Structural basis for selective inhibition of human PKG Iα by the balanol-like compound N46

Liying Qin1, Banumathi Sankaran2, Sahar Aminzadeh1, Darren E. Casteel1, and Choel Kim1*2

From the 1Verna and Marris McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, the 2Berkeley Center for Structural Biology, Lawrence Berkeley National Laboratory, Berkeley, California 94720, the 3Department of Medicine, University of California, San Diego, La Jolla, California 92039, and the 4Department of Pharmacology and Chemical Biology, Baylor College of Medicine, Houston, Texas 77030

Edited by Henrik G. Dohlman

Activation of protein kinase G (PKG) Iα in nociceptive neurons induces long-term hyperexcitability that causes chronic pain. Recently, a derivative of the fungal metabolite balanol, N46, has been reported to inhibit PKG Iα with high potency and selectivity and attenuate thermal hyperalgesia and osteoarthritic pain. Here we determined co-crystal structures of the PKG Iα C-domain and cAMP-dependent protein kinase (PKA) Cα, each bound with N46, at 1.98 Å and 2.65 Å, respectively. N46 binds the active site with its external phenyl ring, specifically interacting with the glycine-rich loop and the αC helix. Phe-371 at the PKG Iα glycine-rich loop is oriented parallel to the phenyl ring of N46, forming a strong π-stacking interaction, whereas the analogous Phe-54 in PKA Cα rotates 30° and forms a weaker interaction. Structural comparison revealed that steric hindrance between the preceding Ser-53 and the propoxy group of the phenyl ring may explain the weaker interaction with PKA Cα. The analogous Gly-370 in PKG Iα, however, causes little steric hindrance with Phe-371. Moreover, Ile-406 on the αC helix forms a hydrophobic interaction with N46 whereas its counterpart in PKA, Thr-88, does not. Substituting these residues in PKG Iα with those in PKA Cα increases the IC50 values for N46, whereas replacing these residues in PKA Cα with those in PKG Iα reduces the IC50 values, consistent with our structural findings. In conclusion, our results explain the structural basis for N46-mediated selective inhibition of human PKG Iα and provide a starting point for structure-guided design of selective PKG Iα inhibitors.

Chronic pain is a debilitating condition that affects nearly 25 million adults in the United States (1). Opioid pain relievers are the most prescribed medication class in the United States (2). The increasing prescription of opioid pain relievers is associated with a dramatic increase in opioid misuse, abuse, overdose, and opioid use disorder, contributing to a $50 billion economic cost in the United States in 2015 and more than 63,600 opioid overdose deaths in 2016 (2–6). Another major category of analgesics, COX inhibitors, has long-term cardiovascular side effects (7). Therefore, a new type of nonopioid-based pain reliever is in demand for effective pain management.

Reversible protein phosphorylation regulates all aspects of cell survival. Consequently, dysregulation of protein kinases is often involved in human diseases such as cancer (8), diabetes (9–11), and chronic pain (12, 13). More than 30 protein kinase inhibitors have been approved by the Food and Drug Administration in the past 23 years, with the majority of them targeting tyrosine kinases for cancer treatment (14).

Beyond its role as a central regulator of smooth muscle tone, cGMP-dependent protein kinase (PKG)3 Iα activation in nociceptive neurons results in long-term hyperexcitability that causes chronic pain (15, 16). PKG Iα is also a crucial modulator of cortical neuronal activity in pathological pain; thus, it represents a novel target for developing analgesic therapeutic agents (17). A recent study demonstrated that N46, a derivative of the fungal metabolite balanol, inhibits PKG Iα with high potency and selectivity, resulting in attenuation of thermal hyperalgesia and osteoarthritic pain in rats (18).

PKG Iα belongs to the AGC kinase family and consists of N-terminal regulatory (R) and C-terminal catalytic (C) domains (Fig. 1A) (19, 20). PKG Iα shares a large degree of sequence similarity with cAMP-dependent protein kinase (PKA). In particular, the PKG Iα C-domain shows 45% sequence identity with the PKA Cα, consistent with their similar structures. The C-domain includes small and large lobes that consist of mostly β strands and α helices, respectively. A highly acidic active site is formed between the two lobes that binds Mg2+, ATP, and substrates. In the absence of cGMP, the activity of PKG Iα is negatively regulated by the interaction between the R- and C-domains (21, 22).

Three classes of small-molecule PKG inhibitors have been widely used for functional studies of PKG (23, 24). The first...
class is the R-diastereomer of the phosphorothioate analogs of cGMP, including Rp-cGMPS (25). This compound binds the R-domain and stabilizes its inactive state without causing conformational changes required for activation (26). The second class consists of small molecules that compete with ATP by directly binding the active site within the C-domain. These reagents include H-89, balanol, and KT-5823 (27–32). The third class includes peptide inhibitors that also bind the active site and prevent substrate binding. However, all of these inhibitors lack potency, specificity, and activity in vivo. For example, Rp-cGMPS is not potent (Kᵢ = 20 μM) and nonselectively inhibits other cyclic nucleotide effectors, such as phosphodiesterase and PKA (23). KT-5823 also inhibits other kinases and may not inhibit PKG in intact cells (33). Despite its high potency in vitro, DT-2 does not inhibit PKG in platelets or in rat mesangial cells (34).

As mentioned, balanol is a potent inhibitor of PKG but also inhibits other serine and threonine kinases such as PKA, most PKC isoforms, and Ca²⁺-dependent protein kinase (30, 35). To improve inhibitor selectivity for PKG Iα, a homology model of PKG Iα docked with balanol was generated based on the crystal structure of the PKA Ca⁺—balanol complex. Several amino acid differences near their binding pockets were identified, and balanol was modified to preferentially interact with PKG Iα-specific residues (18). In particular, the homology model showed that Thr-88 of PKA Ca⁺ corresponds to Ile-406 in PKG Iα (16). To exploit this difference, a propoxy group was added to the external phenyl ring (ring D) of the balanol derivatives to
selectively interact with Ile-406 of PKG Iα. Although one such compound, N46, was reported to have a high selectivity and potency for PKG Iα over PKA Cα, the exact molecular basis of its improved affinity and specificity is unknown.

Results and discussion

Several crystal structures have been solved for mammalian PKG I, but these are of various fragments of the R-domains (36–39). Because N46 directly targets the C-domain of PKG Iα, we first obtained an isolated C-domain that is fully active. To understand the molecular basis of N46’s high selectivity for human PKG Iα, we determined co-crystal structures of N46 bound to the human PKG Iα C-domain and human PKA Cα at 1.98 Å and 2.65 Å, respectively (Fig. 1 and Fig. S1 and Table S1). The PKG Iα C–N46 complex was crystallized in the P4_12_1 space group with one molecule in the asymmetric unit. The molecule shows clear electron density for the bound N46 and the C-domain used for crystallization, excluding the first 10 residues at the N terminus (Fig. 1B). The PKA Cα–N46 complex was crystallized in the P3_21 space group with one molecule in the asymmetric unit (Fig. 1C and Fig. S1). The final model shows clear density for the Cα subunit except for the first 10 residues. Unlike previous PKA Cα structures, the N-terminal αA helix disengages from the catalytic core because of unusual crystal packing interactions (Fig. S2). The αA helix of a neighboring symmetry mate occupies the equivalent position seen in previous structures and provides the same set of interactions with the catalytic core.

The overall structure of the PKG Iα C–N46 complex is similar to the AMP-PNP-bound structure.4 It shows a closed conformation with the fully ordered glycine-rich loop and C-terminal tail (Fig. 1B). N46 binds to a pocket that extends from the hinge region to the inner surface of the αC helix and spans ~20 Å (Fig. 2A). The pocket can be divided into three subsites according to the interaction between the PKG Iα C-domain and AMP-PNP: the adenine, the ribose, and the extended triphosphate subsites. N46 binds to all three subsites in the extended active site of the PKG Iα C-domain (Fig. 2A).

The A-ring (indazole ring) binds the adenine subsite consisting of the hinge (loop between β5-αD) and hydrophobic residues from both small and large lobes (Fig. 2B). Specifically, the protonated 1-N binds the backbone carbonyl of Glu-439, whereas the unprotonated 2-N interacts with the backbone amide of Cys-441 through hydrogen bonds. Additionally, the indazole ring is surrounded by several hydrophobic residues that coat the adenine subsite. These residues include Leu-366, Val-374, Ala-388, Val-422, Met-438, Ile-491, Val-501, and Phe-649.

The B-ring (pyrrolidine ring), which connects the A-ring to the C-ring, interacts with the acidic ribose subsite directly and indirectly through water molecules (Fig. 2B). The ribose subsite consists of the hinge and activation loop residues. The side chains of Glu-445 at the hinge and Asp-502 at the activation loop form hydrogen bonds with the amine groups on either side. Two water molecules bridge the interaction with N46 at this subsite. These water molecules are located adjacent to the amide connecting the B-ring to the A-ring, bridging them to the side chains of Glu-445 and Asp-502 through hydrogen bonds.

The C-ring (phenyl ring) interacts with β1 and the glycine-rich loop through van der Waals (VDW) contacts (Fig. 2B). In particular, Val-368, Gly-369, and Gly-370 are within 3.4–3.8 Å from the C-ring, providing VDW interactions. Because these interactions are through backbone atoms, this region does not provide any PKG-selective contacts.

The D-ring (external phenyl ring) with the propoxy and methoxy groups provides two interactions that are PKG-specific and may explain its high selectivity for PKG Iα over PKA Cα (Fig. 2B). In designing N46, the propoxy group was added to the phenyl ring to provide a preferential interaction with Ile-406 of PKG Iα over PKA Cα, which has a threonine (Thr-88) at the analogous position (Fig. S3)(18). However, the structure shows that the methoxy group points toward the side chain of Ile-406 instead, whereas the propoxy group points toward the

---

4 L. Qin, B. Sankaran, D. E. Casteel, and C. Kim, unpublished data.
hydrophobic pocket consisting of Leu-49, Val-57, Ala-70, Val-104, Met-120, Leu-173, and Phe-327 (Fig. 3B). Tyr-122 at the hinge region provides an additional hydrophobic contact unseen in PKG Iα because Tyr-122 replaces Ala-440 of PKG Iα. Although the B-ring similarly docks onto the ribose subsite, its amine group interacts only with the hinge residue Glu-127 through a hydrogen bond, not with the activation loop residue Asp-184 (Fig. 3B). Unlike Asp-502 of PKG I, which forms a hydrogen bond with N46 (Fig. 2B), the side chain of Asp-184 points away and no longer interacts with N46 in PKA. The C-ring similarly docks to θ1 and the glycine-rich loop and interacts with the backbone atoms of Thr-51, Gly-52, and Ser-53.

The D-ring interacts less strongly with PKA Ca compared with PKG Iα because of two PKA-specific residues, Phe-54 and Thr-88 (Fig. 3B). The structure shows that the side chain of Phe-54 at the tip of the glycine-rich loop rotates ~30° and provides a weaker T-shaped π interaction with the D-ring. Because of this rotation, the interconnecting carbonyl no longer forms a lone pair–π interaction with the aromatic Phe-54. In addition, the side chain of Thr-88 of the αC helix is smaller and less hydrophobic than that of Ile-406 of PKG Iα, thus providing a much weaker hydrophobic interaction with the methoxy group (3.7 Å) (Fig. 3B). The structural alignment with the PKG Iα C–N46 complex suggests that a steric clash between the side chain of the preceding residue, Ser-53, and the propoxy moiety causes the rotation of the Phe-54 side chain. As seen in Fig. S3, N46 moves away slightly from the active site because of the steric clash. This allows more room between the D-ring and the glycine-rich loop, causing the rotation of the Phe-54 side chain.

The reported inhibition constant of balanol for PKA Ca is 1.6 nM, whereas N46 inhibits PKA with an IC_{50} of 1.0 μM, showing an over 600-fold increase (18). Comparing the PKA Ca–N46 complex with the PKA Ca–balanol complex reveals that this reduction is mostly due to loss of hydrogen bonds (Fig. 4). The PKA Ca–balanol complex shows 12 nonsolvent mediated hydrogen bonds and large numbers of VDW interactions between the extended active site and balanol. The PKA Ca–N46 complex shows that, although the most of the VDW contacts are preserved, N46 forms only 6 direct hydrogen bonds because of the modifications on the C and D rings.

Substituting the phenol of balanol (Fig. 4A, ring a) with the indazole ring of N46 (Fig. 4B, ring A) does not reduce the number of hydrogen bonds and VDW contacts with the adenine subsite. In the PKA–balanol complex, the phenol forms hydrogen bonds with the same backbone atoms of Glu-121 and Val-123 at the hinge region (Fig. 4A) as the indazole. However, replacing a more puckered azepane ring of balanol (Fig. 4A, ring b) with a less puckered pyrroline of N46 (Fig. 4B, ring B) results in one additional hydrogen bond at the ribose subsite. The puckered azepane ring interacts mainly with a conserved catalytic loop residue, Glu-170, through its backbone (Fig. 4A). In the PKA–N46 complex, the less puckered pyrrolidine ring brings its amine group within a hydrogen bonding distance of the Glu-127 side chain, forming a new hydrogen bond (Fig. 4B).

Removing two hydroxyl groups from the c-ring of balanol (Fig. 4A) disrupts all four hydrogen bonds with the triphosphate subsite. In the PKA Ca–balanol complex, two hydroxyl groups on the c-ring interact with Gly-55, Lys-72, and Asp-184...
through four hydrogen bonds. In major contrast, the C-ring of N46 (Fig. 4B) no longer binds these residues and interacts with the glycine-rich loop through VDW contacts. Last, substituting a carboxyl group and a hydroxyl group on the d-ring of balanol (Fig. 4A) with a bulky and hydrophobic propoxy group and a fluorine atom, respectively (Fig. 4B, ring D of N46), significantly weakened the interaction with the glycine-rich loop and the αC helix. In the PKA–balanol complex, the carboxyl group on the d-ring forms strong hydrogen bonds with both the side chain and backbone of Ser-53 at the glycine-rich loop, whereas the 3-hydroxy group binds the side chains of Glu-91 and Lys-72 through hydrogen bonds. Additionally, the d-ring is oriented parallel to the side chain of Phe-54, allowing a parallel π-stacking interaction between them as well as a lone pair–π interaction between the carbonyl group and Phe-54. None of these interactions are preserved in the PKA–N46 complex, although a new hydrogen bond forms between the propoxy group and the backbone amide of Ser-53. We noticed that the side chain of Phe-54 remains parallel to the d-ring when bound to balanol and rotates when bound to N46 (35). The balanol-bound PKA structure shows that this is because balanol binds deeper into the pocket, allowing a parallel π-stacking interaction with Phe-54 (Fig. S4A). In contrast, N46 cannot bind as deeply because of its bulky methoxy group, resulting in enough space between the D-ring and Phe-54, which allows Phe-54 to rotate to provide VDW contact with the D-ring (Fig. S4B).

To test the molecular basis for N46’s PKG Iα-selective inhibition over PKA, we mutated the unique contact residues in PKG Iα to those in PKA and vice versa. Specifically, for PKG Iα, we mutated Gly-370 and Ile-406 to the corresponding residues in PKA Cα (i.e. G370S and I406T). We also mutated these two PKA Cα residues into the corresponding PKG Iα residues (S53G and T88I). For PKG Iα, we generated two single mutants (G370S and I406T) and a double mutant (G370S/I406T). For PKA Cα, we only generated a double mutant (S53G/T88I). We then measured IC50 values using in vitro kinase assays (Fig. 5). N46 showed an IC50 of 43 nM for WT PKG Iα, whereas it inhibited PKA Cα with an IC50 of 1030 nM, showing an ~24-fold difference in selectivity. The PKG Iα single mutants were inhibited with higher IC50 values of 90 nM and 142 nM for G370S and I406T, respectively. The double mutant PKG Iα showed an IC50 value of 301 nM, demonstrating a synergistic effect of the two mutations. In contrast, the PKA Cα double mutant showed an IC50 of 552 nM, which is almost half of that seen in WT Cα. The higher IC50 values seen in the PKG Iα mutants and the lower value of the PKA Cα double mutant compared with their respective WT are consistent with our structural findings. Despite lack of data on inhibition constants of N46 for other kinases, our model of a PKCα isoform (PDB code 3IW4) docked with N46 suggests that N46 is a poor inhibitor for the PKCα isoform (Fig. S5) (40). The model shows that the tip of the glycine-rich loop curls in toward the active site and clashes with the C-ring. In particular, Phe-350 at the glycine-rich loop occupies the part of the pocket the C-ring binds, suggesting that N46 would interact poorly with PKCα. Consistent with the model, Sung et al. (18) reported that, at 0.75 μM of N46, PKC8 had 68% residual activity, whereas PKG Iα was completely inhibited with 0% residual activity.

Our structural and biochemical data suggest new strategies for generating N46 derivatives with higher selectivity for PKG Iα over PKA Cα. Amino acid sequences at the hinge region and β7 that make up the left edge and the base of the adenine pocket are different in PKG Iα compared with PKA Cα. PKG Iα has Ala-Cys-Leu (residues 440–442) at the hinge, whereas PKA has Tyr-Val-Pro (residues 122–124) (Fig. S6). This causes PKG Iα to have a wider adenine pocket compared with the PKA Cα subunit (Fig. 6). Additionally, at the base of the adenine pocket, PKG Iα has an isoleucine (Ile-491 at β7) replacing a leucine (Leu-173) of PKA Cα, providing a slightly deeper pocket. Thus, to improve selectivity for PKG Iα, bulkier heterocyclic rings could be engineered in N46 to fill this unique pocket. Also, a reactive group can be placed here to covalently link to Cys-441 because PKA lacks a cysteine residue at the analogous position (Fig. S6). During the initial design of N46, the propoxy group was added to increase its interaction with Ile-406 at the αC helix. However, our structures revealed that this group points in an opposite direction (toward the glycine-rich loop) and interacts with Gly-370 instead. Thus, it may be possible to add an additional ethyl or propyl group here to improve interaction with PKG Iα. This modification should cause steric hindrance with Ser-53 of PKA at the glycine-rich loop while providing additional nonpolar interactions with Gly-370 in PKG Iα. In conclusion, our structural and biochemical data in part explain N46’s selectivity for PKG Iα and provide a starting point for structure-guided design of selective PKG Iα inhibitors.
**Experimental procedures**

### Expression and purification of the hPKG Iα C-domain

The sequence encoding the human PKG Iα C-domain (327–671) was cloned into the pBlueBacHis2A vector. The vector was modified to put a tobacco etch virus (TEV) protease site just before the PKG coding sequence. The protein was expressed in High Five cells. The cells were grown at 28 °C and infected at a multiplicity of infection of 3.0 for 32 h. All cells were lysed in buffer A (25 mM Tris (pH 7.5), 500 mM NaCl, and 1 mM β-mercaptoethanol) with a Constant Systems TS cell disrupter (Daventry Northants, UK) and cleared via ultracentrifugation. The supernatant was loaded onto a Bio-Rad Nuvia nickel affinity column, washed with buffer A and eluted with buffer A containing 300 mM imidazole. The His tag was removed by incubating the sample with TEV protease at 4 °C overnight. TEV was removed from the protein sample by performing a second nickel affinity chromatography and collecting the flow-through fractions. The sample was further purified by anion exchange chromatography (Mono Q 10/100 GL, GE Healthcare) in buffer B (25 mM Tris (pH 7.5) and 1 mM β-mercaptoethanol) with and without 1 M sodium chloride. This was followed by size exclusion chromatography (HiLoad 16/60 Superdex 75, GE Healthcare) in buffer C (25 mM Tris (pH 7.5), 150 mM sodium chloride, and 1 mM tris(2-carboxyethyl)phosphine (TCEP)).

### Expression and purification of hPKA Cα

The pET15b plasmid encoding human PKA Cα was transformed into BL21 (DE3) *Escherichia coli* cells. The cells were grown at 37 °C until OD$_{600}$ = 1.0 was reached. The expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside at 18 °C for 18 h. The cells were then lysed by the Constant Systems TS cell disruptor in buffer A. The lysate was then cleared by ultracentrifugation and membrane filtration. The supernatant was applied onto a GE His-Trap column for nickel affinity purification. The protein was eluted by buffer A containing 300 mM imidazole. The His tag was removed by incubating the sample with TEV protease at 4 °C overnight, followed by a second nickel affinity chromatography. The protein was then further purified by anion exchange chromatography (Mono Q 10/100 GL, GE Healthcare) in buffer B (25 mM Tris (pH 7.5) and 1 mM β-mercaptoethanol) with and without 1 M sodium chloride.
purified by anion exchange chromatography (anion exchange chromatography, Mono Q 10/100 GL, GE Healthcare) in buffer D (25 mM potassium phosphate (pH 7.0) and 1 mM β-mercaptoethanol) with and without 1 mM sodium chloride. This was followed by size exclusion chromatography (HiLoad 16/60 Superdex 75, GE Healthcare) in buffer C.

**Crystallization and structure determination**

To obtain crystals of the PKG Iα C-domain–N46 complex, 14 mg ml⁻¹ of the PKG Iα C-domain solution was incubated with 1 mM N46 for 30 min at room temperature. Crystals were obtained by mixing 1 μl of the C-domain–N46 complex solution with 1 μl of well solution (24% w/v PEG 1500 and 20% v/v glycerol) and 0.2 μl of additive (30% w/v trimethylamine N-oxide dihydrate) at 22 °C. To obtain crystals of the PKA Caα–N46 complex, 12 mg ml⁻¹ of PKA Caα was incubated with 1 mM of N46 for 30 min at room temperature. Crystals were obtained by mixing 0.2 μl of the C-domain–N46 complex solution with 16% (w/v) PEG 8000, 0.04 M potassium phosphate (monobasic), and 20% (v/v) glycerol. PKG Iα C-domain and PKA Ca crystals were cryoprotected by paratone, and diffraction images were collected at the Advanced Light Source (Berkeley, CA). Data were processed using CCP4/Imosfilm (41). The structures of the PKG Iα C-domain–N46 and PKA Caα–N46 complexes were determined by Phaser-MR using AMP-PNP–bound PKG Iα C-domain (PDB code 6BG2) and balanol-bound PKA Ca (PDB code 1BX6) as molecular replacement probes (42). Both final structures were manually built using Coot and refined using Phenix:Refine (43, 44). The figures were generated using PyMOL (Schrodinger, LLC).

**In vitro kinase assays**

FLAG-tagged WT and mutant PKG Iα proteins were purified from transiently transfected 293T cells as described previously (45). PKA Caα WT and its mutant were purified as described above. The purified kinases were diluted in kinase dilution buffer (10 mM potassium phosphate (pH 7.0), 1 mM EDTA, 35 mM β-mercaptoethanol, and 0.1% BSA) so that the reactions produced ~10⁵ counts per reaction (corresponding to about 36 pmol phosphate incorporation). Reactions were initiated by adding 10 μl of diluted kinase to 5 μl of 3× kinase reaction mixture (120 mM HEPES (pH 7.4), 1.56 mg/ml Kemp tide, 30 mM MgCl₂, 300 μM ATP, 360 μCi/ml [γ-³²P]ATP and 30 μM cGMP) containing variable amounts of the N46 inhibitor diluted in DMSO (control assays contained DMSO alone). Reactions were run for 1.5 min at 30 °C and stopped by spotting on P81 phosphocellulose paper. Unincorporated [γ-³²P]ATP was removed by washing P81 paper 4 × 2 liters in 0.45% O-phosphoric acid. ³²P incorporation was measured by liquid scintillation counting. The data were analyzed using GraphPad Prism 7.

**Acknowledgments**—We thank Andrey Kovalevsky and Friedrich W. Herberg for critical reading of the manuscript. We also thank Paul Leonard (MD Anderson Cancer Center) for assistance with the initial screening of the PKG Iα–N46 complex crystals and Ying-Ju Sung (Geisinger Commonwealth School of Medicine) for kindly providing N46. The Berkeley Center for Structural Biology is supported in part by the National Institutes of Health; the NIGMS, National Institutes of Health; and the Howard Hughes Medical Institute. The ALS-ENABLE beamlines are supported in part by NIGMS, National Institutes of Health National Grant P30 GM124169-01. The Advanced Light Source is a Department of Energy Office of Science User Facility under Contract DE-AC02-05CH11231. The Pilatus detector was funded under National Institutes of Health Grant S10OD021832.

**References**

1. Walitt, B., Nahin, R. L., Katz, R. S., Bergman, M. J., and Wolfe, F. (2015) The prevalence and characteristics of fibromyalgia in the 2012 national health interview survey. PLoS ONE 10, e0138024 CrossRef Medline
2. U.S. Department of Health and Human Services (HHS), Office of the Surgeon General (2016) Facing addiction in America: The Surgeon General’s report on alcohol, drugs, and health. U.S. Department of Health and Human Services (HHS), Office of the Surgeon General, Washington, D.C. Medline
3. Ryen, S. A. (2018) Calculating the real costs of the opioid crisis. Pediatrics 141, e20174129 CrossRef Medline
4. The Council of Economic Advisers (2017) The Underestimated Cost of the Opioid Crisis. The Council of Economic Advisers, Washington, D.C.
5. Marsico, F., Paolillo, S., and Filardi, P. P. (2017) NSAIIDs and cardiovascular risk. J. Cardiovasc. Med. (Hagerstown) 18, Suppl. 1, e90–e43 CrossRef Medline
6. Koya, D., and King, G. L. (1998) Protein kinase C activation and the development of diabetic complications. Diabetes 47, 859–866 CrossRef Medline
7. Arora, A., and Scholar, E. M. (2005) Role of tyrosine kinase inhibitors in cancer therapy. J. Pharmacol. Exp. Ther. 315, 971–979 CrossRef Medline
8. Fountas, A., Diamantopoulos, L. N., and Tsatsoulis, A. (2015) Tyrosine kinase inhibitors and diabetes: a novel treatment paradigm? Trends Endocrinol. Metab. 26, 643–656 CrossRef Medline
9. Tsuda, M., Mizokoshi, A., Shimamoto-Mogami, Y., Koizumi, S., and Inoue, K. (2004) Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. Glia 45, 89–95 CrossRef Medline
10. Milligan, E. D., and Watkins, L. R. (2009) Pathological and protective roles of glia in chronic pain. Nat. Rev. Neurosci. 10, 23–36 CrossRef Medline
11. Berndt, N., Karim, R. M., and Schönbrunn, E. (2017) Advances of small molecule targeting of kinases. Curr. Opin. Chem. Biol. 39, 126–132 CrossRef Medline
12. Liao, C., Gangadharan, V., Bai, K. K., Xie, R. G., Agarwal, N., Nuretjo, M., Tappe-Theodor, A., Tegeder, I., Feil, S., Lewin, G., Polgar, E., Todd, A. J., Schlossmann, J., Hofmann, F., Li, D. L., et al. (2012) Presynaptically localized cyclic GMP-dependent protein kinase 1 is a key determinant of spinal synaptic potentiation and pain hypersensitivity. PLoS Biol. 10, e1001283 CrossRef Medline
13. Sung, Y. J., Chiu, D. T., and Ambron, R. T. (2006) Activation and retrograde transport of protein kinase G in rat nociceptive neurons after nerve injury and inflammation. Neuroscience 141, 697–709 CrossRef Medline
17. Gangadharan, V., Wang, X., and Luo, C. (2017) [EXPRESS] Cyclic GMP-dependent protein kinase-I localized in nociceptors modulates noiceptive cortical neuronal activity and pain hypersensitivity. *Mol. Pain* **13**, 1744806917701743 Medline

18. Sung, Y. J., Sofoluke, N., Nkamany, M., Deng, S., Xie, Y., Greenwood, I., Farid, R., Landry, D. W., and Ambron, R. T. (2017) A novel inhibitor of active protein kinase G attenuates chronic inflammatory and osteoarthritis pain. *Pain* **158**, 822–832 CrossRef Medline

19. Hofmann, F., and Wegener, J. W. (2013) in *Guanylate Cyclase and Cyclic GMP: Methods and Protocols* (Krieg, T., and Łukowski, R., eds.), pp. 17–50. Humana Press, Totowa, NJ

20. Francis, S. H., Busch, J. L., Corbin, J. D., and Sibley, D. (2010) cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol. Rev.* **62**, 525–563 CrossRef Medline

21. Alverdi, V., Mazon, H., Versluis, C., Hemrika, W., Esposito, G., van den Heuvel, R., Scholten, A., and Heck, A. J. (2008) cGMP-binding prepares PKG for substrate binding by disclosing the C-termdinal. *J. Mol. Biol.* **375**, 1380–1393 CrossRef Medline

22. Wall, M. E., Francis, S. H., Corbin, J. D., Grimes, K., Richie-Jannetta, R., Kotera, J., Macdonald, B. A., Gibson, R. R., and Trehwellia, J. (2003) Mechanisms associated with cGMP binding and activation of cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2380–2385 CrossRef Medline

23. Butt, E. (2009) in *cGMP: Generators, Effectors and Therapeutic Implications* (Schmidt, H. H. W. H., Hofmann, F., and Stasch, J.-P., eds.) pp. 409–421. Springer, Berlin

24. Wolfertstetter, S., Huetttner, J. P., and Schlossmann, J. (2013) cGMP-dependent protein kinase inhibitors in health and disease. *Pharmaceuticals* **6**, 269–286 CrossRef Medline

25. Butt, E., van Bemmelen, M., Fischer, L., Walter, U., and Jastoff, B. (1990) Inhibition of cGMP-dependent protein kinase by (Rp)-guanosine 3’5’-monophosphorothioates. *FEBS Lett.* **263**, 47–50 CrossRef Medline

26. Campbell, J. C., VanSchouwen, B., Lorenz, R., Sankaran, B., Herberg, F. W., and Kim, C. (2016) Crystal structure of PKG IcGMP complex reveals a cGMP-mediated dimeric interface that facilitates cGMP-induced activation. *Structure* **24**, 710–720 CrossRef Medline

27. Qin, L., Reger, A. S., Guo, E., Yang, M. P., Zwart, P., Casteel, D. E., and Kim, C. (2015) Structures of cGMP-dependent protein kinase (PKG) Iα leucine zippers reveal an interchain disulphide bond important for dimer stability. *Biochemistry* **54**, 4419–4422 CrossRef Medline

28. Gustafsson, A. B., and Brunton, L. L. (1999) Differential and selective inhibitors of protein kinase C isotypes. *Acta Crystallogr. D Biol. Crystallogr.* **56**, 33536–33541 CrossRef Medline

29. Farid, R., Landry, D. W., and Ambron, R. T. (2017) A novel inhibitor of cGMP-dependent protein kinase C isotype Cα modulates nociceptive pain. *BMC Chem. Biol.* **17**, 50–59 CrossRef Medline

30. Koide, K., Bunnage, M. E., Gomez Paloma, L., Kanter, J. R., Taylor, S. S., Brunt, L. L., and Nicolau, K. C. (1995) Molecular design and biological activity of potent and selective protein kinase inhibitors related to balanol. *Chem. Biol.* **2**, 601–608 CrossRef Medline

31. Bader, B., Heermeier, K., Lincoln, T. M., Walter, U., and Palmetshofer, A. (2000) KTS823 inhibits cGMP-dependent protein kinase activity in vitro but not in intact human platelets and rat mesangial cells. *J. Biol. Chem.* **275**, 33536–33541 CrossRef Medline

32. McLauchlan, H., and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. *Biochem. J.* **371**, 199–204 CrossRef Medline

33. Boyne, S., and Soto, L. C. (2015) Structures of cGMP-dependent protein kinase (PKG) Iα leucine zippers reveal an interchain disulfide bond important for dimer stability. *Biochemistry* **54**, 4419–4422 CrossRef Medline

34. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) The specificities of protein kinase inhibitors. *Mol. Pain* **9**, 2388–2398 CrossRef Medline

35. AEB071, a potent and selective inhibitor of protein kinase C isotype Cα modulates nociceptive pain. *Mol. Pain* **9**, 2388–2398 CrossRef Medline