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Cyclic Adenosine 5′-Diphosphate Ribose Analogs without a “Southern” Ribose Inhibit ADP-ribosyl Cyclase–Hydrolase CD38

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ABSTRACT: Cyclic adenosine 5′-diphosphate ribose (cADPR) analogs based on the cyclic inosine 5′-diphosphate ribose (cIDPR) template were synthesized by recently developed stereo- and regioselective N1-ribosylation. Replacing the base N9-ribose with a butyl chain generates inhibitors of cADPR hydrolysis by the human ADP-ribosyl cyclase CD38 catalytic domain (shCD38), illustrating the nonessential nature of the “southern” ribose for binding. Butyl substitution generally improves potency relative to the parent cIDPRs, and 8-amino-N9-butyl-cIDPR is comparable to the best noncovalent CD38 inhibitors to date (IC50 = 3.3 μM). Crystallographic analysis of the shCD38:8-amino-N9-butyl-cIDPR complex to a 2.05 Å resolution unexpectedly reveals an N1-hydrolyzed ligand in the active site, suggesting that it is the N6-imino form of cADPR that is hydrolyzed by CD38. While HPLC studies confirm ligand cleavage at very high protein concentrations, they indicate that hydrolysis does not occur under physiological concentrations. Taken together, these analogs confirm that the “northern” ribose is critical for CD38 activity and inhibition, provide new insight into the mechanism of cADPR hydrolysis by CD38, and may aid future inhibitor design.

INTRODUCTION

Cyclic adenosine 5′-diphosphate ribose (cADPR, 1, Figure 1)1,2 is synthesized in biological systems from nicotinamide adenine dinucleotide (NAD+) by ADP-ribosyl cyclases (ADPRCs). It acts as a second messenger, mobilizing intracellular calcium.3−6 In humans, formation of cADPR is catalyzed by the multifunctional transmembrane glycoprotein ADPRC CD38.7 CD38 also acts as an NAD+ glycohydrolase (NADase) and as a cADPR hydrolase to generate adenosine 5′-diphosphate ribose (ADPR), another Ca2+-releasing second messenger.8−10 Under acidic conditions, CD38 generates the most potent Ca2+-releasing second messenger known to date, nicotinic acid adenine dinucleotide phosphate (NAADP) from nicotinamide adenine dinucleotide phosphate (NADP).11−13 CD38 knockout studies have revealed the importance of this pathway in a range of diseases. CD38 is a marker in AIDS progression12 and a negative prognostic marker of chronic lymphocytic leukemia.13 It acts to regulate intracellular levels of NAD+, being implicated in energy homeostasis, signal transduction, and aging,14−16 and recently has been shown to be critical for social behavior in mice.17 The emerging role of CD38 in disease states is stimulating the search for modulators of activity for chemical biological studies and to provide structural clues for drug design and potential therapeutic candidates.18 To date, CD38 inhibitors fall broadly into two categories: mechanism-based covalent inhibitors that bind to the catalytic residue, and reversible, competitive, noncovalent inhibitors. Most are derived from NAD+ and designed as inhibitors of the predominant NADase activity of CD38. Nicotinamide-based derivatives have demonstrated nanomolar inhibition of NADase activity by covalent modification of CD38.19 Low millimolar activity when designed as membrane permeable analogs.20 Competitive inhibitors are more diverse in structure, with a nonhydrolyzable NAD+ analog reported (IC50 ≈ 150 μM).21 Flavonoids showing low micromolar inhibition22 and the development of a hit from...
commercial libraries generating the most active reported noncovalent inhibitor to date (IC\textsubscript{50} = 4.7 μM).\textsuperscript{33}

As both cADPR and ADPR are derived from a common intermediate,\textsuperscript{24} we chose to design product-like inhibitors based on the cADPR structure to exploit inhibition of CD38 cADPR hydrolase activity. cADPR itself is unattractive for inhibitor design, since it is hydrolyzed at the N1 link in both neutral aqueous solution and under physiological conditions.\textsuperscript{25–27} More stable analogs have been accessed by one of two routes: either a chemoenzymatic route modeled on the biosynthesis of cADPR from NAD\textsuperscript{+} or by total chemical synthesis. We have previously reported a chemoenzymatic route to N1-cyclic inosine 5′-diphosphate ribose (cIDPR, 2) via its 8-bromo derivative (8-Br-cIDPR, 3, Figure 1).\textsuperscript{28} Chemically and biologically stable, 2 and 3 both inhibit cADPR hydrolysis by the catalytic domain of CD38 (shCD38; IC\textsubscript{50} of 276 and 158 μM, respectively). Furthermore, 2 acts as an agonist for Ca\textsuperscript{2+}-release with almost equivalent potency to cADPR in permeabilized T-cells and 3 is a membrane permeant agonist.\textsuperscript{29,30}

Until recently, total synthetic approaches have required modification of the “northern” ribose to generate a more stable N1-link by introduction of a carbocyclic “northern” ribose,\textsuperscript{31–33} replacement of the “northern” ribose, or both ribose sugars, by an alkyl or ether bridge\textsuperscript{34,35} or by attaching the “northern” ribose to the base at C-2.\textsuperscript{36} We recently reported the use of modified Vorbrüggen conditions to effect stereo- and regiospecific introduction of an acetylated ribose at the N1-position of a protected inosine and demonstrated the utility of this method in the first total synthesis of 3.\textsuperscript{37} This paves the way for further exploration of the structure–activity relationship (SAR) of cADPR, using the cIDPR template, with the opportunity to retain an intact “northern” ribose and without the structural limitations of using enzymatic cyclization.\textsuperscript{38} We report here the synthesis of the first analogs in which the “southern” ribose is selectively replaced, the activity of these analogs as inhibitors of cADPR hydrolysis by shCD38, crystallography of the most potent inhibitor with shCD38, and HPLC studies to examine the ability of shCD38 to hydrolyze the cIDPR scaffold.

RESULTS AND DISCUSSION

Crystallography of shCD38 has revealed the mechanism by which NAD\textsuperscript{+} is either cyclized to cADPR or hydrolyzed to ADPR.\textsuperscript{39–41} Crystal structures obtained with shCD38 and unnatural ligands 2 (PDB code 2PGJ),\textsuperscript{42} cyclic adenosine 5′-diphosphate carbocyclic ribose (cADPCr, 3UHI),\textsuperscript{43} and 8-NH\textsubscript{2}-cIDPR (3U4H) suggest a critical role for the base and “northern” ribose in the binding of cADPR analogs to CD38, as might be predicted for the locus of both cADPR formation and degradation. Glu146 hydrogen-bonds to N6 and N7 and is critical in regulating the ADPRC multifunctionality.\textsuperscript{44} Glu226 is the catalytic residue and interacts with the 2′- and 3′-OH of the “northern” ribose.\textsuperscript{45} It has recently been highlighted as crucial in orientating NAD\textsuperscript{+} for cleavage of nicotinamide.\textsuperscript{46} The “southern” ribose appears to be accommodated in a more flexible fashion across the open face of the pocket. Thus, we designed analogs in which the “southern” ribose is replaced with an N9-butyl linker (N9-butyl-cIDPRs 4−7, Figure 1) to explore whether it is required for binding to CD38. Such analogs would be predicted to have the further advantage that the acid sensitivity of the N9-riboyl link is eliminated.\textsuperscript{47}

Molecular Modeling. To predict the binding mode of analogs 4−7, which might be more flexible because of the linear N9-alkyl chain, they were first docked into the 2PGJ crystal structure of shCD38 in complex with cIDPR. The docked and minimized poses were almost identical to that of cIDPR (Figure 2A), suggesting that these analogs would mimic the critical interactions with the binding site. Minimization of the protein resulted in very little movement of the amino acid residues forming the binding site.

The docked ligands display face-to-face stacking between the hypoxanthine base and Trp189. There is one predicted hydrogen bond from the ribose 3′-OH\textsuperscript{a} hydroxyl group to the catalytic acid (Glu226), although the 2′-OH does not appear to be as closely located, and from the side chains of Ser126 and Arg127 to the phosphates. The 8-amino group of 7 is predicted to form an additional hydrogen bond to the side chain of Asp155. The N9-butyl chain of the docked ligand lies across the entrance of the binding pocket in an almost identical position to the “southern” ribose of cIDPR (Figure 2B).

Synthesis of N9-Butyl-cIDPR Analogs 4−7. 6-Chloropurine (11, Scheme 1) was alkylated with 4-chlorobutylacetate
in the presence of 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU).48 The desired major product, the N9-isomer 12, was obtained in high yield after separation from the minor N7-isomer. Treatment of 12 with methanolic ammonia at 80 °C efficiently effected both amination at the 6-position and deprotection of the N9-butyl acetyl ester to afford N9-hydroxybutyladenine 13.49 The alkyl group was then reprotected as the TBDPS ether using tert-butyldiphenylsilyl chloride (TBDDS-Cl) and imidazole to give 14. Introduction of the 8-bromo substituent to 14 is crucial at this stage to promote the subsequent N1-ribosylation.50 Attempted bromination of 14 using a solution of bromine in sodium hydrogen phosphate buffer and dioxane was not successful, and no reaction was observed. Similarly, treatment of 14 with N-bromosuccinimide or N-bromosaccharimide afforded only starting material. Applying the conditions developed by Lauffer et al.50 for the 8-bromination of N9-methyl chloropurines, we first deprotonated 14 using lithium diisopropylamide (LDA), followed by addition of 1,2-dibromomethanol, which allowed selective introduction of the 8-bromo substituent to 14. However, when this reaction was scaled up (2.5 g), an inseparable mixture of two products was obtained after column chromatography. Mass spectrometry (m/z = 480.1970 and 524.1476) containing characteristic [M + Cl]− and [95/99]Br isotope patterns confirmed this was both the 8-bromo and 8-chloro substituted products. It was only possible to recover the starting material by treating the mixture of products with Pd/C under an atmosphere of H2. We therefore sought an alternative source of bromine that would avoid this complication and found that direct addition of Br2 to deprotonated 14 generated 15 in 66% yield, with the remainder as starting material, which could be separated by column chromatography and reused. Treatment of 15 with excess sodium nitrite converted 8-bromoadenine 15 to 8-bromohypoxanthine 16, which was a suitable substrate for N1-ribosylation. Deprotonation of 16 with DBU followed by addition of tetraacetyl-D-ribose and trimethylsilyl triflate (TMSOTf)57 afforded stereo- and regiospecific N1-coupling to generate only the desired N1-ribosylated product 17 in high yield. Deprotection of the three acetyl esters using methanolic ammonia generated triol 18.

The 2',3'-diol was reprotected as an isopropyldiene ketal to afford 19, which allowed selective introduction of the first phosphate ester to the 5'-OH by treatment of 19 with N,N-diisopropylidenebenzylphosphoramidite and 5-phenyl-1-H-tetrazole as activator, followed by oxidation of the intermediate phosphate with hydrogen peroxide and triethylamine (Scheme 2). The silyl ether of 20 was then removed under neutral conditions to reveal the hydroxyl group. The second protected phosphate ester was introduced by treatment of 21 with cyclohexylammonium S,S-diisopropylphosphorothioate (PSS), 2,4,6-triisopropylbenzenesulfonyl chloride (TIPS-Cl), and 5-phenyl-1-H-tetrazole in pyridine under strictly dry conditions to generate the fully protected precursor for cyclization. 22. Sequential deprotection of the phosphates was carried out as previously described using first 50% aqueous TFA to simultaneously remove the tert-butyl esters and the isopropyldiene ketal, then 0.1 M sodium hydroxide in dioxane, to afford 24. The N9-butyl chain shows increased stability toward acidic conditions, compared to the natural "southern" ribose of cIDPR, which had initially suffered partial hydrolysis at the N9-glycosidic bond during deprotection of the isopropylidene acetal and tert-butyl phosphate esters.54,57 No degradation of the N9-butyl analogs was observed even over prolonged periods in 50% TFA at room temperature, since the ribose oxygen has been removed.

Cyclization of 24 was promoted using iodine and activated 3 Å molecular sieves in pyridine,58 under very dilute conditions, to afford 5 (21% yield over two steps, Scheme 3). Because of the stability of our cIDPR-based analogs, modifications at the 8-position were possible after cyclization, which allowed us to generate three further analogs; 8-H, 8-N3, and 8-NH2. Subjecting 5 to an atmosphere of hydrogen with Pd/C catalysis generated the 8-H analog, N9-butyl-cIDPR (4). Treatment of 5

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**Scheme 1. Synthesis of the N1-Ribosyl-N9-butyl-8-bromohypoxanthine Building Block**

![Scheme 1](image)

Reagents: (i) DBU, 4-Cl-butyl-OAc, 77%; (ii) NH3, MeOH, 96%; (iii) imidazole, TBDPS-Cl, 92%; (iv) (a) diisopropylamine, n-BuLi, (b) Br2, 66%; (v) NaNO2, AcOH (aq), 84%; (vi) (a) DBU, (b) tetraacetyl-D-ribose, TMSOTf, 86%; (vii) NH3, MeOH, 98%.

**Scheme 2. Sequential Introduction of the Phosphate Esters**

![Scheme 2](image)

Reagents: (i) pTsOH, H2C(OOMe)2CH3, acetone, 100%; (ii) (a) (BuO)2PN(Pr)2, 5-Ph-1H-tetrazole, DCM; (b) H2O2, Et3N, 80%; (iii) TBAF-3H2O, AcOH, 100%; (iv) FSS, TPS-Cl, 5-Ph-1H-tetrazole, pyridine, 100%; (v) 50% TFA (aq), 100%; (vi) 0.1 M NaOH–dioxane.

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with NaN₃ in an attempt to generate the 8-N₃ analog 6 was not successful, despite conversion to the triethylamine salt or the free acid to improve the solubility of 5 in DMF. In each case, the DMF solution became cloudy over time as the sodium salt of the starting material was formed and became insoluble in the reaction media. However, upon treatment of the free acid form of the starting material was formed and became insoluble in the DMF solution became cloudy over time as the sodium salt of 6 was converted into the 8-azido product [8-N₃-7].

Inhibition of CD38-Mediated cADPR Hydrolysis. The ability of the novel compounds 4–7 to inhibit shCD38-mediated hydrolysis of cADPR was assessed in a dose–response manner using a fluorometric cycling assay. The novel N9-butylo-cIDPR analogs 4–7 inhibit cADPR hydrolysis in a concentration-dependent manner, with half maximal inhibition in the low micromolar range (Figure 3 and Table 1). They are all more potent than 2, showing an almost 100-fold increase in inhibition. The introduction of an 8-bromo substituent (5) gave a marginally improved inhibition (IC₅₀ = 27 μM) compared to the parent 4 (33 μM), which was then further improved by substitution to the 8-azido (6) or 8-amino (7) group (IC₅₀ of 6.4 and 3.3 μM, respectively). The trend of improved inhibition upon 8-H → 8-Br → 8-NH₂ substitution mimics that which was observed for the cIDPR series, the 8-NH₂ derivative benefiting from an additional binding site interaction with Asp155. This suggests that the new analogs are binding similarly in the active site (vide infra). They are of increased flexibility afforded by an alkyl chain linker, compared to a ribose sugar. This might allow the ligand to align other key residues more favorably with their binding targets, resulting in a tighter affinity for CD38. Another factor may be the hydrophobic nature of the butyl chain, which may prefer to sit further into the binding pocket, in a less polar environment away from bulk solvent.

Structure of 8-NH₂-9-butylo-cIDPR (7) Complexed with Wild-Type CD38. Preformed crystals of wild-type shCD38 were soaked with a cryoprotectant solution containing 5 mM 8-NH₂-9-butylo-cIDPR (7). X-ray diffraction data were collected to a resolution of 2.05 Å. The structure was solved by the molecular replacement method revealing two molecules of CD38 in the asymmetric unit with a ligand present in each active site (Figure 4A). Previous crystallographic work has generally shown occupancy of only one site by noncovalent inhibitors.

To our surprise, the electron density within each active site did not fit the cycloprod 7. However, hydrolysis at N1 to generate a linear compound, 7a, gave a structure with a good fit (Figure 4B). After refinement, the complex clearly showed that the catalytic residue Glu226 was still hydrogen-bonded to the ribose. For molecule A, Glu226 H-bonds to the 1′- and 3′-OH of the ribose (both 2.7 Å), which has previously been observed in hydrolyzed intermediates or products. In molecule B, Glu226 forms H-bonds with the 3′-OH (2.6 Å) and longer distance interactions with the 1′-OH and 2′-OH (3.3 and 3.5 Å). In both molecules, the 2′-OH interacts with an active site water molecule (Figure 4C). Addition of water to C-1′ has occurred to generate the hydrolyzed product, and the -OH group is attached to the α-face of the ribose, as has been observed previously for NAD⁺ hydrolysis products captured by crystallization. Such observations are at odds with both the proposed ionic or covalent hydrolysis mechanisms, which predict a predominantly or entirely β-product, respectively.
Notably, the hydrolyzed ligand still occupies the catalytic site in the same manner as the cADPR, except that the hypoxanthine base has rotated in the binding pocket after hydrolysis. While maintaining the stacking interaction with Trp189, it has also formed new hydrogen bonds with Glu146 and Asp156 via its 8-NH$_2$ and 6-O substituents, respectively (Figure 4C). It is unusual to capture this snapshot of the ligand directly after hydrolysis, before diffusion out of the binding site. Previously reported crystal structures of the hydrolysis products of N$_7$-cGDPR and cADPR, GDPR and ADPR, respectively, have also captured products that were not bound in a conformation directly resulting from hydrolysis.$^{53}$

**Proposed Mechanism of Hydrolysis by CD38.** Finding a hydrolyzed ligand in the active site of CD38 was unexpected, as the cIDPR template has previously been considered "non-hydrolyzable".$^{42}$ The absence of a partial positive charge at N1, compared to that in the N6-amino form of cADPR, was thought to contribute to this stability (Figure 5).

However, a mechanism in which the anomeric oxygen assists in breaking the N1-link could still be envisaged (Scheme 4). This would be without prejudice as to whether the role of Glu226 is to capture the resulting oxonium ion formally or interact with it as an ion pair before insertion of water to generate the final linear product. Examination of the crystal structures 2PGJ and 3U4H suggests that both binding sites...

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Figure 4. Crystal structure of PDB code 4TMF. (A) Ligand (shown as sticks) occupies each active site. (B) The electron density fits that of a hydrolyzed ligand (carbons in cyan, key residues that interact with the ligand in the binding site shown as green sticks). (C) H-bonds (shown as black dotted lines) are formed between the rotated hypoxanthine base and both Glu146 and the backbone nitrogen of Asp156. Interacting residues are shown as pink sticks, and other residues are shown as green lines. The interacting binding site water is shown as a red sphere, and the other waters as red crosses.
contain two water molecules close to the N1-link of the cyclic ligand. One of these waters forms H-bonds to Leu123, Leu145, and the ribose 2′-OH that is consistent with other reported CD38 crystal structures. Notably, in the 3U4H crystal with 8-NH₂-cIDPR, the remaining water molecule lies within 3 Å of the hypoxanthine carbonyl group. This water is not observed in the complex of the hydrolyzed ligand 7a (PDB code 4TMF); thus, we postulate that donation of a proton from this water molecule could be enzyme-assisted in the active site. Further examination reveals a proximal histidine residue (His133) that may potentially contribute to overall catalysis (Figure 6).

For the corresponding cADPR ligand to be hydrolyzed via such a mechanism would require the ligand to be present in the N6-imino form. Indeed, an earlier report indicated that cADPR exists in two forms, the N6-imino and N6-amino, the latter with a pKₐ of 8.3. Although at physiological pH the amino form will be predominant, the active form for Ca²⁺ release remains unresolved and crystallization of cADPR originally suggested that the C6–N6 bond displayed double bond characteristics (length 1.33 Å, cf. 1.47 Å expected for an amino group) with only one hydrogen atom bound to N6. This is somewhat surprising given that the molecule was crystallized as its free acid, presumably well below pH 8.3. The C6–N6 bond length in the cADPcR-shCD38 complex, the only cocrystal structure of a cyclic ligand with a starting C6-substituent in the amino form yet determined, measures 1.35 Å, also suggesting substantial double bond character. The authors measured the pKₐ of the N6-amino dissociation in this case as 8.9. However, cADPcR is a completely nonhydrolyzable ligand due to the absence of the ribose oxygen. Since crystallization of cADPR itself with shCD38 is not possible because of its very rapid hydrolysis, it has been impossible to capture directly the intermediates in this process. Previously, the surrogate substrate NGD⁺ revealed the role of Glu226 in activating and stabilizing the intermediate precursor for hydrolysis or cyclization. We originally showed that cIDPR, as expected, has no ionization between 6.8 and 10.9 and it is not likely that 7 will be any

Scheme 4. Proposed Mechanism for Hydrolysis of 7 by a High Concentration of shCD38
different. Thus, since it is unlikely that cIDPR or any related analog will exist in the 6-phenolic form at the pH used with shCD38 (vide infra) and the enzyme would not adopt a different mechanism to hydrolyze an analog vs cADPR itself, we can conclude that the complex of 7a with shCD38 provides the first evidence that CD38 can bind cADPR in the N6-imino form. This could have future implications for inhibitor design. We clearly cannot comment upon the ability of CD38 to bind the N6-amino form of cADPR.

**Study of Ligand Hydrolysis by CD38.** Following the discovery of hydrolyzed ligand 7a in the crystal complex, it was important to determine whether it is the cyclic or the hydrolyzed linear compound that inhibits hydrolysis of cADPR by shCD38. Incubation of 7 (1 mM final concentration) with 4 mg/mL shCD38 was monitored using RP-HPLC. The peak corresponding to 7 (t_½ = 9.1 min) reduced in intensity over time, alongside the appearance of a new peak (t_½ = 10.6 min), which was characteristic of an ADPR analog (see Supporting Information, Figure S1). The rate of conversion depended on the concentration of CD38 present in solution (Figure S2), and no change in the original peak was observed in a parallel control experiment containing no shCD38 (data not shown). Novel analogs 4, 5 and cIDPR (2) were also analyzed in this way (Figure 7). Surprisingly, we found that all the compounds, including cIDPR, are hydrolyzed by the enzyme at 4 mg/mL but at a slower rate than 7. The increased rate of hydrolysis of 7 could conceivably be promoted by the additional hydrogen bond generated between the 8-NH₂ substituent and Asp155 promoting elongation of the N1-bond toward the transition state. This faster hydrolysis of 7 under these conditions (50% hydrolysis for 7 at T = 105 min and for cIDPR at T = 420 min), also reinforced by the extra interaction afforded by the 8-amino group after cleavage, perhaps explains why the hydrolyzed ligand 7a is captured in the 8-NH₂-N9-butylicDPR:CD38 complex, but a cyclic ligand is observed in the previously reported cIDPR:CD38 complex 2PGJ.12

These conditions are representative of crystallization concentrations of shCD38 (10 mg/mL) and are 10,000-fold more concentrated than those used in the enzyme assay (1 μg/ mL). Therefore, further HPLC experiments were carried out using shCD38 concentrations of 1 μg/mL and either cIDPR or 7 to observe the effect of CD38 under conditions that reflect the enzyme assay (Supporting Information, Figure S3). Under these conditions the ligand is not hydrolyzed, confirming that the inhibition observed in the CD38 assay is a consequence of the cyclic compounds, not their hydrolyzed counterparts.

**CONCLUSION**

Two of the analogs developed in this study are potent (≤10 μM) inhibitors of cADPR hydrolysis by CD38. Analogs 4—7 illustrate that the “southern” ribose can be modified to considerably improve inhibitory activity of CD38 mediated cADPR hydrolysis. This agrees with modeling predictions based on the shCD38-ligand crystal structure complex interactions and confirms that the “northern” ribose and base are key features for the interaction of cADPR analogs with the CD38 binding pocket. The investigation of SAR in this region in isolation was not possible prior to development of a method for N1-ribosylation. This method thus opens up new avenues and the potential to introduce desirable characteristics, such as membrane permeability, into cADPR analogs by modifications in this region, without loss of activity at CD38. Crystallization of 8-NH₂-N9-butylicDPR with shCD38 revealed that the cIDPR scaffold can unexpectedly be hydrolyzed at high concentrations of shCD38, resulting in a cocystal structure with linear 8-NH₂-N9-butylicDPR (7a) in the active site. Hydrolysis of the hypoxanthine-linked scaffold offers new insight into the mechanism by which CD38 hydrolyzes cADPR to ADPR and suggests for the first time that the imino form is active in this process. However, HPLC studies suggest that hydrolysis would not occur under assay or physiologically relevant concentrations. Thus, this observation does not detract from the established use of cIDPR or its derivatives as either pharmacological tools or as nonhydrolyzable templates for future CD38 inhibitor design or indeed of 8-Br-cIDPR (3) as a stable membrane permeant agonist of Ca²⁺ release.

**EXPERIMENTAL SECTION**

**General.** All reagents and solvents were of commercial quality and were used without further purification, unless described otherwise. Unless otherwise stated, all reactions were carried out under an inert atmosphere of argon. 1H, 13C, and 31P NMR spectra were collected on a Varian Mercury 400 MHz or Bruker Avance 500 MHz spectrometer. All 1H and 2D NMR assignments are based on gCOSY, gHMBC, gHSQC, and DEPT-135 experiments. Abbreviations for splitting patterns are as follows: b, broad; s, singlet; d, doublet; t, triplet; m, multiplet. Coupling constants are given in hertz (Hz). High resolution time-of-flight mass spectra were obtained on a Bruker Daltonics microOTOF mass spectrometer using electrospray ionization (ESI). The purity of new tested compounds was determined to be ≥95% by analytical HPLC. Analytical HPLC analyses were carried out on a Waters 2695 Alliance module equipped with a Waters 2996 photodiode array detector (210–350 nm). The chromatographic system consisted of a Hichrom Guard column for HPLC and a Phenomenex Synergi 4 μm MAX-PR 80A column (150 mm × 4.60 mm), with elution at 1 mL/min with the following ion-pair buffer: 0.17% (m/v) cetrime and 45% (v/v) phosphate buffer (pH 6.4) in MeOH. Synthetic phosphates were assayed and quantified by the Ames phosphate test.

6-Chloro-9-(4-acetoxybutyl)purine (12).68 To 6-chloropurine 11 (2.50 g, 16.2 mmol) and DBU (2.91 mL, 19.4 mmol) in DMF (22 mL) was added 4-chlorobutylacetate (4.54 mL, 32.3 mmol). After the mixture was stirred at 60 °C for 14 h, the DME was removed under reduced pressure, and the residue was purified by column chromatography on silica gel, eluting with PE/ EtOAc (1:0 → 0.1 v/v) to afford the title compound (3.07 g, 71%) as a colorless oil. R_f = 0.61 (DCM/MeOH 9:1 v/v); 1H NMR (400 MHz, MeOD) δ 8.78 (s,
1H), 8.16 (s, 1H), 4.37 (t, 2H, J = 7.2, CH2), 4.15 (t, 2H, J = 6.4, CH2), 2.08–2.04 (m, 5H, CH2 and OAc), 1.74–1.67 (m, 2H, CH2) ppm.

9-(4-Hydroxylbutyl)adenine (13). A solution of 12 (3.00 g, 11.2 mmol) in MeOH (70 mL) was cooled to 0 °C and saturated with NH4Cl (g) for 1 h at 80 °C. After cooling, 1.5% aq. solution of Na2S2O3 was added to the mixture solution to allow the formation of Na2SO3, and the solution was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with PE/EtOAc (1:0 v/v) to afford the title compound (4.02 g, 98%) as a colorless mass solid. Rf = 0.61 (DCM/acetone 1:1 v/v); 1H NMR (400 MHz, MeOD) δ 8.31 (s, 1H, H-2), 7.64–7.62 (m, 4H), 7.70–7.73 (m, 6H), 5.85 (bs, 2H, CH2), 4.22 (t, 2H, J = 7.4, CH2), 3.70 (t, 2H, J = 6.1, CH2), 2.00–1.93 (m, 2H, CH2), 1.62–1.55 (m, 2H, CH2), 1.03 (s, 9H) ppm; 13C NMR (100 MHz, CDCl3) δ 154.2, 153.0, 151.3, 135.5 (4C), 133.7 (2C), 129.6 (2C), 127.8 (4C), 127.3 (2C), 127.0; 119.9, 63.0, 43.8, 29.5, 26.9 (3C), 26.7, 19.2 ppm; HRMS (ESI) calc'd for C24H32N4O2Si81Br 697.2072, found 699.2031.

9-(4-tert-Butyldiphenylsilyl)butyl)-8-bromoadenine (15). To 13 (2.00 g, 9.65 mmol) in DMF (20 mL) were added imidazole (1.71 g, 25.09 mmol) and TBDPS-CI (3.5 mL, 12.55 mmol). After 1 h at rt, all solvents were evaporated and the residue was purified by column chromatography on silica gel, eluting with DCM/acetone (1:0 v/v) to afford the title compound (3.96 g, 92%) as a white solid. Rf = 0.35 (DCM/acetone 1:1 v/v); 1H NMR (400 MHz, CDCl3) δ 8.31 (s, 1H, H-2), 7.64–7.62 (m, 4H), 7.70–7.73 (m, 6H), 5.85 (bs, 2H, CH2), 4.22 (t, 2H, J = 7.4, CH2), 3.70 (t, 2H, J = 6.1, CH2), 2.00–1.93 (m, 2H, CH2), 1.62–1.55 (m, 2H, CH2), 1.03 (s, 9H) ppm; 13C NMR (100 MHz, CDCl3) δ 154.2, 153.0, 151.3, 135.5 (4C), 133.7 (2C), 129.6 (2C), 127.8 (4C), 127.3 (2C), 127.0; 119.9, 63.0, 43.8, 29.5, 26.9 (3C), 26.7, 19.2 ppm; HRMS (ESI) calc'd for C35H42N4O2Si81Br 753.2227, found 751.2219.

13.5% (4C), 133.7 (2C), 129.6 (2C), 127.8 (4C), 127.3 (2C), 127.0; 119.9, 63.0, 43.8, 29.5, 26.9 (3C), 26.7, 19.2 ppm; HRMS (ESI) calc'd for C24H32N4O2Si81Br 697.2072, found 699.2031.

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9-(4-tert-Butyldiphenylsilyl)butyl)-8-bromohypoxanthine (19). To 18 (430 mg, 0.634 mmol) in acetone/2,2-dimethoxypropane (5 mL, 4:1 v/v) was added p-TsOH (124 mg, 0.654 mmol). After 30 min, DCM and NaHCO3 solution were added. The organic layer was washed with saturated Na2S2O3 solution, and all solvents were evaporated. The residue was taken up in MeOH (2 mL), and Dowex 50W×8 H+ resin (50 mg) was added. After 30 min the resin was removed by filtration under gravity and the solvent was evaporated to afford the title compound (455 mg, 100%) as a colorless oil. Rf = 0.65 (PE/Hex 1:3 v/v); 1H NMR (400 MHz, CDCl3) δ 8.78 (s, 1H, H-2), 7.55 (dd, 4H, J = 5.9, 1.5), 7.35–7.26 (m, 6H, 6H) (10 × Ar-H), 5.62 (dd, 1H, J = 2.8, H-1'), 5.23 (dd, 1H, J = 6.5, 2.8, H-2'), 5.08 (dd, 1H, J = 6.5, 3.6, H-3'), 4.28 (dd, 1H, J = 3.6, 2.4, H-4'), 4.09 (t, 2H, J = 7.5, CH2), 3.85 (dd, 1H, J = 12.2, 2.4, H-S'), 3.75 (bs, 1H, J = 12, 2.2, H-S'), 3.61 (t, 2H, J = 6.0, CH2), 1.89–1.82 (m, 2H, CH2), 1.55–1.49 (m, 2H, CH2), 1.02 (s, 9H) ppm; 13C NMR (100 MHz, CDCl3) δ 155.3, 153.0, 151.0, 140.5, 135.5 (4C), 133.7 (2C), 129.7 (2C), 127.7 (4C), 127.1, 124.6, 91.5, 86.2, 76.9, 70.6, 64.1, 61.7, 45.7, 30.4, 27.4 (3C), 27.1, 19.9 ppm; HRMS (ESI) calc'd for C33H42N4O6Si81Br 765.1739 [(M + H)+], found 767.1734, calc'd for C33H42N4O6Si81Br 765.1718 [(M + H)+], found 765.1729.
The solution was allowed to warm to rt and stirred for a further 2 h, after which DCM (20 mL) and H2O were added. The organic layer was washed with NaHCO3 (sat. aq), then dried (Na2SO4), and evaporated to dryness. The residue was purified by column chromatography on silica gel, eluting with PE/EtOAc (1:0 → 1:0 v/v), where both solvents contained 0.5% v/v pyridine, to afford the title compound (35% yield, 80%) as a colorless glass. Rf = 0.45 (EtOAc 1:3 v/v); H NMR (400 MHz, CDCl3) δ 0.02 (s, 1H, H-2), 7.63–7.60 (m, 4H), 7.40–7.32 (m, 6H) (10 × Ar-H), 5.97 (s, 1H, J = 1.8, H-1′), 5.08 (dd, 1H, J = 6.4, 1.8, H-2′), 4.99 (dd, 1H, J = 6.4, 3.9, H-3′), 4.41 (dd, 1H, J = 9.6, 4.3, H-4′), 4.27–4.13 (m, 4H, both H-5′ and CH2), 3.67 (t, 2H, J = 6.0, CH2), 1.93–1.89 (m, 2H, CH2), 1.57 (s, 3H), 1.55–1.44 (m, 4H, CH2), 1.448 (s, 9H), 1.447 (s, 9H), 1.33 (s, 3H), 1.02 (s, 9H) ppm; 13C NMR (100 MHz, CDCl3) δ 153.0, 147.2, 144.1, 133.7 (4C), 131.9 (2C), 127.8 (2C), 125.8 (4C), 124.2, 122.7, 112.5, 92.5, 85.1 (d, J = 7.9), 83.2, 80.9 (d, 2C, J = 7.2), 79.7, 64.7 (d, J = 6.2), 61.1, 42.8, 28.0 (d, 3C, J = 2.8), 27.9 (d, 3C, J = 2.9), 27.5, 25.3, 25.0 (3C), 24.4, 23.5, 17.3 ppm; 31P NMR (162 MHz, H2O decoupled, CDCl3) δ = –10.2 ppm; HRMS (ESI+), calc'd for C41H58N4O9PSi79BrNa 797.41141 [{(M + H)+}], found 797.4147, calc'd for C41H58N4O9PSi79BrNa 939.1420 [{(M + Na)+}], found 939.1440.

**N1-(2′,3′-O-Isopropylidene-5′-O-(di-tert-butylphosphoryl)-β-D-ribofuranosyl)-N9-(4-hydroxybutyl)-8-bromohydropyranoxane (23)**.

Intermediate 22 (40 mg, 0.044 mmol) was stirred in 50% TFA (2 mL) at 0 °C for 4 h. All solvents were evaporated, and the residue was coevaporated with MeOH (x4). The residue was purified by column chromatography on silica gel, eluting with EtOAc/MeOH/H2O (1:0 → 4:2 → 7:2:1 v/v) to afford the title compound (33 mg, 100%) as a colorless glass. Rf = 0.20 (EtOAc/MeOH/H2O:7:2:1 v/v); H NMR (400 MHz, MeOD) δ 8.59 (s, 1H, H-2), 7.43 (dd, 4H, J = 7.1, 0.7), 7.33–7.28 (m, 6H) (10 × ArH), 6.21 (dd, 1H, J = 3.8, H-1′), 4.24 (d, 2H, J = 2.6, H-2′ and H-3′), 4.16–4.07 (m, 7H, H-4′, both H-5′ and 2 × CH2), 1.70 (app quintet, 2H, J = 7.1, CH3), 1.55 (app quintet, 2H, J = 5.7, CH2) ppm; 13C NMR (125 MHz, MeOD) δ 157.2, 150.6, 147.1, 136.6 (d, J = 4.3, 5.3), 131.1 (d, J = 3.2, 3.4), 130.7 (d, J = 4.3, J = 2.5), 127.6, 127.0 (d, J = 2.7, 2.6), 124.5, 89.8, 85.6 (d, J = 8.6), 77.1, 71.4, 69.2 (d, J = 8.9), 65.3 (d, J = 4.8), 45.2, 28.3 (d, J = 7.0), 26.8 ppm; 31P NMR (202 MHz, H2O decoupled, MeOD) δ 50.8, 0.8 ppm; HRMS (ESI+) calc'd for C42H60N4O9PSi80Br 760.9911 [{(M – H)+}], found 760.9878, calc'd for C42H60N4O9PSi80Br 760.9912 [{(M + H)+}], found 760.9876. Conversion to TEA salt: The Na+ salt was passed through prewashed Dowex H+ resin. Acidic fractions were neutralized with TEAB (2 mL, 1 M). All solvents were evaporated, and the residue was coevaporated with H2O to remove excess buffer. The colorless glass obtained was used directly for cyclization.

**N1-Cyclic-8-bromohydropyranoxane-N9-hydroxybutyl Diphosphoryl Ribose (8-Br-N9-butyl-cdPCR, 5)**.

Intermediate 24 (0.026 mmol) was evaporated from pyridine (2 × 2 mL). The residue was taken up in pyridine (10 mL) and placed in a syringe. This solution was added over 15 h to a solution of iodine (70 mg, 0.30 mmol) and 3 Å molecular sieves (0.5 g) in pyridine (20 mL), in the dark. The solution was filtered through Celite, washed with H2O. After addition of TEAB (2 mL) all solvents were evaporated, and the residue was partitioned between H2O and CHCl3. The aqueous layer was washed with CHCl3 and evaporated to dryness. The residue was purified by semipreparative HPLC (1.1 cm × 25 cm C18 column), eluting with acetonitrile/0.1 M TEAB (1:19 → 13:7 v/v) over 25 min. Fractions were analyzed by analytical HPLC and appropriate fractions collected and evaporated under vacuum to give the title compound (3.0 mg, 21% over two steps). UV (H2O, pH 7), λmax = 256 nm (ε = 19900); 1H NMR (500 MHz, D2O) δ 8.81 (s, 1H, H-2′), 6.08 (d, 1H, J = 1.8, H-1′), 4.43 (dd, 1H, J = 4.7, 1.8, H-2′), 4.38 (dd, 1H, J = 6.9, 4.7, H-3′), 4.35–4.19 (m, 4H, 4.17 (app septet, 1H, J = 5.1, CH2H), 4.11 (dd, 1H, J = 3.9, H-5′), 3.82–1.80 (m, 7H, CH2), 1.56 and 1.48 (d, 3H, J = 7.0), 1.46 (app quintet, 2H, J = 6.3, CH3), 1.36 (s, 3H), 1.454 (s, 9H), 1.449 (s, 9H), 1.32 (s, 9H) ppm; 13C NMR (100 MHz, D2O) δ 154.7, 149.0, 146.0, 135.2 (d, 4C, J = 5.2), 129.4 (d, 2C, J = 3.2), 129.3 (d, 4C, J = 2.5), 126.3 (d, 2C, J = 6.7), 125.8, 124.5, 114.3, 94.3, 86.8

(d, J = 7.9), 85.0, 82.8 (d, 2C, J = 7.3), 81.4, 67.0 (d, J = 8.7), 66.5 (d, J = 6.2), 43.9, 29.77 (d, 3C, J = 4.2), 29.74 (d, 3C, J = 4.2), 27.1, 27.0 (d, J = 6.7), 25.6, 25.2 ppm; 31P NMR (162 MHz, H2O decoupled, CDCl3) δ = 49.0, –103.3 ppm; HRMS (ESI+) calc'd for C54H50N4O9PSi81BrNa 939.1420 [{(M + H)+}], found 939.1440, calc'd for C54H50N4O9PSi81BrNa 939.1420 [{(M + H)+}], found 939.1440.
taken up in Milli-Q (3 mL) and NaHCO₃ (225 mg, 2.77 mmol) was added. When all material had dissolved, EtOH (1.5 mL) and then Pfizer C (6 mg) were added and the solution was placed under an atmosphere of He. After 5 h stirring, the solution was filtered and all solvents evaporated. The residue was purified by semipreparative HPLC (1.1 cm × 25 cm C₁₈ column), eluting with MeCN:0.1 M TEAB (1:19 v/v) over 25 min. Fractions were analyzed by analytical HPLC and appropriate fractions collected and evaporated under vacuum to give the title compound (4 mg, 76% over two steps). UV (H₂O, pH 7), δ_m _ax = 252 nm (ε = 14 400); ¹H NMR (500 MHz, D₂O) δ 8.79 (s, 1H, H-2), 8.03 (s, 1H, H-8), 6.10 (s, 1H, H-1'), 4.49 (d, 1H, J = 3.0, H-2'), 4.40 (app t, 1H, J = 3.0, H-3'), 4.33–4.42 (4H, dd, 1H, J = 11.8, 4.4, H-S'), 4.05 (app septet, 1H, J = 5.2, CH₂H), 3.81 (app septet, 1H, J = 5.2, CH₂H), 1.96–1.84 (4H, 2H, CH₂), 1.41–1.35 (1H, 1H, CH₂H), 1.11–1.05 (1H, 1H, CH₂H) ppm; ¹³C NMR (125 MHz, D₂O) δ 158.0, 148.9, 147.4, 142.3, 122.2, 91.1, 83.6 (δ, J = 8.9), 75.2, 68.3, 65.1 (δ, J = 5.9), 63.0 (δ, J = 4.5), 42.3, 25.4, 25.0 (δ, J = 9.5) ppm; ³¹P NMR (202 MHz, D₂O, 4H-decoupled) δ 10.3 (d, J = 14.1), −11.2 (d, J = 14.2) ppm; HRMS (ESI⁺) calc for C₁₉H₂₅N₅O₇P, 511.1839 [M – H]⁺ found 511.1822.

¹H-NMR (D₂O) δ 7.76 (app d, 1H, J = 7.5, H-6'), 7.45 (app d, 1H, J = 7.5, H-5'), 7.36 (app d, 1H, J = 7.5, H-4'), 4.80 (d, 1H, J = 14.1, H-S'), 3.95 (app septet, 1H, J = 5.3, CH₂H), 2.10–1.85 (4H, CH₂), 1.53–1.50 (1H, H-1′), 1.31–1.28 (1H, H-2′) ppm; ¹³C NMR (125 MHz, D₂O) 155.3, 152.2, 148.3, 143.2, 116.7, 91.1, 83.5 (δ, J = 8.9), 75.2, 68.1, 65.1 (δ, J = 5.6), 62.9 (δ, J = 3.1), 39.7, 24.8 (δ, J = 9.6), 22.5 ppm; ³¹P NMR (202 MHz, D₂O, 4H-decoupled) δ 10.39 (br, J = 11.44 (br); HRMS (ESI⁺) found m/z [M – H]⁺ 496.0637; C₁₉H₂₅N₅O₇P requires 496.0640.

Enzyme Assay for cADPR Hydrolysis. The inhibition of cADPR hydrolysis by various concentrations of analog (0–1 mM) was determined by incubating 1 μM cADPR with 1 μg/mL CD38 catalytic domain for 10 min at 20–24 °C in 25 mM sodium acetate, pH 4.5. The reaction was stopped by the addition of 150 mM HCl. The precipitated protein was filtered, and the pH was neutralized with Tris base. After the mixture was diluted 20-fold, the concentration of the unhydrolyzed cADPR present in the diluted reaction mixture was assayed by the fluorometric cycling assay as described previously.52

Modeling of the Butyl Compounds. The 2PGJ crystal structure of human CD38 with cADPR was passed through the Protein Preparation Wizard in the Schrödinger software (http://www.schrodinger.com/). Four N-buty1 compounds, where the cADPR “southern” ribose is replaced by a four carbon linker, were built using the Schrödinger software. The four compounds differed in the substituent at the 8-position: hydrogen, bromine, azido, or an amino group. The compounds were docked into the 2PGJ structure using GOLD.56 The binding site was defined as a sphere of 5 Å radius centered on the centroid of the cADPR ligand: the centroid of the docked ligands was to lie within this sphere. Each ligand was docked 25 times. The best ranked pose of each of the ligands was merged with the protein structure, and the resulting complex was passed through a minimization procedure using the Schrödinger software.

Crystallization, Diffraction Data Collection, and Structure Refinement. The human CD38 catalytic domain was expressed and purified as described previously.45,54 The protein was diluted to 10 mg/mL for crystallization trials. Crystals of wild type CD38 were obtained by the hanging drople vapor diffusion method with the reservoir buffer in 0.1 M sodium acetate, pH 4.0, 15% PEG 10K, 0.2 M ammonium acetate, and 3% isopropanol. They were harvested and soaked in 0.1 M sodium acetate, pH 4.0, 15% PEG 10K, 16% ethylene glycol, and 5 mM compound 7 for 1 h at 295 K and then flash-frozen in liquid nitrogen. The diffraction data were collected at 100 K on beamline BL17U at the Shanghai Synchrotron Radiation Facility and processed with HKL2000.59 Molecular replacement was performed using the program Phaser60 from the CCP4 suite,61 and the wild-type human CD38 (PDB code 1YH3) was used as the search model. The model was refined with Refmac62 and then cycled with manual building in Coot.63 Hydroxylated compound 7 was built into positive difference electron-density maps of the CD38 model after a few restrained refinement runs with the stereochemical restraints generated from the program PRODRG.64 TLS refinement65 was incorporated into the later stages of the refinement process. Solvents were added automatically in Coot and then manually inspected and modified. The final model was analyzed with MolProbity,66 showing that 98% residues were in the Ramachandran favored region with only one residue (D202 in chain B) in the Ramachandran outlier region. Data collection and model refinement statistics are summarized in Supporting Information Table 1. The coordinates and structure factors are deposited in the Protein Data Bank with the code 4TMF.

HPLC Studies. The solution containing the CD38 catalytic domain was adjusted to the desired concentration using Tris-HCl buffer (20 mM, pH 8), and 50 μL of the mixture was then added to the inhibitor (0.05 μmol) in an Eppendorf tube at room temperature. At a given time point, a sample of 5 μL was removed and diluted with 95 μL of Milli-Q water. Then 10 μL of this sample was injected directly into the analytical HPLC system (see section General in Experimental Section), eluting at 1 mL/min with an isocratic ion-pair buffer: 0.17% (v/v) cimetidine and 45% (v/v) phosphate buffer (pH 6.4) in MeOH.
ADPR, adenosine 5′-diphosphate ribose; ADPRC, adenosine 5′-diphosphate ribosyl cyclase; cADPR, cyclic adenosine 5′-diphosphoribose; cCDPR, cyclic adenosine 5′-diphosphoribose; CD38, cyclic ADP-ribose carboxyribicicose; CD38, cyclic adenosine 5′-diphosphoribose; cDPR, cyclic inosine 5′-diphosphoribose; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD+, nicotinamide adenine 5′-dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; N-mybutyl-CDPR, cyclic-N- mybutylhypoxanthine 5′-diphosphate ribose; shCD38, CD38 catalytic domain

ADDITIONAL NOTE
“Note that for the N9-butyl analogs, the prime notation refers to the N1-ribose, in contrast to the natural products where the prime notation refers to the N9-ribose.

REFERENCES


