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An Aminopyridazine-Based Inhibitor of a Pro-apoptotic Protein Kinase Attenuates Hypoxia-Ischemia Induced Acute Brain Injury

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Abstract—Death associated protein kinase (DAPK) is a calcium and calmodulin regulated enzyme that functions early in eukaryotic programmed cell death, or apoptosis. To validate DAPK as a potential drug discovery target for acute brain injury, the first small molecule DAPK inhibitor was synthesized and tested in vivo. A single injection of the aminopyridazine-based inhibitor administered 6 h after injury attenuated brain tissue or neuronal biomarker loss measured, respectively, 1 week and 3 days later. Because aminopyridazine is a privileged structure in neuropharmacology, we determined the high-resolution crystal structure of a binary complex between the kinase domain and a molecular fragment of the DAPK inhibitor. The co-crystal structure describes a structural basis for interaction and provides a firm foundation for structure-assisted design of lead compounds with appropriate molecular properties for future drug development.

Acute brain injuries, such as stroke, are leading causes of death and disability in the US, with a continuing unmet medical need for effective therapies.[1] Evaluation of recent failures in clinical trials has allowed a consensus to evolve for features required of new compounds and potential therapeutic targets.[2] Key among these are the requirement that treatment be amenable to use within the therapeutic time window of patient presentation to the clinic, which is usually hours after injury, and that targeted tissue and cellular responses be events occurring within this time window. One such response is neuronal apoptosis in the penumbra surrounding the brain injury core. However, early steps in the apoptotic pathway prior to cell commitment to death must be targeted for potential use in therapeutic development. DAPK functions early in neuronal apoptosis and the levels of DAPK increase in the brain prior to the onset of neuronal death, raising the possibility that DAPK could be a potential drug discovery target for acute brain injury.[3] However, there are no selective, bioavailable small molecule inhibitors of DAPK to test this hypothesis.

We report here the development of the first DAPK small molecule inhibitor, an alkylated 3-amino-6-phenylpyridazine. Remarkably, a single intraperitoneal (ip) injection of the inhibitor is able to diminish in vivo brain injury in an animal model when administered 6 h after the insult, a minimal desired therapeutic time window. These proof of principle results demonstrate the potential utility of targeting protein kinases that function at early steps in apoptosis. Further, aminopyridazine is a privileged structure in neuropharmacology,[4] yet there is no structure available for how this chemotype interacts with a protein kinase target. Therefore, we determined the high-resolution co-crystal structure of the DAPK catalytic domain in the presence of a 3-amino-6-phenylpyridazine fragment of the inhibitor.

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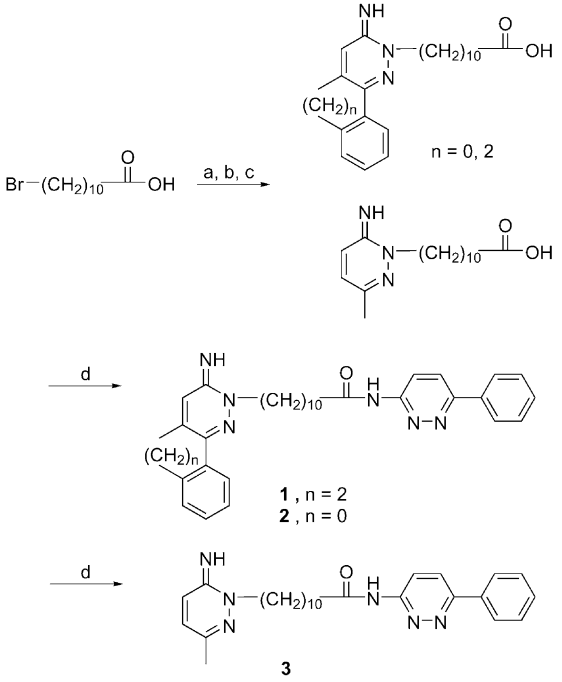
The novel co-crystal structure provides a precedent for how this privileged structure can interact with a protein kinase active site, and a firm foundation for future development of lead compounds using as a starting point the fragment-based, structure guided synthesis approach.[5]

DAPK inhibitor discovery was done using previously described experimental approaches.[6,7] Initially, we developed a quantitative DAPK enzyme assay[8] and screened a proprietary, pharmacology-focused library of compounds.[6] An aminopyridazine with non-selective kinase inhibitory activity[6,7,9] was the only DAPK inhibitor detected (IC_{50} 100 μM). Subsequently, the parent structure was diversified through parallel synthesis,[7] and in vitro screening for inhibition of DAPK activity.[7,9] The procedure for the synthesis of compounds **1**–**3**[10–12] is illustrated in Scheme 1. Briefly, esterification of the commercially available 11-bromoundecanoic acid (Aldrich) yielded the corresponding acid ethyl ester, which was then alkylated with 5,6-dihydro-benzo[*h*]cinnolin-3-ylamine, 3-amino-6-phenyl-pyridazine or 3-amino-6-methyl-pyridazine as previously described.[7] The resulting ester was hydrolyzed to generate the corresponding carboxylic acid, which was coupled to 3-amino-6-phenyl-pyridazine by the HOBt/EDC method. 3-amino-6-phenyl-pyridazine and 3-amino-6-methyl-pyridazine were prepared from the corresponding 3-chloro-pyridazines following previously described procedures.[7,9] All products were screened for their ability to inhibit DAPK and closely related protein kinases.[8,9] The latter included signaling kinases key to CNS function, such as protein kinase C (PKC), protein kinase A (PKA), and the closely related, calmodulin-dependent protein kinase II (CaMKII). Assays were performed and data analyses done as previously described.[7,8]



Scheme 1. Reagents and conditions: (a) EtOH, 1 equiv of 4 N HCl, 48 h, 95%; (b) 0.75% equiv of aminopyridazine, DMF, 80 °C, 94%; (c) 20% concentrated HCl in AcOH, 100 °C, 10 h, 90–98%; (d) 1 equiv of 3-amino-6-phenyl-pyridazine, HOBt/EDC, NMP, 0–22 °C, 21 h, 60–98%.

Compounds **1** and **2** are 3-amino-6-phenylpyridazine derivatives that differ only at C(5) on the pyridazine ring. Compound **1** exhibited (Table 1) more selective inhibition for DAPK compared to compound **2**. The common feature of a 6-phenyl group among compounds **1** and **2** (Table 1) is also found in previously described[4] aminopyridazine drugs. To test the importance of this common feature, compound **3**, which has a methyl in place of the phenyl group, was synthesized. Compound **3** did not inhibit DAPK at concentrations up to 100 μM (Table 1), indicating that the 6-phenyl group is required for DAPK inhibitory activity. Derivatives of 3-amino-6-phenylpyridazine have been shown[7] to be effective cell-permeable inhibitors whose pharmacology depends on the nature of the chemical diversification[4] of the aminopyridazine privileged structure. These precedents and our finding of a common requirement for the 6-phenyl modification of the 3-aminopyridazine raised the possibility that currently approved drugs for CNS disorders[4,13] might be undiscovered protein kinase inhibitors, similar to the discovery that experimental anti-inflammatory compounds are protein kinase inhibitors.[6,14] To address this possibility, the commercially available (Aldrich) compound **4**, (4-methyl-6-phenyl-pyridazin-3-yl)-(2-morpholin-4-yl-ethyl)-amine, also known as minaprine,[4] was tested for protein kinase inhibitory activity. Compound **4** does not show protein kinase inhibitory activity comparable to compounds **1** and **2** (Table 1). The basis of why compound **4** is not a protein kinase inhibitor was not pursued as part of this investigation. Regardless, we concluded that the diversification of 3-amino-6-phenylpyridazine required for selective protein kinase inhibition is distinct from that of previously developed drugs that use this chemotype. Based on these initial results, compound **1** was chosen for further analysis.

The potential of a small molecule inhibitor of an intracellular signal transduction enzyme to function as an in vivo modulator of disease or injury depends on the upstream activating events and the downstream effector pathways. Based on this regulatory paradigm, extended in vitro testing of a new compound for inhibition of protein kinases past those with structural and regulatory similarity (e.g., CaMKII), or those previously implicated in the same tissue injury and recovery (e.g., PKC, PKA), has limited utility if the ultimate goal is in vivo modulation of disease relevant biological responses.[15] Further, protein kinase inhibitors with inhibition

Table 1. IC_{50} values for compounds on protein kinase activities

Compd	DAPK IC_{50} (μM)[a]	CAMKII IC_{50} (μM)[a]	PKC IC_{50} (μM)[a]	PKA IC_{50} (μM)[a]
1	13	>100	>100	>100
2	22	>100	3	>100
3	>100	>100	31	>100
4	>100	>100	>100	>100

[a]Values are averages of two or more determinations. The SEM between assays was <4.

constants that approximate the K_m value of substrates have proven to be effective drug discovery compounds,[15] indicating that feasibility experiments to evaluate compound **1** in vivo should be considered. Kinetic analysis revealed mixed inhibition by compound **1** against ATP or peptide substrate, with apparent K_i values of 20 and 15 μM, respectively. The kinetics are consistent with prior docking studies[9,16] which showed that a 3-amino-6-phenylpyridazine can occupy the ATP site of DAPK. Therefore, compound **1** has the minimal in vitro properties to justify proceeding immediately to in vivo screening.

Our initial goal was to probe the ability of compound **1** to exhibit in vivo neuroprotective function when administered outside the CNS in a standard screening protocol. We used an established rodent model[16] that measures brain hemisphere weight loss 1 week after injury as a quantitative end point.[17] Animals were treated with compounds (5 mg/kg body weight) or solvent vehicle administered by IP injection just prior to HI injury. In the first set of experiments (Fig. 1A), control animals given solvent ($n=14$) showed a 30.7±5.5% loss of brain hemisphere weight (data expressed as mean% hemisphere weight loss±SEM). Compound **4** (minaprine) lacks kinase inhibitory activity (Table 1), allowing its use as a structural analogue control with CNS bioavailability for in vivo studies. Control animals ($n=10$) given compound **4** (minaprine) were not protected from brain injury (Fig. 1A). These animals showed a loss (29.3±5.5%) similar to that seen with vehicle treated animals. In contrast, animals ($n=30$) given compound **1** had only a 17.3±3.2% loss. The brain tissue loss in the compound **1** treated animals is significantly ($p<0.05$) less than that seen in the two control groups. To obtain information about neuronal viability using an established biomarker, western blot analysis was done 3 days after injury using a monoclonal antibody selective for microtubule associated protein, MAP-2. As shown in the insert of panel A, levels decreased as expected in the injured right (R) hemisphere of vehicle-treated mice, whereas animals administered compound **1** did not exhibit decreases in the neuronal biomarker.

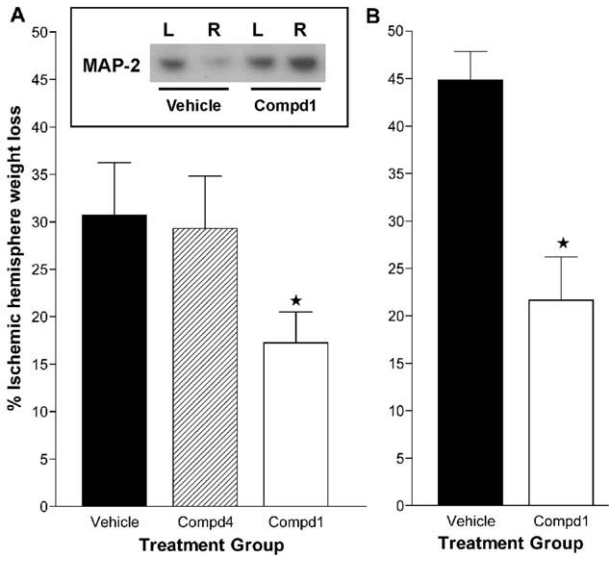


Figure 1. Fig. 1. (A) Reduction of HI induced brain injury by 3-amino-6-phenylpyridazine DAPK inhibitor compd **1** but not by the 3-amino-6-phenylpyridazine analogue compd **4** (the CNS active drug minaprine). The weight of the ischemic (right) cerebral hemisphere from treated (vehicle control, compd **1**, or compd **4**) rats was compared seven days after injury. Compd **4** does not attenuate brain tissue loss as seen with compd **1**. *$p<0.05$ by student's two-tailed t-test. The insert shows protection afforded by compd **1** treatment using a neuronal viability biomarker, microtubule associated protein-2 (MAP-2). Homogenate supernatants from the left, L, or right, R, brain hemispheres of animals were analyzed by western blot analysis (1:500 dilution of monoclonal antibody) three days after injury and treatment with either vehicle or compd **1**. (B) The attenuation of ischemic brain tissue loss afforded by compd **1** is also seen when the DAPK inhibitor is given 6 h after HI injury. The percent reduction in hemispheric weights was compared by one-way analysis of variance (StatView 5.0, SAS, Cary, NC, USA).

Although minaprine has been shown previously[18] to protect against brain ischemia induced neuronal death in other animal models when administered prior to injury, this was not observed with the vigorous injury induced by HI in this study. In addition, no behavioral changes were observed in response to administration of Compound **1**, and no overt signs of toxicity in these groups were seen over a 7-day recovery period after exposure to compound **1**. In separate experiments,[27] no cardiotoxicity was found, determined by analysis on the QT interval in the mouse, with exposure to compound **1** with doses up to 50 mg/kg. Specifically, the corrected Q-T (Q-Tc) interval (values pressed in ms±SEM) in control mice was 17.32±0.45, and that from mice treated with Compound **1** was 18.52±0.33.

To determine if the in vivo function of compound **1** could be observed in a more therapeutically relevant scenario, we next administered the compound 6 h after the HI insult. Again, neurological injury was attenuated in animals treated with a single injection of compound **1** (Fig. 1B). Control animals ($n=8$) had a 44.0±3.3% loss of brain hemisphere weight. In contrast, animals ($n=8$) treated with compound **1** had only a 21.6±4.6% loss of brain hemisphere weight. This difference in brain injury between control animals and those treated with compound **1** six h after injury is significant ($p<0.05$).

These results are remarkable in that the protection is afforded by only a single treatment given after injury and at a site remote from the CNS.

Our proof of concept results with compound **1** are consistent with DAPK being a potential therapeutic target for acute brain injury, and raise the significance of future investigations that focus on drug development using the privileged aminopyridazine structure. However, the molecular properties of compound **1** do not make it an ideal candidate for consideration in drug development based on the history of the development process.[19] For example, the molecular weight is already over 500 (534.6) and the calculated LogP (ACD logD suite, v5.11, Toronto, ON, Canada) is over 5 (6.26 ±0.76). Although oral biovailability is not a primary consideration in critical care medicine, where administration is often intravenous, the future development of compounds with attractive molecular properties and potential for oral

bioavailability should be possible based on the prior use of aminopyridazines in drug development and their potential for chemical diversification at multiple positions.[4] In this regard, an aminopyridazine fragment would make an excellent starting point for lead compound development using structure-assisted fragment based approaches.[5] In this approach, a low molecular weight fragment containing the chemotype of interest is used in determination of a high-resolution co-crystal structure with the protein target as a starting point for structure-guided synthesis. The starting fragment does not need to have detectable activity. The goal is to use structure guided design and in silico filtering for desired molecular properties in the synthesis of compounds in activity-based discovery. Therefore, we determined the crystal structure of the complex between the DAPK catalytic domain and the 3-amino-6-phenylpyridazine fragment of compound **1** to obtain experimental evidence for how this chemotype interacts with the enzyme and to provide a firm foundation for future research using the fragment-based approach. Crystal growth and data processing were done essentially as previously described[20] but with the exceptions noted.[21]

The structure of the DAPK:aminopyridazine complex was determined at 1.9 Å resolution. The orientation of the aminopyridazine fragment is stabilized by hydrophobic and hydrogen bond interactions with the protein, and is well-defined within the electron density (Fig. 2). Direct comparisons of the 3-amino-6-phenylpyridazine complex to the previously described[20] AMPPnP complex of DAPK (PDB file 1JKL) revealed that the 3-amino-6-phenylpyridazine occupies part of the ATP binding site. For example, the phenyl ring of the fragment is in the same plane and occupies part of the space filled by the adenine ring of ATP, although interactions with the adenine-binding region of the protein are not observed. The 3-amino group of the pyridazine ring occupies the area used by the alpha phosphate of ATP, in proximity to the catalytic Lys42, and is also within potential H-bond distance of Asp161. The smaller aminopyridazine molecular fragment, therefore, occupies space in the enzyme active site that is used by the ATP substrate, but atoms on the aminopyridazine fragment are oriented such that they are amenable to chemical diversifications with standard chemistries and generation of products that could exploit space not used by ATP.

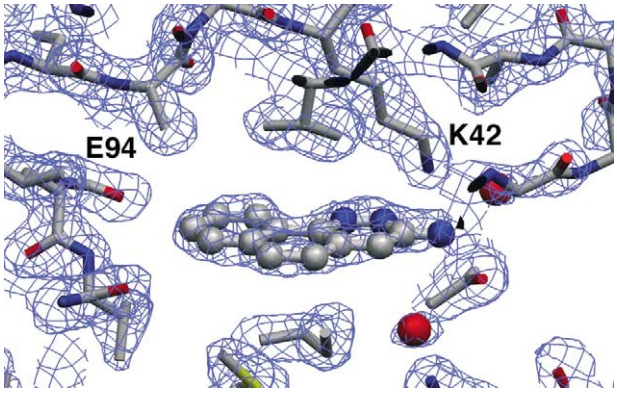


Figure 2. Co-crystal structure of 5,6-dihydro-benzo[*h*]cinnolin-3-yl-amine inhibitor fragment bound in the ATP site of DAPK catalytic domain. The 2Fo-Fc electron density map for the binary complex between DAPK and the aminopyridazine fragment is shown for the region proximal to the conserved catalytic lysine-42 (K42) and glutamic acid-94 (E94) in the ATP site. DAPK amino acids are shown in stick representation and the aminopyridazine is shown in ball and stick representation. The structure is deposited in the Protein Data Bank with PDB ID code 1P4F.

Several areas around the fragment can be potentially filled by synthetic diversification that exploits complementary hydrophobic or polar interactions. One example is the available space between the phenyl ring of the fragment and the carbonyl group of Glu94 in DAPK (see Fig. 2). This protein carbonyl oxygen is hydrogen-bonded to the exocyclic amino group of the adenine ring of ATP,[20] a conserved interaction found in kinases. This interaction could be potentially exploited in aminopyridazine derivatives that have H-bond donor groups on the phenyl ring of the fragment. Combined use of such conserved features with various unique aspects of the structure within this area of the active site can clearly be used in the design of future syntheses. Current investigations are focused on the use of this crystal structure of the binary complex as a starting point for the development of DAPK inhibitors that utilize potential chemical complementarity and available space. Structure-assisted synthetic design can then be filtered for the potential of the proposed products to be rule-of-five compliant[19] in their molecular properties, and, therefore, more attractive candidates for further development.

In conclusion, compound **1** is the first DAPK small molecule inhibitor described. A single administration at a tissue site remote from the CNS and at a therapeutically relevant time window of hours after injury is able to attenuate CNS damage measured 1 week later. The crystal structure of the binary complex between this privileged chemotype and the target protein kinase reveals regions of potential chemical complementarity to be exploited in future syntheses of compounds with desired molecular properties. The results provide support for the hypothesis that targeting protein kinases which function early in programmed cell death pathways could identify new therapeutic approaches to acute brain injury.

Acknowledgements

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10. The extent of reactions was monitored by analytical HPLC and final compounds were purified by RP-HPLC chromatography on a preparative Microsorb (Rainin Instruments; Woburn, MA, USA) C18 column using gradients of 0.1% (v/v) TFA in water and 80% aqueous acetonitrile containing 0.08% TFA. Mass spectrometry was done using a PerSeptives (Foster City, CA, USA) Voyager DE-Pro system. ^{1}H NMR spectrum for compound **1** was obtained on an INOVA 500 (^{1}H, 20–500 MHz tunable) spectrometer in CD_3OD.
11. Synthesis and characterization of compounds: For the synthesis of compound **1**, 11-(3-imino-5,6-dihydro-3H-benzo[*h*]cinnolin-2-yl)-undecanoic acid (0.88 g, 2.3 mmol) reacted with 3-amino-6-phenyl-pyridazine (0.4 g, 2.3mmol) in 2 mL DMF. HOBt (0.35 g, 2.3 mmol) and EDC (0.44 g, 2.3 mmol) were used. 11-(3-imino-5,6-dihydro-3H-benzo[*h*]cinnolin-2-yl)-undecanoic acid-(6-phenyl-pyridazin-3-yl)-amide was prepared as a light yellow solid. The mass (*m*/*z*) of 534.9 was that expected for the product (calcd for $C_{33}H_{38}N_6O$ 534.3). NMR analysis was consistent with the expected product. ^{1}H NMR (CD_3OD): δ 1.262–1.4 (t, 16), 1.675–1.690 (t, 2), 1.916–1.929 (t, 2), 2.461–2.490 (t, 2), 2.974–3.030 (m, 4), 4.307–4.336 (t, 2), 5.468 (s, 4), 7.285–7.524 (m, 4), 7.971–8.087 (m, 1), 8.484–8.502 (m, 2). For the synthesis of compound **2**, 11-(6-imino-3-phenyl-6H-pyridazin-1-yl)-undecanoic acid (0.88 g, 2.3 mmol) reacted with 3-amino-6-phenyl-pyridazine (0.4 g, 2.3 mmol) exactly as described for compound **1**. The mass (*m*/*z*) of 509.1 was that expected for the product (calcd for $C_{31}H_{36}N_6O$ 508.3). For the synthesis of compound 3, 11-(6-imino-3-methyl-6H-pyridazin-1-yl)-undecanoic acid (0.1 g, 0.35 mmol) reacted with 3-amino-6-phenyl-pyridazine (0.06 g, 0.35 mmol) exactly as described for compound **1**. The mass (*m*/*z*) of 447.4 was that expected for the product (calcd for $C_{26}H_{34}N_6O$ 446.3).
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17. **Quantification of brain injury**: All experiments were performed in accordance with the relevant National Institutes of Health guidelines, and protocols were approved by the Institutional Animal Care and Use Committee of Northwestern University, Chicago. The comparison of disparities in hemispheric weight as a valid outcome measure of neurologic injury in the newborn rat has been demonstrated in studies[26] correlating changes in hemispheric weight following hypoxia-ischemia with other measures of brain injury. Animals were sacrificed seven days after injury and differences in weight between the HI injured and control contralateral hemisphere were calculated for each animal both directly and using the formula: $100 \cdot \left(\frac{C-I}{C}\right) = \%Damage$ where I represents the weight of the ipsilateral and C the weight of the contralateral hemisphere. All data are expressed as mean ± SEM. The degree of HI induced brain injury in each treatment group was expressed as the percentage of reduction in the tissue weight of the hemisphere ipsilateral to carotid ligation. This was calculated as a ratio of the right (ipsilateral, ischemic) to the left (contralateral, non-ischemic) hemispheric weights. The percent reduction in hemispheric weights was compared by one-way analysis of variance (StatView 5.0, SAS, Cary, NC, USA). The Mann–Whitney test was used to determine significance for nonparametric data. Statistical significance was assumed when $p < 0.05$. In the first set of experiments where animals were treated prior to HI induced injury, the weight of the ischemic hemisphere in the vehicle-treated animals was 0.2878 ± 0.0242 g. The weight of the ischemic hemisphere in the animals treated with compound **4** (minaprine) was 0.3181 ± 0.0328 g. The weight of the ischemic hemisphere in the animals treated with compound **1** was 0.3869 ± 0.0165 g. There was no significant difference in the weight of the non-ischemic (left) hemisphere between vehicle-treated (0.4168 ± 0.1248 g), compound **4** (minaprine)-treated (0.4462 ± 0.0172 g) and compound **1**-treated (0.4672 ± 0.0083 gm) animals. In the second experiment where treatments were done 6 h after HI injury, the weight of ischemic hemisphere in the vehicle-treated animals (0.2484 ± 0.0103 g) was significantly different from the compound **3**-treated animals (0.3191 ± 0.0228 g).
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21. Crystals were grown at 20 °C by mixing the protein and aminopyridazine solutions (6 mg mL^{-1} protein, 30 mM 5,6-dihydro-benzo[*h*]cinnolin-3-ylamine) with an equal volume of 1.1 M ammonium sulfate, 100 mM Hepes pH 7.5. The crystals were transferred to a non-polar stabilization buffer of 20% PEG 8000, 100 mM Hepes pH 7.5, containing 15 mM of the 5,6-dihydro-benzo[*h*]cinnolin-3-ylamine to ensure the binding of the low affinity fragment. The crystals were cryoprotected with 20% glycerol in the stabilization buffer and flash-cooled in liquid nitrogen. Diffraction data were measured at the DuPont-Northwestern-Dow Collaborative Access Team (DND-CAT) beamline 5-ID-B, at the Advanced Photon Source (APS), Argonne, IL, USA and processed with the HKL suite of programs.[22,23] The initial model, 1JKL[20] with waters and ligands removed, was positioned by rigid body refinement using CNS.[24] Model building was done using O.[25] Further refinement included simulated annealing, positional minimization and temperature factor protocols using CNS, employing the bulk solvent correction.[22] The structure has been deposited in the Protein Data Bank and the PDB ID code is 1P4F.
22. Data collection and refinement statistics for the crystal structure of the complex of DAPK catalytic domain with 5,6-Dihydro-benzo[*h*]cinnolin-3-ylamine: Space group $P2_12_12_1$, unit cell $a = 47.24$ $b = 62.53$ $c = 88.93$ Å, resolution range 30–1.9 Å (highest bin 1.97–1.90 Å), completeness 97.9% (97.3%), $R_{merge} = 0.049$ (0.213), Average $I/\sigma(I) = 25.8$ (5.5). The final model contains 2275 protein, 193 water, and 15 aminopyridazine fragment atoms, has an R_{cryst} of 20.7% and R_{free} of 25.6%, and mean temperature factors of 20.8 Å^2 for protein atoms, 26.6 Å^2 for waters and 23.1 Å^2 for aminopyridazine atoms.
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27. Mice were anesthetized using ketamine and isoflurane with temperature monitored by rectal probe and maintained at 37.0 °C by surface heating and cooling. Anode, cathode and ground leads were placed subcutaneously and the electrocardiogram (ECG) recorded using commercially available software (Maclab, AD Intstruments, Colorado Springs, CO, USA). After stabilization, compound **1** or control solutions was administered and the ECG recorded for up to 60 min. Mice treated with quinidine (positive control) showed an increase in the Q-Tc to 21.48 ± 1.11, significantly different ($p < 0.001$ by ANOVA) from the control mice (17.32 ± 0.45) or the compound **1** treated mice (18.52 ± 0.33).