_{50} of 3.2 μM for (R,S)-rolipram, k_{cat} of 2.7 s^{-1}, and K_{m} of 2.2 μM for cAMP. All three kinetic parameters are very close to 0.55, 3.9, and 1.5 for the wild type PDE4D2, respectively.

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Extensive structural studies have shed light on the substrate specificity and inhibitor selectivity of PDEs (25, 31–40). The structures of PDE4 in complex with rolipram (25, 32, 39) showed that an invariant glutamine (Glu-369 in PDE4D2)
forms two hydrogen bonds with rolipram, implying a critical role of the glutamine in the inhibitor binding. On the other hand, the opposite orientation of the side chain of the glutamine in the PDE4 and PDE5 structures (36, 38) also suggests a key role of glutamine in the substrate specificity. Comparison of the structures of PDE4, PDE5, and PDE9 in complex with IBMX leads to identification of a common pocket for non-selective binding for all PDE inhibitors (36, 37). The structures of PDE4 and PDE5 in complex with various inhibitors support the proposal of the common pocket and identify other subpockets that are important for inhibitor binding (39). The structural and kinetic studies on the mutants in this report quantitatively show the role of several residues, thus a further insight into the inhibitor selectivity.

PDE7 was originally named as a family of “rolipram-insensitive PDE”, but it has remained unknown why PDE7 is not sensitive to PDE4 inhibitors. To explain the poor inhibition of PDE7 activity by PDE4 inhibitors, we previously proposed a rolipram-recognition triad (Tyr-329, Thr-333, and Gln-369 in PDE4D2, or Ser-373, Ser-377, and Gln-413 in PDE7A1), based on the observation that the side chain conformation of invariant Gln-369 is stabilized by a hydrogen bond to Tyr-329 in the crystal structures of PDE4D2 (25). It was also predicted that the replacement of Tyr-329 with Ser-373 in PDE7A1 would switch the side chain of Gln-413 away from Ser-373 to form a hydrogen bond with Ser-377, thus demolishing the hydrogen bonds between the glutamine and rolipram (25).

The PDE7A1-IBMX structure reveals the hydrogen bond between Gln-413 and Ser-377, confirming the earlier prediction (25). However, the invariant glutamine (Gln-413 in PDE7A1) does not change its side chain conformation but its position, thus suggesting that the positioning of the glutamine is critical for inhibitor binding. In addition, our study shows that two groups of residues are critical for the inhibitor selectivity. One is the scaffolding residues such as Tyr-329 in PDE4D2 and Ser-377 in PDE7A1, which support the conformation and position of the invariant glutamine. Another is residues such as Ile-412 in PDE7A1 that affect the shape and size of the binding pocket.

The significant change of inhibitor sensitivity by the single mutations of PDE7A1 and PDE4D2 can be explained on a structural basis. The S373Y mutation in PDE7A1 does not change its side chain conformation but its position, thus suggesting that the positioning of the glutamine is critical for inhibitor binding. In addition, our study shows that two groups of residues are critical for the inhibitor selectivity. One is the scaffolding residues such as Tyr-329 in PDE4D2 and Ser-377 in PDE7A1, which support the conformation and position of the invariant glutamine. Another is residues such as Ile-412 in PDE7A1 that affect the shape and size of the binding pocket.

Therefore, the understanding of the inhibitor selectivity of PDE4 and PDE7 in this report should be applicable to other PDE families and provide a general guideline for design of highly selective inhibitors of PDE families.

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