Crystal structure of human E-cadherin-EC1EC2 in complex with a peptidomimetic competitive inhibitor of cadherin homophilic interaction.

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ABSTRACT: Cadherins are transmembrane cell adhesion proteins whose aberrant expression often correlates with cancer development and proliferation. We report the crystal structure of an E-cadherin extracellular fragment in complex with a peptidomimetic compound that was previously shown to partially inhibit cadherin homophilic adhesion. The structure reveals an unexpected binding mode and allows the identification of a druggable cadherin interface, thus paving the way to a future structure-guided design of cell adhesion inhibitors against cadherin-expressing solid tumors.

INTRODUCTION

Cadherins are transmembrane calcium-dependent cell adhesion molecules that are mostly localized at the intercellular junctions and are known to play a crucial role in tissue morphogenesis and architectural integrity.[1,2] Many functional studies have shown that aberrant expression of epithelial E-cadherin (CDH1) and neuronal N-cadherin (CDH2) often correlates with tumor progression.[3,4] In fact, the switch in expression from E-to N-cadherin is a key event in the cellular epithelial-to-mesenchymal transition (EMT) that takes place as cancer progresses. Despite its tumor repressor role in the majority of carcinomas, in epithelial ovarian cancer (EOC) cells E-cadherin shows a high level of expression during tumor progression and facilitates EOC cell proliferation.[5] Interestingly, most cadherin family members are now being considered important pharmaceutical targets. For instance, a role for P-cadherin (CDH3) in different types of cancer, such as in malignant melanoma, breast, gastric, lung, colorectal and pancreatic cancer has also been clearly identified.[6,7] Therefore, efforts to develop therapeutic agents against P-cadherin have recently intensified.[8] Likewise, increased expression of cadherin-11 (CDH11) on poorly differentiated highly invasive breast cancer cell lines is also associated with EMT and tumor progression.[9,10] Furthermore, cadherin-11 is a validated therapeutic target in rheumatoid arthritis (RA).[11] Hence, efforts to develop effective therapeutic agents against cadherin-11 are also under way.[12]

Cadherins mediate cell-cell adhesion by means of a concerted mechanism whereby proteins protruding from opposing cells interact with each other at the cellular adherens junctions. All classical cadherin family members share a high degree of homology and structural similarity. Classical cadherins comprise an elongated extracellular portion formed by five immunoglobulin-like extracellular cadherin domains (ECs) arranged in tandem, and an intracellular portion whose dynamic association with cytoplasmic molecules called catenins provides a physical link between the membrane-bound cadherins and the actin cytoskeleton. Structural and mutational studies on several type-I classical cadherin family members such as E-, N-, P- and C-cadherin have clearly shown that dimerization of two cadherin molecules protruding from the surface of two neighboring cells at the adherens junction occurs through a highly dynamic strand-exchange mechanism involving the opening of a 6-residue long N-terminal portion of the protein, usually referred to as the adhesion arm, and the mutual insertion of the side chain of the conserved Trp2 residue into the highly conserved acceptor pocket in the EC1 of the partner molecule (**Fig. 1, A and C**).[13-19] Likewise, in type-II classical cadherins, such as cadherin-11, cadherin-8 and MN-cadherin, a similar

mechanism involves the simultaneous mutual insertion of the side chain of the conserved Trp2 and Trp4 residues into the binding pockets in the partner molecule's EC1, providing a tighter link and a larger interface between the two interacting monomers relative to type-I cadherins.[20,21]

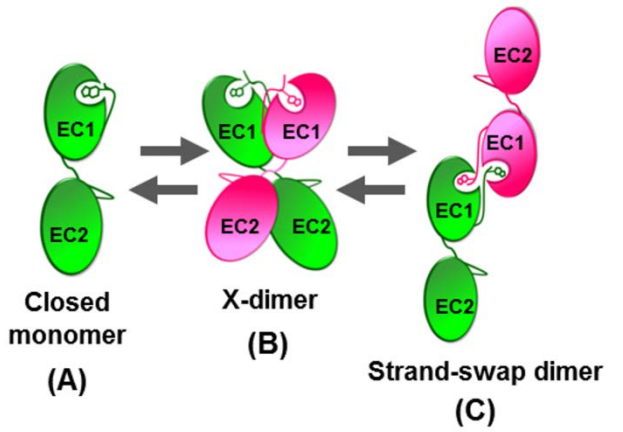

Figure 1 Schematic of the dynamic dimerization mechanism that leads from monomeric cadherin (A) to strand swap dimer formation (C) and back. The mechanism is characterized by the opening of the adhesion arm of two interacting cadherins and the mutual insertion of the Trp2 side chain in the binding pocket of the partner molecule. This process occurs via the formation of a weakly adhesive intermediate configuration which is usually referred to X-dimer (B) and has been shown to facilitate arm opening by lowering the energy barrier associated with strand swap dimer formation.

Interestingly, the highly dynamic cadherin dimerization pathway that leads from monomeric cadherin to strand-dimer formation and back involves a number of crucial intermediate steps that are critical not only for cell adhesion but also for conferring specificity to the cellular recognition process during tissue morphogenesis. Along the complex trajectory that leads from monomers to strand-swap dimers and back, cadherins have been shown to go through an intermediate dimeric state characterized by stabilizing contacts between two cadherin molecules at the level of their Ca^{2+}-binding site between the EC1 and EC2 domains (**Fig. 1, B**). This arrangement, usually referred to as the X-dimer[14,15,22-24], provides a weak adhesive force between cadherin molecules[25] and facilitates subsequent strand-swap dimer formation by lowering the energy barrier associated with the strand swapping process, suggesting that X-dimer formation is one of the major structural "checkpoints" along the cadherin dimerization pathway that leads from monomeric cadherin to strand dimer formation.[26] A K_d value in the sub-millimolar range has been estimated for the homophilic adhesion of classical type I cadherins.[23,27]

Owing to this intrinsic dynamic behavior, the rational design of small ligands targeting cadherin homophilic interactions has proved difficult and, to date, no crystal structure exists of a complex between a cadherin molecule and an inhibitor. Early structural studies suggested an important contribution of the His79-Ala80-Val81 (HAV) and the Ile53-Asn54-Pro55 (INP) sequences to the cadherin homophilic binding mechanism.[13] As a result, libraries of cyclic peptides based on HAV or INP sequences and of non-peptide HAV mimics were developed and tested for their ability to inhibit the cadherin dimerization process.[28-30] Based on these studies, the antagonist peptide N-Ac-CHAVC-NH2 (ADH-1 or Exherin™) is now in phase I clinical trials in patients with N-cadherin-expressing solid tumors.[31-33] However, its binding interface and mechanism of inhibition are still totally unknown.

Protein-protein interfaces, especially homo-oligomers, are usually flat and featureless. Therefore, they are generally either completely undruggable or difficult to target with efficient antagonists.[34,35] This is especially true for highly dynamic systems that undergo major conformational changes as part of their substrate recognition mechanism. The cadherin homo-dimerization and activation mechanism falls within this realm and it is not surprising that, to date, almost no modulators or inhibitors of cadherin-mediated cell-cell adhesion have been developed. Moreover, no actual structural evidence regarding the mechanism through which successful modulation of cadherin homo-association may actually occur has yet been provided, thus making the development of effective antagonists particularly difficult.

RESULTS AND DISCUSSION

In one of the first attempts to target the cadherin strand dimer interface identified earlier by X-ray diffraction analysis, we have previously designed and synthesized a library of small peptidomimetic molecules featuring a conformationally constrained tetra-peptide scaffold from the native N-terminal DWVI sequence where a

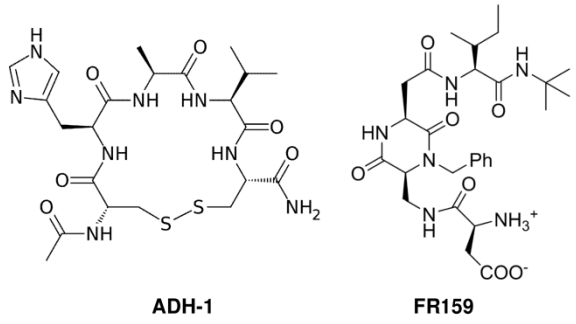

Figure 2 Structure of ADH-1 and FR159. Given their structural differences, it is unlikely that they share identical binding sites.

phenyl ring was introduced to mimic the indole moiety of the crucial Trp2 residue.[36] By a combination of biochemical and functional assays, we have also shown that, even at μM concentrations, some of these peptidomimetic compounds have a significantly higher ability to inhibit N- and, to a slightly lower extent, E-cadherin homophilic adhesion compared to ADH-1.[36] For instance, FR159 (**Fig. 2**) is more effective than ADH-1 in inhibiting both N- and E-cadherin homophilic binding. In ELISA tests using N-cadherin-expressing epithelial ovarian cancer (EOC) cells SKOV3 and N-cadherin-Fc chimeric protein, FR159 inhibited N-cadherin homophilic binding by 84% and by

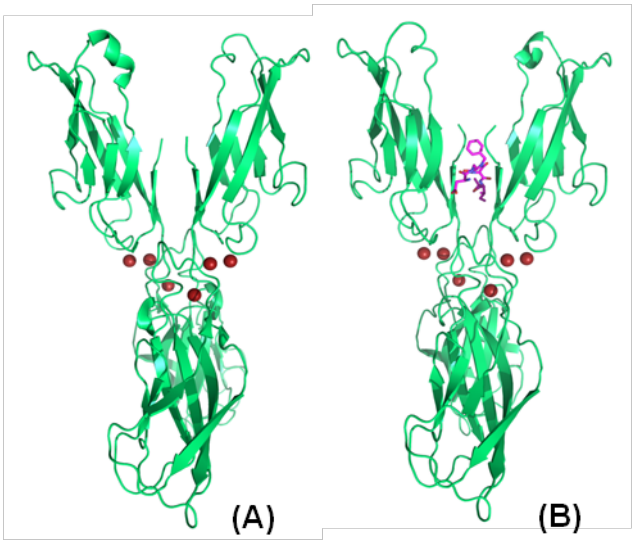

Figure 3 Crystal structure of the human E-cadherin-(Val3)-EC1EC2 fragment (residues 3-213) in its un-ligated form (PDB id: 4ZT1) **(A)** and in complex with FR159 (PDB id: 4ZTE) **(B)**.

65% at 2mM and at 1mM concentration, respectively, while ADH-1 showed 50% inhibition at 2mM concentration and virtually no inhibition at 1mM. Cell-free Surface Plasmon Resonance (SPR) experiments using the N-cadherin-Fc recombinant protein showed that at 10μM concentration FR159 provides 55% inhibition of N-cadherin homophilic binding, compared to 28% for ADH-1. In ELISA tests conducted using the E-cadherin-expressing EOC cell line OAW42 FR159 provided about 50% inhibition at 2mM concentration, while ADH-1 showed only 30% inhibition. Here, we show the crystal structure of the complex between the E-cadherin-EC1EC2 fragment and FR159, one of the best inhibitory peptides that have been reported to date. The structure allows the identification of a novel druggable interface and provides, for the first time, a structural framework to guide the design of modulators of cadherin-mediated cell adhesion.

Previous attempts to co-crystallize the *wt*-human E-cadherin-EC1EC2 fragment (residues 1-213) with several small molecule ligands did not yield results, mostly owing to the highly dynamic behavior of the protein. We then removed the first two amino acids (Asp-Trp) at the N-terminus of the EC1EC2 construct and produced the E-cadherin-(Val3)-EC1EC2 fragment (residues 3-213), where the Trp2-binding pocket is intrinsically un-occupied and, in principle, more easily accessible to the ligand. Crystallization of this shorter protein fragment with the same panel of peptidomimetic compounds yielded co-crystals only with FR159, likely owing to the intrinsic conformation of the ligand.

Fig.3 shows a side by side comparison of the E-cadherin-(Val3)-EC1EC2 structure in the absence (**A**) and in the presence (**B**) of FR159. As expected, due to the removal of the key Trp2 residue that is used by the protein in the final stage of its complex dimerization mechanism, the protein is unable to promote strand swap dimer formation and it is found in the X-dimer configuration, an arrangement that has been previously shown to represent a crucial intermediate along the full cadherin dimerization trajectory. In essence, by removing the Asp1-Trp2 N-terminal fragment, we reduced the degree of conformational freedom of the cadherin association complex by preventing the X-dimer to progress towards strand swap dimer formation. A comparison of the two structures shows that the protein dimers superimpose with an r.m.s.d. of 0.4 Å for main chain atoms.

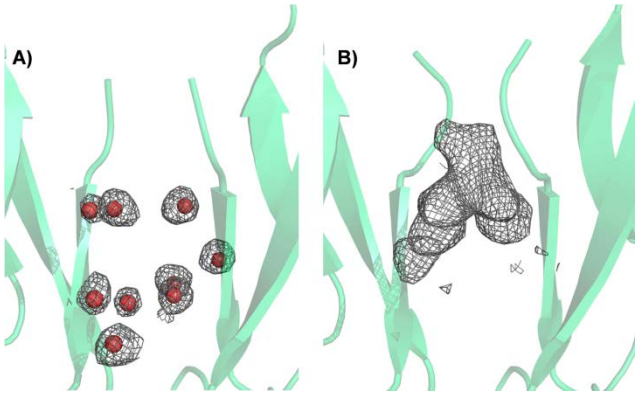

Figure 4 Side by side comparison of the electron density map in the binding region between the un-ligated (**A**) and the ligated (**B**) structure (PDB id: 4ZT1 and 4ZTE, respectively).

The preliminary computational studies that we carried out using both E- and N-cadherin suggested that the ligand may bind the protein by inserting the scaffold benzyl ring into the hydrophobic pocket of Trp2 and receive further stabilization by a number of additional contacts with the protein (see the Supplementary Information for the best computational pose of FR159 into the E- and the N-cadherin binding site).[36] However, in the crystal the ligand does not bind in the expected Trp2 pocket and the X-ray structure described here combined with previous cell adhesion inhibition data reveals a new target site for the design of cadherin inhibitors. In fact, the ligand binds across the two interacting cadherin molecules at the level of the di-proline motif of their adhesion arm. In the unligated structure, several water molecules occupy the space that is taken by the peptidomimetic molecule in the ligated structure (**Fig. 4**). The phenyl ring of the peptidomimetic ligand fits into an hydrophobic cavity formed by the side chains of residues Ile4, Pro5, Ile7 and Val22 from both cadherin molecules. The Val3 residues from the two interacting molecules are not visible in the map but it is conceivable that they may transiently cap the hydrophobic cavity, thus providing further stabilization energy to the complex. The hydrophobic cavity is symmetrical as it is formed by exactly the same group of residues from the two interacting proteins. The electron density map of the ligand in the crystal is shown in **Fig. 5, A** while a schematic representation of the interactions between the phenyl ring of the ligand and the surrounding molecules is shown in **Fig. 5, B**. Interestingly, all the residues involved in ligand stabilization are conserved across most type-I classical cadherins, suggesting a common general inhibitory mechanism associated with this peptidomimetic compound for this cadherin subset.

Further hydrophobic contacts are formed by the tert-butyl moiety that was introduced as a protective group at the C-terminus of the ligand and residues such as Thr97 and Pro10 of the protein. However, this moiety of the ligand is rather disordered and therefore other contacts may be dynamically formed with neighboring residues. The isoleucine side chain of the peptide forms hydrophobic contacts with the side chain of Leu21 and Ile7 in the protein, providing further stabilization energy to the complex. At the other moiety of the ligand, the Asp1 side chain of the peptidomimetic forms water-mediated contacts with the carboxy group of Glu13 of the protein. Finally, in the central part of the peptidomimetic ligand, the carbonyl group of the heterocycle forms an hydrogen bonding interaction with the amide of Ser8 of the protein (2.81 Å).

the ligand through its phenyl ring moiety as part of a competitive mechanism with the Trp2 residue of the intact adhesion arm of the protein itself,[36] the crystal structures shown here provides clear evidence of an inhibitory mechanism whereby the ligand binds across the weakly adhesive X-dimer conformation at the level of the adhesion arms of the two interacting cadherins. This suggests that in intact proteins, which unlike our N-terminally truncated fragment can form strand swap dimers, the binding of FR159 across the intermediate X-dimer provides a physical barrier against the progression of the system towards strand dimer formation and blocks the system at the weakly adhesive X-dimer conformation, as suggested by the binding inhibition data previously reported. In line with these data, we have previously shown[36] that FR159 can partially inhibit adhesion of E-

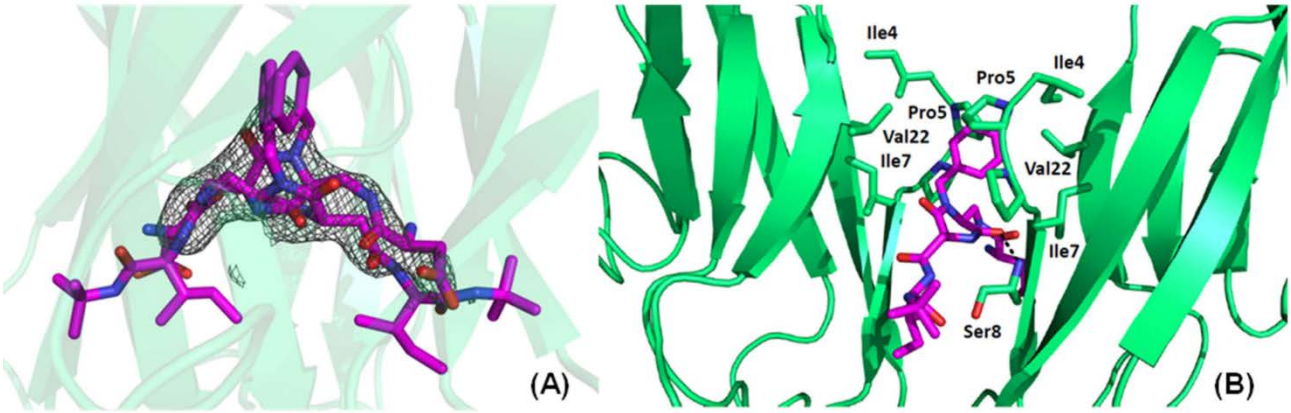

Figure 5 Electron density map of the ligand in the crystal of the complex between human E-cadherin-(Val3)-EC1EC2 and FR159 (PDB id: 4ZTE). The 2Fo-Fc map is shown at the 0.7 σ-level. A double conformation was found for the ligand, the two orientations forming exactly the same set of contacts with each of the two interacting protein fragments owing to the C2 internal symmetry of the complex **(A)**. The stabilizing contacts between the ligand and the protein. For simplicity, only one of the two alternative orientations of the ligand are shown in the picture. Regardless of the orientation, the contacts formed by the ligand with the two interacting proteins are the same. Most contacts are hydrophobic in nature and are within the typical range distance for hydrophobic interactions (4.2-4.5 Å) **(B)**.

Each cadherin X-dimer has one inhibitor molecule bound between them. However, in the crystal the peptidomimetic ligand is found in two alternative conformations (each refined with 50% occupancy in the crystal structure), one rotated relative to the other around the approximate C2 axis of the complex (**Fig. 5, A**). Interestingly, in the two alternative conformations the two terminal moieties of the ligand are found in the same environment and are stabilized by the same set of contacts with the protein. This is due to the C2 symmetry of the X-dimer configuration and to the constrained "cis" conformation of the ligand.

CONCLUSION

Here, we provide the first crystal structure of a cadherin fragment in complex with a small molecule inhibitor. This ligand has been previously shown to modulate and partially inhibit cadherin-mediated cell adhesion better than ADH-1, a small cyclic peptide that is currently in phase I clinical trials in cancer patients. Whereas we predict that at some stage in the course of the dimerization process the cadherin binding pocket is also transiently targeted by cadherin-expressing EOC cells, where E-cadherin plays a pro-proliferative role by mediating PI3K/AKT signaling activation.[5] Interestingly, in the same E-cadherin-expressing EOC cells, FR159 affects the activation of the PI3K/AKT signaling pathway (see Supporting Information), indicating that indeed FR159 may help prevent or modulate the growth of EOC cells.

Clearly, the novel structural details shown here allowed for the identification of a druggable pocket formed by two cadherin proteins in the weakly adhesive X-dimer conformation and provide unprecedented opportunities for a structure-based approach to the design and development of inhibitor compounds with enhanced efficacy against cadherin-expressing solid tumors. This could be especially relevant for relapsed EOC patients, in which tumor growth in the peritoneum may be inhibited by targeting up-regulated E-cadherin. Moreover, effective and selective cell-cell junctions modulator peptides may represent potential pharmaceutical excipients to improve drug delivery across biological barriers.[37,38]

EXPERIMENTAL SECTION

Cloning, expression and purification of human E-cadherin-EC1EC2

A DNA fragment encoding for the EC1EC2 portion of human E-cadherin and lacking the first two N-terminal residues (Asp1-Trp2) was cloned into a pET-3a expression vector (Novagen) using the NdeI and BlpI restriction sites. This fragment, corresponding to the amino-acid sequence 3-213 of the protein, was fused at its N terminus to a 6His-tag, a spacer peptide (Ser-Ser-Gly-His-Ile) and the enterokinase recognition site (Asp-Asp-Asp-Asp-Lys). Overnight protein expression at room temperature in the BL21(DE3)pLysS *E.coli* strain (Invitrogen) afforded soluble protein in very high yield. Cells were lysed by sonication in TBS, pH 7.4 + 1 mM $CaCl_2$. The cell lysate was first purified on a Ni-NTA column and then through a Sephacryl 100 HR HiPrep 26/60 size exclusion column (GE Healthcare). Then, the protein was dialyzed in TBS buffer + 20 mM $CaCl_2$, digested with enterokinase (New England Biolabs) at 25° C, and passed over a Ni-NTA column to remove all traces of the cleaved 6His-tag, as well as any residual un-cleaved protein. The flow-through was then collected and further purified by size exclusion chromatography with TBS + 1 mM $CaCl_2$ and finally brought to a concentration of 12 mg/ml for crystallization experiments.

Peptidomimetic design and synthesis

The synthesis and characterization of FR159 has been described previously.[36] Copies of the ^1H and ^{13}C NMR spectra and the HPLC-MS chromatogram are reported in the Supporting Information. HPLC-MS data were collected with an Agilent 1100 HPLC connected to a Bruker Esquire 3000+ ion trap mass spectrometer through an ES interface. FR159 was found to be ≥95% pure by LC-MS.

Co-crystallization and data collection

Crystals of the protein alone and the protein-ligand complex were obtained by the vapor diffusion method at room temperature from a 1.3 M ammonium sulfate, 80 mM$CaCl_2$, 0.1 M Tris pH 8.5 crystallization solution. Crystals were then frozen in 25% (v/v) glycerol for X-ray diffraction data collection. A 1.9 Å resolution data set and a 2.13 Å data set were collected from a E-cadherin-(Val3)-EC1EC2 crystal of 0.20 x 0.15 x 0.15 mm size and from a 0.15 x 0.15 x 0.10 mm crystal of E-cadherin-(Val3)-EC1EC2 in complex with FR159, respectively. In both cases, a λ = 1.000 Å radiation in the X06DA-PXIII beamline at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland) was used. Diffraction images were processed and scaled using XDS.[39] Data collection and refinement statistics are shown in Table 1.

Structure determination

The structure was determined by molecular replacement using Molrep[40] from the CCP4 package[41] and the human E-cadherin-EC1EC2 crystal structure (PDB id: 2O72) as the search probe. Model refinement was carried out using Refmac5 and Phenix.[42,43] Water molecules were added both automatically using the program Phenix refine[43] and manually from visual inspection of the electron density map. Figures 3, 4 and 5 in the paper were generated using PyMOL ("www.pymol.org,"). The refinement converged to a final R/R_{free} = 19.4/22.1 % for the un-ligated structure and R/R_{free} = 18.5/23.0 % for the complex .

CRYSTALLOGRAPHIC TABLE

	E-cadherin-EC1EC2	E-cadherin-EC1EC2 / FR159
Space group	C 1 2 1	C 1 2 1
Cell dimensions		
***a* (Å)**	120.45	120.58
***b* (Å)**	76.42	76.83
***c* (Å)**	73.02	72.77
Wavelength (Å)	1.000	1.000
Resolution (Å)	42.66-1.92	42.26-2.13
R_{sym} **or** R_{merge} **(%)**	4.5 (17.6)	12.9 (12.3)
$I/\sigma I$	8.4 (2.9)	7.3 (0.6)
Completeness (%)	98.1 (96.2)	98.7 (93.5)
Multiplicity	2.0	6.0
No. of reflections	45679	33775
R_{work}/R_{free} **(%)**	19.4 / 22.1	18.5 / 23.0
No. of atoms		
Protein	3180	3196
Ligand (Ca^{2+}/FR159)	6	6 / 82
Water	382	229
Average B-factors (Å²)		
Protein	46.20	46.86
Ligand: Ca^{2+}/FR159	30.87	32.18 / 73.68
Water	47.68	47.54
r.m.s.d.		
Bond lengths (Å)	0.003	0.003
Bond angles (°)	0.513	0.487
Ramachandran		
Most favored (%)	97	97
Allowed (%)	3	3
Disallowed (%)	0	0

Table 1. Crystallographic data of the free and the ligand-bound crystal structures of E-cadherin-EC1EC2 (PDB id: 4ZT1 and 4ZTE, respectively).

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the ACS Publications website: Best Calculated poses for FR159 in complex with E- and N-cadherin, characterization of compound FR159, inhibition of phosphorylated AKT by FR159.

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Author Contributions

± These authors contributed equally to this work.

Notes

The authors declare no competing financial interests.

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ABBREVIATION USED

Surface Plasmon Resonance (SPR); epithelial-to-mesenchymal transition (EMT); Epithelial ovarian cancer (EOC); extracellular cadherin domain (EC).

ACCESSION CODES

Final crystallographic coordinates are available in the RCSB PDB (4ZT1 and 4ZTE).

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