Catalysis-associated Conformational Changes Revealed by Human CD38 Complexed with a Non-hydrolyzable Substrate Analog*§

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Cyclic ADP-ribose (cADPR) is a calcium mobilization messenger important for mediating a wide range of physiological functions. The endogenous levels of cADPR in mammalian tissues are primarily controlled by CD38, a multifunctional enzyme capable of both synthesizing and hydrolyzing cADPR. In this study, a novel non-hydrolyzable analog of cADPR, N1-cIDPR (N1-cyclic inosine diphosphate ribose), was utilized to elucidate the structural determinants involved in the hydrolysis of cADPR. N1-cIDPR inhibits CD38-catalyzed cADPR hydrolysis with an IC50 of 0.26 mM. N1-cIDPR forms a complex with CD38 or its inactive mutant in which the catalytic residue Glu-226 is mutated. Both complexes have been determined by x-ray crystallography at 1.7 and 1.76 Å resolution, respectively. The results show that N1-cIDPR forms two hydrogen bonds (2.61 and 2.64 Å) with Glu-226, confirming our previously proposed model for cADPR catalysis. Structural analyses reveal that both the enzyme and substrate cADPR undergo catalysis-associated conformational changes. From the enzyme side, residues Glu-146, Asp-147, and Trp-125 work collaboratively to facilitate the formation of the Michaelis complex. From the substrate side, cADPR is found to change its conformation to fit into the active site until it reaches the catalytic residue. The binary CD38-cADPR model described here represents the most detailed description of the CD38-catalyzed hydrolysis of cADPR at atomic resolution. Our structural model should provide insights into the design of effective cADPR analogs.

Cyclic ADP-ribose (cADPR)§ is a novel cyclic nucleotide derived from NAD. It is metabolized by a family of proteins called ADP-ribose cyclases (1, 2). This cyclic nucleotide features a head-to-tail type of glycosidic linkage (N1-C1′) between N1 of its adenine and the anomeric carbon (C1′) of the terminal ribose (3, 4). Results obtained in the past decade have established the second messenger role of cADPR in mobilizing calcium stores in various cell types (reviewed in Refs. 5, 6). Its calcium signaling function has since been found to be more versatile and has additionally been shown to modulate calcium influx across the plasma membrane (7, 8) as well as regulate calcium homeostasis within the nucleus (9, 10).

Human CD38 is a type II transmembrane glycoprotein containing a small N-terminal tail, a single transmembrane helix, and a large extra-membranous portion that possesses ADP-ribose cyclase activity. As a member of the cyclase family, CD38 not only can synthesize cADPR from NAD but also can hydrolyze NAD and cADPR to produce ADP-ribose (2, 11–14). At acidic conditions, CD38 can also catalyze the formation and hydrolysis of NAADP, another calcium mobilization messenger (15, 16). Although the mechanism of how these various activities of CD38 are regulated inside cells remains to be determined, we have previously identified residues Glu-146 as being critically important for controlling the relative synthesizing and hydrolyzing activities (16, 17). The enzymatic portion of human CD38 can be subdivided into two domains, the α-helix-rich N-domain and the β-strand-rich C-domain. Site-directed mutagenesis and x-ray crystallography studies have identified a single active site in the enzymatic domain that is located in a pocket at the central cleft separating the C- and N-domains (16, 18–20). This active site is responsible for all of the multiple enzymatic activities of CD38 (also reviewed in Ref. 21).

Studies using knock-out mice have established that CD38 is the major mammalian enzyme responsible for regulating endogenous levels of cADPR (22, 23). Multiple defects have been seen in the knock-out mice, indicating the important roles of CD38 in regulating physiological functions (23–27). In addition to its cADPR-synthesizing activity, CD38 is in fact the only characterized enzyme that can effectively degrade cADPR. To

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The atomic coordinates and structure factors (code 2PGL, 2PGJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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3 The abbreviations used are: cADPR, cyclic ADP-ribose; cGDPR, cyclic GDP-ribose; N1-cIDPR, N1-cyclic inosine diphosphate ribose; MES, 4-morpholineethanesulfonic acid.
better understand CD38-cADPR-dependent calcium signaling, it is important to know the structural determinants and molecular interactions involved in the synthesis and hydrolysis of cADPR catalyzed by CD38.

We have previously obtained the structure of a CD38-cADPR complex by using a catalytically inactive mutant of CD38. This was done by mutating the catalytic residue Glu-226 to glutamine (Q), and the inactive mutant (E226Q) was used to form a complex with cADPR (20, 28). The structure shows that Gln-226 is too far away from the binding site of cADPR to be involved in catalysis. An analog of cADPR, cyclic GDP-ribose (cGDPR), which is cyclized at the N7 position instead of the N1 position of cADPR, however, is able to bind close to Gln-226. These structural results have led us to propose that in wild-type CD38, cADPR may, in fact, bind close to Glu-226 in a manner similar to cGDPR so that catalysis can occur. It has been further proposed that the catalytic residue is involved not only in attacking the scissile glycosidic bond but also in stabilizing cADPR at an active position close to the residue for catalysis. To prove our proposal, a binary system consisting of the active wild-type CD38 and an inactive substrate analog is necessary.

Recently, an analog of cADPR, N1-cIDPR, has been synthesized that is totally resistant to hydrolysis by CD38 (29). It is also a close structural analog of cGDPR so that catalysis can occur. The only difference is an oxo group at position 6 of the purine ring replacing the imino group of cADPR. CD38, cADPR may, in fact, bind close to Glu-226 in a manner similar to cGDPR so that catalysis can occur. It has been further proposed that the catalytic residue is involved not only in attacking the scissile glycosidic bond but also in stabilizing cADPR at an active position close to the residue for catalysis. To prove our proposal, a binary system consisting of the active wild-type CD38 and an inactive substrate analog is necessary.

In the current study, we have succeeded in obtaining a complex of N1-cIDPR with the wild-type CD38. The results show that the cyclic nucleotide indeed binds close to the catalytic residue Glu-226, confirming our original proposal. Structural analyses of the complex reveal that both the active site of CD38 and the substrate are flexible and multiple conformational changes accompany substrate binding and the catalytic process.

**EXPERIMENTAL PROCEDURES**

**Protein Production, Crystallization, Complex Formation, and Data Collection**—Expression, purification, and crystallization of wild-type CD38 and E226Q mutant proteins were performed using procedures as previously described (18, 20, 28). N1-cIDPR was synthesized by a chemo-enzymatic procedure as published (29). The CD38-N1-cIDPR complex was obtained by incubating preformed wild-type CD38 crystals with 30 mM N1-cIDPR for 4 min at 4 °C in 3 mM MES, pH 6.0. The preformed CD38 crystals were grown at room temperature as described previously (18, 20, 28). The incubation conditions required to obtain the complex did not alter the crystals as they were stable for as long as several hours at 4 °C. Similarly, the E226Q-N1-cIDPR complex was obtained by incubating preformed E226Q mutant crystals with 30 mM N1-cIDPR for 4 min at 4 °C in 3 mM MES, pH 6.0. X-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS) A1 station under the protection of a liquid nitrogen cryo-stream at 100 K. A total of 360 images were collected from a single crystal with an oscillation angle of 1°. Raw images were integrated, scaled, and merged by using the program HKL2000 (31). The crystallographic statistics are listed in Table 1.

**Structure Refinement**—The N1-cIDPR complex structures were refined by REFMAC (32) with the starting model derived from the CD38 E226Q-cGDPR complex (PDB accession code 2O3T). There are two CD38 molecules in the crystallographic asymmetric unit. Only the electron densities in the active site of one of the two molecules were modeled as N1-cIDPR. In the

**TABLE 1**

| Data collection | CD38-N1-cIDPR | E226Q-N1-cIDPR |
|-----------------|---------------|---------------|
| Cell dimensions | a, b, c (Å)   | a, b, c (Å)   |
| a, b, c (Å)     | 41.891        | 41.866        |
| Root mean square | 16.10 (1.88)  | 16.00 (1.88)  |
| Root mean square | 8.2 (1.18)    | 7.1 (1.547)   |
| Completeness (%) | 92.7 (71.0)   | 96.6 (90.6)   |

**Refinement**

| Resolution (Å) | CD38-N1-cIDPR | E226Q-N1-cIDPR |
|----------------|---------------|---------------|
| Resolution (Å) | 30-1.70       | 30-1.75       |
| Resolution (Å) | 30-1.70       | 30-1.75       |
| Resolution (Å) | 30-1.75       | 30-1.75       |
| Multiplicity   | 3.6 (2.5)     | 3.7 (2.9)     |
| Multiplicity   | 14.82 (2.74)  | 16.10 (1.88)  |
| Rmerge (%)     | 8.2 (31.8)    | 7.1 (54.7)    |
| Rmerge (%)     | 92.7 (71.0)   | 96.6 (90.6)   |

**Ramachandran plot**

| Most favored (%) | CD38-N1-cIDPR | E226Q-N1-cIDPR |
|------------------|---------------|---------------|
| Most favored (%) | 88.6          | 88.4          |
| Additional allowed (%) | 10.1 | 10.5 |
| Generally allowed (%) | 1.1  | 0.9  |
| Disallowed (%) | 0.2           | 0.2           |

Values in parentheses are from the highest resolution shell.

**FIGURE 1.** Schematic diagram for the structure of cADPR and N1-cIDPR. N1-cIDPR (right panel) is a structurally close analog of cADPR (left panel). The only difference between cADPR and N1-cIDPR is shown as shadowed. The C6-imino group (NH) in cADPR was substituted by a C6-keto group (O) in N1-cIDPR.
other molecule, the electron densities are too noisy to be useful. The N1-cIDPR molecule was built in O (33) based on the structure of cGDPR and the weighted $F_o - F_c$ difference electron densities. Solvents were added automatically by Arp/warp (34) and manually inspected/modified under the program O. At the end of refinements, translation, libration, screw rotation (TLS) refinement implemented in REFMAC was introduced to model the data anisotropy. The refinement results and model statistics are listed in Table 1.

Modeling the cADPR Structure—The cADPR model was built based on the wild-type CD38-N1-cIDPR complex. The O6 atom on the purine ring was changed to NH, and the new CD38-cADPR model was energetically minimized with the C6-N6 distance restrained at 1.33 Å. To keep the overall structure unchanged, we included the diffraction data during the energy minimization. Before running energy minimization with the program CNS (35), the occupancies for residues 129, 133, 143–148, and 155 in molecule A were set to 0. These residues showed structural differences from their corresponding residues in the cGDPR complex and were supposed to undergo structural changes during cADPR binding. Setting their occupancies to 0 excludes the x-ray term restraints on these selected residues so that they can move during the energy minimization. A control, in which x-ray terms for these indicated residues were turned off, was also performed in an equivalent refinement of the original CD38-N1-cIDPR complex.

Enzymatic Assay for cADPR Hydrolysis—The inhibition of cADPR hydrolysis by various concentrations of N1-cIDPR (0–1 mM) was determined by incubating 1 μM cADPR with 2 μg/ml of CD38 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 diluting the mixture 20-fold, the concentration of the unhydrolyzed cADPR present in the diluted reaction mixture was assayed by the fluorimetric cycling assay as described previously (36).

RESULTS

Structure of N1-cIDPR Complexed with Wild-type CD38—The structure of the CD38-N1-cIDPR complex refined at 1.7 Å contained two molecules of CD38 in the asymmetric unit, with N1-cIDPR present in only one of them. This is also true for the cGDPR complex as previously reported (28). N1-cIDPR bound to a pocket at the cleft that separated the N- and C-domains of CD38 (Fig. 2, A and B). The 2'-, 3'-OH groups on the N1-cIDPR northern ribose form two hydrogen bonds with the carboxylate group of Glu-226 (Fig. 2C). The C1'-carbon of the bound N1-cIDPR, is 3.43 Å from Glu-226, a suitable distance for catalytic attack of the N1-glycosidic bond by the residue. Similar distances between the catalytic residue and the bound nucleotide ribose were also found in CD38 soaked in NGD (19) and CD157 bound with NMN (37).

In addition to Glu-226, N1-cIDPR interacted with the substrate binding motif (Trp-125, Ser-126, Arg-127, Thr-221, Phe-222) and the activity regulatory motif (Glu-146 and Trp-189) identified previously (28). The oxygen at position 6 of the purine ring (6-oxo) was 2.24 Å from Glu-146 (Fig. 2C). Additionally, Ser-193, a residue previously identified as having an auxiliary catalytic role (19, 28), was 3.18 Å from the N1-glycosidic bond of N1-cIDPR. The geometry of the anomeric carbon (C1') and its proximity to the OH group of Ser-193 suggest the formation of a hydrogen bond between C1' and the OH group with C1' being the hydrogen donor.

Comparison between the N1-cIDPR and the cADPR Complexes—Comparing the CD38-N1-cIDPR structure with the previously published E226Q-cADPR structure (28) showed that N1-cIDPR can bind much deeper into the active site pocket (Fig. 3A). The binding of a non-hydrolyzable analog into the catalytic pocket is expected to block entry of cADPR. Indeed, we have observed inhibition of cADPR hydrolysis by N1-cIDPR with a half-maximal effect at 0.26 mM.

In the case of N1-cIDPR, the N6 is replaced with oxygen (O6), resulting in altered interactions with both Glu-146 and Asp-155. N1-cIDPR is now closer to catalytic residue (Glu-226) by N1-cIDPR with a half-maximal effect at 0.26 μM.

Fig. 3B compares the binding of cADPR to the inactive E226Q mutant and N1-cIDPR to the wild-type CD38. It can be seen that, in the case of the cADPR-E226Q complex, the N6 of the cADPR was H-bonded not only to Glu-146 but also to Asp-155. Additionally, Glu-146 was H-bonded to one of the ribosyl hydroxyl groups of cADPR. Hydrophobic overlap between the CD38 adenosine moiety and Trp-189 indole moiety was also extensive, involving both rings of the adenine. The mutation of the catalytic residue Glu-226 to Glu-226 trapped cADPR in an unproductive site, 6.12 Å away from the catalytic residue. In the case of N1-cIDPR, the N6 is replaced with oxygen (O6), resulting in altered interactions with both Glu-146 and Asp-155. N1-cIDPR is now closer to catalytic residue (Glu-226)
and forms two H-bonds with its carboxyl group. Consistently, we have previously shown that cGDPR (28), which also has O6 instead of N6 in its purine ring, likewise can bind deep into the pocket in a manner similar to N1-cIDPR.

Comparison with E226Q-N1-cIDPR Complex—To directly compare with the E226Q-cADPR complex, we determined the crystal structure of the E226Q-N1-cIDPR complex at 1.76 Å. Similar to that seen with the wild-type CD38 complex as described above, N1-cIDPR not only can bind to the equivalent site in the E226Q mutant but can also bind very close to Gln-226 deep inside the site. The alignment of the E226Q-N1-cIDPR structure and wild-type CD38-N1-cIDPR structure shows slight differences for their equivalent sites (Fig. 4A). In the E226Q-N1-cIDPR structure, Gln-226 was 2.84 and 2.61 Å from the ribosyl 2'-O11032-3'-O11032-OH groups, respectively, suggesting the formation of two hydrogen bonds. The carbonyl group of Gln-226 is less electronegative than the carboxyl group of Glu-226 in the wild-type CD38 and thus is expected to form weaker hydrogen bonding interactions. Consistently, Gln-226 was 3.71 Å to the C1'-H11032-carbon in the E226Q complex, farther away than the distance of 3.43 Å between Glu-226 and C1'-H11032-carbon of N1-cIDPR in the wild-type complex. These observations are all consistent with the proposal that the catalytic residue Glu-226 is also important in stabilizing the substrate, such as cADPR or N1-cIDPR, at its catalytic position.

Two other residues, Ser-193 and Glu-146, also showed mildly altered interactions between N1-cIDPR and the enzyme upon Glu-226 mutation as shown in Fig. 4A. The consequence of the E226Q mutation can also be seen in the electron densities of N1-cIDPR in the E226Q mutant and wild-type CD38. Comparing the $\text{Fo}_\text{H11002} - \text{Fc}_\text{H11002}$ electron densities for N1-cIDPR are shown as gray isomesh contoured at 1.5 $\sigma$. The densities for the protein residues are good in both structures. The poorer densities for N1-cIDPR in the E226Q mutant are the consequence of the E226Q mutation that weakens the interactions to the ribose of N1-cIDPR.
that the electron density for O6 was absent in the E226Q-N1-cIDPR complex (Fig. 4B), whereas it was very clear in the CD38-N1-cIDPR complex (Fig. 4C).

**DISCUSSION**

**A Proposed Nonproductive Site along the cADPR Entry Pathway**—CD38 is the only known protein that can hydrolyze cADPR to ADP-ribose and thus is important for the metabolism of cADPR. In our prior study, we have demonstrated the trapping of cADPR in a nonproductive site when it formed a binary complex with mutants of CD38, E226Q, E226G, or E226D (28). The effort to form a complex between cADPR and wild-type CD38 resulted in only ADPR, the product, in the active site due to the hydrolysis of cADPR. N1-cIDPR, which is not hydrolyzable by the wild-type CD38, is thus more suitable for complex formation.

The crystal structure of CD38-N1-cIDPR complex determined in this study demonstrates that N1-cIDPR can reach the catalytic residue and be stabilized by forming two H-bonds between them (Fig. 2C). During the hydrolysis of cADPR by the wild-type CD38, these two H-bonds are likely to be also present, since they are likely to be important in both substrate activation and the stabilization of the reaction intermediate.

There is likely a nonproductive site on the wild-type CD38 during substrate binding, similar to that seen in the E226Q-cADPR complex (Fig. 3). The superposition of apo-CD38 with apo-E226Q shows that the nonproductive site seen in E226Q is structurally conserved with the corresponding site in the wild-type CD38, with a root mean square difference of only 0.27 Å for residues Trp-125, Ser-126, Arg-127, Glu-146, Asp-155, Trp-189, Ser-193, Thr-221, Phe-222, and Glu-226 (see supplemental Fig. S1). It is logical to propose that during the entry of cADPR into the active site, the substrate may interact with this same site. As the site is close to the mouth of the active site pocket, it can represent the first and transitory contact site of cADPR with the wild-type CD38 before reaching the catalytic residue Glu-226. The E226Q-cADPR complex thus is likely to represent a transient state during the binding of cADPR. Fig. 5B superimposes this transient state (gray) and the final state (green) of cADPR binding inside the active site. These results represent the most detailed description of the catalytic hydrolysis of cADPR at atomic resolution and are fully consistent with all available biochemical data.

**Model of the CD38-cADPR Complex**—The close similarity between N1-cIDPR and cADPR makes it a suitable analog for elucidating the structural determinants important for cADPR hydrolysis by the wild-type CD38. To obtain a model for the wild-type CD38-cADPR complex, the 6-oxo group (O6) on the purine ring was replaced by an imino group (NH) followed by energy minimization to construct the hydrogen bond interac-
Conformational Changes Associated with Human CD38 Catalysis

The results show that the side chain of residue Glu-146 undergoes a large movement and forms two hydrogen bonds to purine N6 and N7 at distances of 2.6 and 2.9 Å, respectively (Fig. 5A, green).

To judge the significance of the revealed molecular motions, we also performed a control refinement of the CD38-N1-cIDPR complex in which x-ray terms for residues (129, 133, 143–148, and 155) were turned off. The refined N1-cIDPR-CD38 and cADPR-CD38 models were superimposed on these residues. Results show that the root mean square deviation between those indicated residues is 0.17 Å for the side chain atoms and 0.06 Å for the main chain atoms, suggesting that the protein side chain atoms are more flexible than the main chain during the transition from cIDPR to cADPR. The most obvious movement seen in the side chains is for Glu-146, the residue involved in forming two H bonds with the cADPR N6 and N7 atoms.

The cADPR-CD38 model obtained should represent the final position of cADPR in the Michaelis complex before the catalytic attack. Superimposed on the model is the structure of the apo-CD38 (Fig. 5A, yellow) to illustrate the conformational change of the residue Glu-146 during catalysis.

Conformational Changes Accompanying Substrate Binding and Catalysis—The first change involves residues Glu-146 and Asp-147. During cADPR entry, the two residues need to retreat from the yellow positions (apo-CD38, Fig. 5A) to the green positions (Fig. 5A) of the final states, respectively, to make room for the adenine ring of cADPR. The backward movements of the residues Glu-146 and Asp-147 were also observed for the binding of both N1-cIDPR and cGDPR but not for the binding of either NAD, nicotinamide guanine dinucleotide, or nicotinamide mononucleotide (16, 19, 28). They are thus specific for cyclic substrates but not for linear substrates.

The important role of Glu-146 in regulating the enzymatic activities of CD38 has been well established by site-directed mutagenesis (17). This is also structurally obvious as it forms direct hydrogen bonds with the N6 of cADPR and the ribosyl 2’-OH group (Fig. 5B, gray) to facilitate the recruitment of cADPR. As the substrate moves to the final catalytic position, Glu-146 constructs sustained hydrogen bonding interactions with the N6 and N7 nitrogen on the substrate purine ring (Fig. 5, A and B, green).

The second change is the indole ring of Trp-125. Comparison of the cADPR-E226Q complex and the cADPR-CD38 model shows that the side chain of Trp-125 rotates toward the active site, accompanying the entry of cADPR from the nonproductive site to the productive site. This results in a 1.16 Å movement for the tip (CH2 atom) of the indole ring (Fig. 5B). The conformational change of the Trp-125 side chain is necessary; otherwise, cADPR would have a close hydrophobic contact with Trp-125 at a distance of 2.40 Å, which is stereochemically unlikely. In other words, the conformational change of the side chain relieves the contact between Trp-125 and the substrate from 2.40 to 3.42 Å, a more favorable distance for hydrophobic interaction. The conformational change for Trp-125 during the catalysis is consistent with the reduced intrinsic fluorescence changes upon substrate binding (38).

The third change is the side chain of Arg-127, which also undergoes a small movement (0.93 Å) during the substrate binding process to optimize its interactions with the substrate (Fig. 5A). Similar to Trp-125, the movement of Arg-127 is essential, or cADPR will be too close to Arg-127 with a distance of only 1.80 Å.

Enzyme-induced Structural Changes of the Substrate—Corresponding to the conformational changes observed in the active site of the enzyme, cADPR itself also undergoes changes. This can be shown by comparing the structures of cADPR determined at its free acid form by x-ray crystallography (4) (Fig. 5C, yellow, Free form) and cADPR bound to the wild-type CD38 derived from the N1-cIDPR complex (Fig. 5C, green, Activated form). The crystal structure of cADPR is shown by NMR to be the same as its natural form adopted in solution (39).

For easy visualization of the substrate transformation, we aligned the adenine rings of the two cADPR molecules (Fig. 5C). Comparing the free form cADPR with the one that bound deep into the active site of the wild-type CD38, it can be seen that the northern ribose was altered to fit the active site as it forms two H-bonds with the catalytic residue Glu-226. Concomitantly, the phosphate groups also change their conformations to adopt more comfortable interactions with the enzyme’s substrate recognition motif defined by residues Trp-125, Ser-126, Arg-127, Thr-221, and Phe-222.

Implications for Inhibitor Design of cADPR Analogues—Studies using CD38 knock-out mice have firmly established the important role of CD38 in a wide range of physiological functions. Most recently, CD38 and its metabolite, cADPR, have been shown to be critical for social behavior in mice by regulating oxytocin secretion (40). It is clearly desirable to develop effective antagonists and agonists of CD38 for physiological studies as well as for possible clinical intervention. The detailed structures of the complexes present in this study should provide valuable clues for their design. One possible approach would be to model such compounds based upon N1-cIDPR. Here we showed that binding of the non-hydrolyzable substrate to the active site indeed results in inhibition of the enzymatic activity of CD38. Modifications on N1-cIDPR to increase its affinity for the active site should produce much more effective inhibitors. An obvious possibility is to introduce an amino group at the 8-position of the purine ring, which could potentially form a hydrogen bond with Asp-155, increasing its affinity for the active site relative to N1-cIDPR (41). The availability of the high resolution structure of the active site provided in this study together with structural approaches to the design of novel potential non-hydrolyzable ligands should also allow the use of computer docking programs to design molecules that fit better into the active pocket. It is anticipated that effective regulator molecules for CD38 will be forthcoming.

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