Small-Molecule Inhibitors

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Cortistatin A is a High-Affinity Ligand of Protein Kinases ROCK, CDK8, and CDK11**

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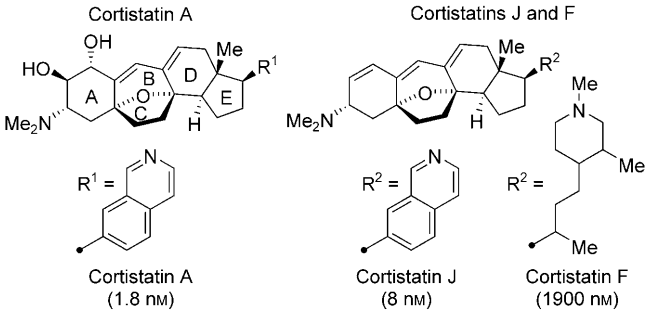
Dedicated to Professor Gary E. Keck on the occasion of his 60th birthday

The cortistatins are a family of eleven steroidal alkaloids isolated from the marine sponge *Corticium simplex* (Scheme 1).[1] The demonstration that some cortistatins show potent and selective antiproliferative properties against human umbilical vein endothelial cells (HUVECs) spurred a surge of research activities in chemistry and biology that resulted in chemical synthesis or approaches toward cortistatin A[2] (**1**) and the construction of several biologically active analogues[3] of this intriguing structure. The intense interest in the cortistatins stems from their potential to serve as lead compounds for drug discovery in antiangiogenic cancer chemotherapy, as demonstrated by the impressive antiproliferative activity of cortistatin A against HUVECs (IC_{50} = 1.8 nM) versus normal human dermal fibroblast (NHDF, IC_{50} = 6.0 μM, selectivity index = 3300), and its ability to inhibit vascular endothelial growth factor (VEGF)-induced migration and tubular formation of HUVECs. However, the biological target of cortistatin A and its siblings has not yet been identified. Here, we report that cortistatin A is a high-affinity ligand for a small set of protein kinases including Rho-associated, coiled-coil containing protein kinase (ROCK), cyclin-dependent kinase 8 (CDK8), and cyclin-dependent kinase 11 (CDK11). Models of cortistatin A bound to a crystallographic structure of ROCK and a homology model of CDK8 are presented.



Scheme 1. Cortistatins A, J, and F. Numbers in parenthesis denote IC_{50} values for HUVEC proliferation as reported in Ref. [1b].

Of the eleven cortistatin family members, cortistatin A was shown to be the most potent inhibitor in an assay measuring proliferation of cultured HUVECs with an IC_{50} of 1.8 nM (Scheme 1).[1b] The importance of the isoquinoline moiety for potent antiproliferative activity is evident by comparison of the related isoquinoline cortistatin J (IC_{50} = 8 nM) with the piperidine-containing cortistatin F (IC_{50} = 1900 nM). This structure–activity relationship (SAR), combined with numerous reports of isoquinoline-based ATP-competitive kinase inhibitors,[4] led us to hypothesize that the biological activity of cortistatin A is due to inhibition of one or more protein kinases.

Cortistatin A was tested at 10 μM in a high-throughput binding assay (KINOMEscan, Ambit Biosciences, San Diego, California) against a panel of 402 kinases.[5] The resulting TREEspot interaction map (Figure 1) shows that cortistatin A has affinity [POC (percent of control) < 35] for 16 kinases of the AGC, CAMK, CMGC, and TK families. The highest-affinity binding at this single concentration was to ROCK II (POC = 0), CDK11 (POC = 0.1), and CDK8 (POC = 0.95). The calculated selectivity score[6] $S(35)$ of cortistatin A is 0.045, indicating a high level of selectivity (only 4.5% of 359 non-mutant kinases inhibited at 35 POC).

Due to limited quantities of synthetic cortistatin A, full K_d determinations were possible only for five kinases (Table 1). These data confirm high affinity for CDK11 (K_d = 10 nM), CDK8 (K_d = 17 nM), and ROCK I and II (K_d = 250 nM and 220 nM, respectively). Due to its high homology with ROCK I and II, binding to PKACα was also assessed, with the measured K_d found to be 14–16 fold higher than that of ROCK I and II/cortistatin A, respectively.

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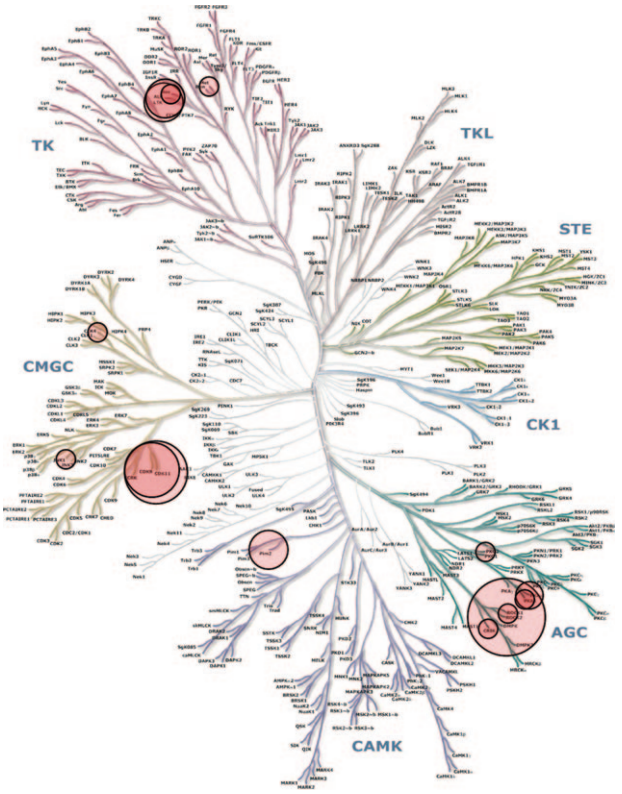


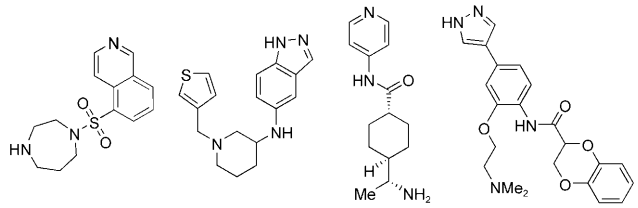
Figure 1. TREEspot interaction map for synthetic cortistatin A tested at 10 μM, with kinases found to bind (POC < 35) marked with red circles, with larger circles indicating higher-affinity binding (Ambit Biosciences, San Diego, California). POC = percent of control. [In this assay, the ability of a test compound to compete with an immobilized, active-site-directed ligand is quantitatively measured and reported as percent of DMSO control (POC), with lower numbers indicating higher binding affinity.] For a larger sized version of this figure see the Supporting Information.

Table 1: Kinase affinity of synthetic cortistatin A.

Kinase	POC (10 μM)[a]	K_d [nM][b]
ROCK II	0	220 ± 7
CDK11	0.1	10 ± 2
CDK8	0.95	17 ± 2
LTK	2.9	ND
ALK	4.4	ND
PIM2	4.4	ND
PKACα	8.7	3500 ± 212
PKACβ	13	ND
MET	18	ND
PRKG2	21	ND
RIOK2	21	ND
ROCK I	21	250 ± 35
CLK4	26	ND
ROS1	26	ND
CIT	28	ND
JNK1	29	ND

[a] Kinases with POC < 35 are shown. [b] Average of two determinations ± SD; ND = not determined.

ROCK I and II are the best characterized of the high-affinity kinase targets of cortistatin A. Discovered in the mid-1990's, ROCK kinases are key effectors of Rho (Ras homolog gene family) GTPases, which regulate cell proliferation, motility, and apoptosis.[7] A variety of potent small-molecule ROCK inhibitors originating from medicinal chemistry programs have been reported (Scheme 2), including those containing isoquinoline[8] (fasudil, **5**), indazole[9] (**6**), pyrimidine[10] (Y-27632, **7**), and pyrazole[11] (SR3677, **8**) heterocycles. Fasudil (Eril Injection S) is the first ROCK inhibitor in clinical use and has established the utility of ROCK inhibition for the treatment of cerebral vasospasm.[12] ROCK inhibitors may also be useful in the treatment of other pathological conditions, including cancer, neuronal degeneration, kidney failure, asthma, glaucoma, osteoporosis, erectile dysfunction, and insulin resistance.[7b]



Scheme 2. Selected ROCK inhibitors from medicinal chemistry programs. K_d reported as an average of two determinations ± SD (Ambit Biosciences, San Diego, California).

RhoA is known to be a key mediator of vascular endothelial growth factor (VEGF) signaling in endothelial cells,[13] and experiments with fasudil (**5**) and Y-27632 (**7**) have established that these molecules inhibit VEGF-stimulated endothelial cell behaviors necessary for angiogenesis, including proliferation, migration, and tube formation, at concentrations in the range of 1–10 μM.[14,15] The dose-dependent inhibition of myosin light chain (MLC) phosphorylation in studies of fasudil over the same concentration range supports the assumption that the observed endothelial cell effects are due to inhibition of ROCK.[14c] The similarity of these observations to that reported for cortistatin A is striking, but we caution that cortistatin A binds to ROCK I and II approximately 7-fold weaker than fasudil, yet impacts endothelial cell behaviors at concentrations two to three orders of magnitude lower than fasudil. Further experiments in HUVECs will be necessary to characterize the dose–response relationship of cortistatin A for ROCK inhibition in a cellular setting.

Cortistatin A also binds with high affinity to CDK8 and CDK11 (K_d = 17 and 10 nM, respectively), with high selectivity over cell cycle regulatory cyclin-dependent kinases (CDK2 and CDK3, POC = 91 % at 10 μM cortistatin A). This selectivity appears to be quite unique relative to other natural product or medicinal chemistry derived CDK inhibitors,[16] although, since CDK8 and CDK11 are the only members of the CDK family that share an extended C-terminal domain beyond the catalytic domain in common with the ROCK kinases, this unprecedented divergence in activity within the

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CDK family is not surprising. In what little is functionally known about CDK8 and CDK11, they have been identified as subunits of mammalian mediator complexes, which are essential regulators of RNA polymerase II transcriptional machinery.[17] CDK8 has also been recently identified as a colorectal cancer oncogene and regulator of β-catenin activity.[18] As the understanding of CDK8/11 biology is at a very early stage, it is not possible to ascertain whether the reported endothelial cell specific effects of cortistatin A are consistent with inhibition of CDK8/11. We do note that the binding K_d for cortistatin A and CDK8/11 (10–17 nM) is in a similar range to the reported IC_{50} values for inhibiting HUVEC proliferation, migration, and tube formation (ca. 2 nM).[1a] Further experiments in HUVECs will be necessary to characterize the dose–response relationship for cortistatin A and CDK8/11 in a cellular setting.

We investigated the binding of cortistatin A to ROCK I, CDK8, and CDK11 through molecular modelling. Figure 2 A–C illustrates the overall topology of ROCK I, CDK8, and CDK2, with residues C-terminal to the catalytic domain colored in red. Similarity in sequence length can be seen between ROCK I and CDK8, as well as the dissimilarity between CDK8 and CDK2. Figure 2 structures A and C are crystallographic structures of ROCK I[19] and CDK2,[20] respectively. Figure 2 B is a homology model of CDK8 that was built based on alignment to ROCK I; because an extended C-terminal domain is absent in other members of the CDK family, illustrated in the co-crystal structure of CDK2 in complex with a non-hydrolyzable ATP analogue (Figure 2 C), ROCK I emerged as a more relevant template than another CDK.

The co-crystal of fasudil in complex with ROCK I,[19] shown in Figure 2 structure A and model D, demonstrates that the extended C-terminal domain constitutes an essential component of the ligand-binding site, encapsulating this region of the kinase, particularly the aromatic Phe 368 sidechain. Similarly, in the CDK8 homology model, Tyr 334 functions as a hydrophobic residue that encloses the ligand-binding pocket. A number of other aromatic sidechains are present on the C-terminal domain of CDK8 and its specific

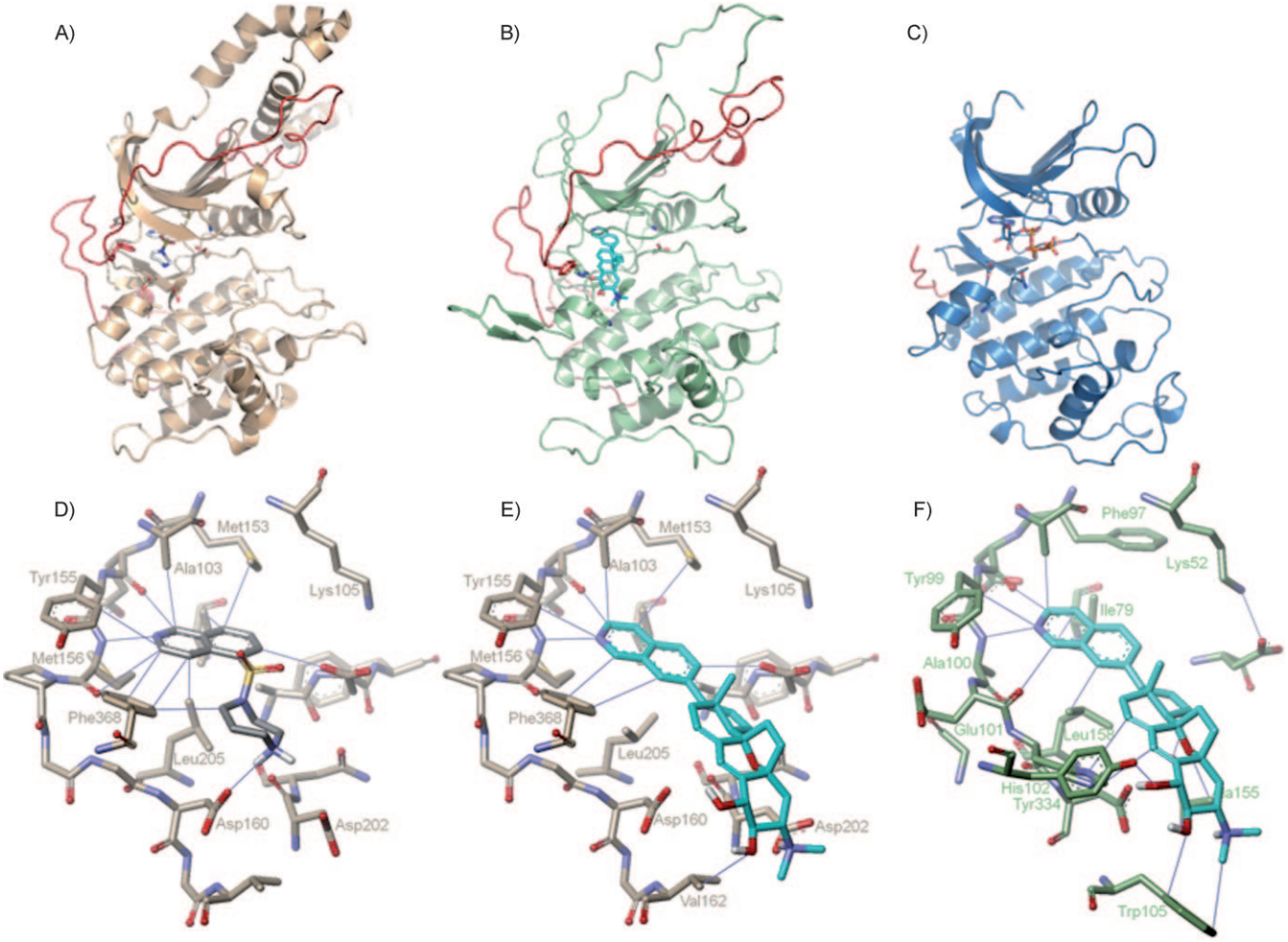

Figure 2. Structures and modeling of ROCK I, CDK8, and CDK2: A) ROCK I in complex with fasudil (PDB entry 2ESM[19]); B) cortistatin A docked into a CDK8 homology model that was constructed using ROCK I as the template; C) CDK2 in complex with ANP-PNP (PDB entry 1FIN[20]), showing lack of an extended C-terminal domain; D) binding site of the ROCK I/Fasudil co-crystal structure; E) binding site of the cortistatin A docked model in ROCK I; F) binding site of the cortistatin A docked model in the CDK8 homology model. Thin solid blue lines in panels D–F indicate both van der Waals (dispersion) contacts and hydrogen bonds between ligand and protein.

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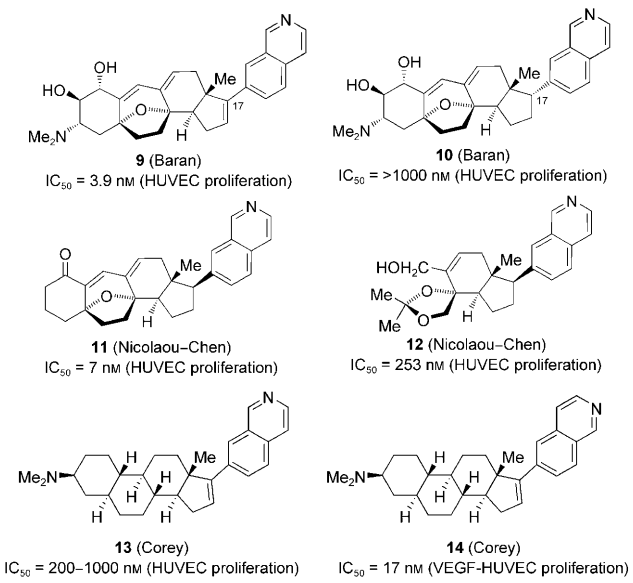
packing against the catalytic domain in a homology model depends on the alignment of the C-terminal domain, which is not unambiguous. However, while there may be uncertainty in the precise packing of the C-terminal domain, the kinase selectivity profile of cortistatin A suggests that the presence of this uncharacteristically long domain is responsible for effecting more potent inhibition; among the panel of 402 kinases in the KINOMEscan, cortistatin A appears to have a much weaker inhibitory effect on those members of the CDK family lacking an extended C-terminal domain.

The docking of cortistatin A into the protein structure from the fasudil/ROCK I co-crystal required minor rotation of the sidechain rotamers for Leu 204 and Asp 202, but was otherwise rather clear, largely because of the highly rigid nature of this small molecule and the compulsory satisfaction of the Met 156 NH with a hydrogen-bond acceptor. The resulting model (Figure 2 E) suggests that the isoquinoline and D/E steroid rings of cortistatin A occupy similar space as the isoquinoline and homopiperazine of fasudil, respectively, with the A, B, and C rings of cortistatin A extended to a region of space not occupied by fasudil. The isoquinoline rings of these two small molecules, while not in the same orientation, are both positioned to place the nitrogen in the same location to serve as the obligatory acceptor for the Met 156 NH. The binding of cortistatin A to ROCK is proposed to be further facilitated by van der Waals contacts with Phe 368 of the C-terminal domain, Ile 82 and Val 90 of the P-loop (not displayed in Figure 2), Ala 103 above, Leu 205 below, and the Met 153 gatekeeper residue behind, as well as a CH···O hydrogen bond with Asp 216 and an N···CH hydrogen bond with Tyr 155. The angular methyl (C18) of cortistatin A is proposed to reside in similar space as the sulfonamide SO_2 of fasudil, wedged up in a cleft between the β1 and β2 strands of the P-loop. The C3 dimethylamino substituent is proposed to form a salt bridge with Asp 202, and the C1 and C2 vicinal diols of cortistatin A are proposed to face solvent. The corresponding homology model of CDK8/cortistatin A (Figure 2 F) suggests a similar binding mode with some differences in residues proposed to contact the ligand, including Phe 368 to Tyr 334, Met 153 to Phe 97, and Asp 202 to Ala 155. While the former substitutions appear to provide van der Waals surfaces that are more complementary to the structure of cortistatin A, the lack of an acidic residue in position 202 in CDK8 is somewhat surprising, given that this residue is proposed to form a salt bridge to the C3 dimethylamino substituent of cortistatin A. However, it must be noted that the A ring of cortistatin A is proposed to occupy a region of the kinase that has considerable solvent exposure, particularly in the case of CDK8, where the carboxylate sidechain of Asp 202 in ROCK 1 is instead replaced by a single methyl group in Ala 155, with solvation of the dimethylamino substituent apparently no less favorable.

Several research groups have synthesized cortistatin A analogues (Scheme 3) and tested these for activity against endothelial cell behaviors.[3] With the caveat that HUVEC proliferation is a distal readout, we do find that the emerging SAR is consistent with the ROCK/CDK8 modeling presented in Figure 2. In general, the most potent analogues contain isoquinoline rings; as described above, the interaction of



Scheme 3. Selected cortistatin analogues capable of inhibiting proangiogenic endothelial cell behaviors.

isoquinoline nitrogen and kinase hinge NH is a key feature of the modeled cortistatin A/kinase binding. Of the analogues containing the abeo-9(10–19)-androstane skeleton of the cortistatins, Baran and co-workers have shown that Δ^{16} unsaturation (**9**) is well tolerated, but epimerization at C_{17} (**10**) abolishes activity.[3b] The cortistatin A/kinase models support this SAR, with Δ^{16} unsaturation serving as a reasonable mimic of the pseudoequatorial disposition of the isoquinoline in cortistatin A; in contrast, the pseudoaxial position of the isoquinoline, as a result of inversion at C17, substantially alters the relative orientations of the isoquinoline and steroid rings, making it very unlikely to bind to ROCK/CDK8 because of steric clashes with the kinase. Work at the opposite end of the molecule by Nicolaou, Chen, and co-workers has established that the A ring can be considerably simplified with only 3-fold (**11**) loss in potency.[3d] Quite remarkably, compound **12**, lacking both A and B rings, is still reasonably potent ($IC_{50} = 253$ nM, 127-fold loss in potency). Our model suggests that loss of solvent-exposed hydroxy groups is not likely to be detrimental to activity, but removal of the C3 basic amine could lead to an attenuation of ROCK binding, because of the proposed interaction of the amine with Asp 202 in ROCK (Ala 155 in CDK8). Kiyota and co-workers[3a] were the first to report analogues based on a simplified estrane skeleton, and Corey and co-workers have produced the most potent analogues in this structural class to date (**13**, **14**).[3c] These analogues show an interesting SAR with respect to the isoquinoline connectivity, with the 6-substituted isoquinoline **14** showing better potency than the cortistatin A-like 7-substituted isoquinoline **13**. We find that due to the estrane ring system, compound **13** models slightly differently in ROCK I, with the dimethylamino group instead forming a salt-bridge with Asp 369 of the extended C-terminal domain, resulting in partial desolvation of Asp 202. In contrast, compound **14** is capable of forming the same salt bridge with Asp 202 in ROCK I as cortistatin A, leaving

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Asp369 largely solvent exposed (models not shown). The putative desolvation of Asp202 by compound **13** may be the cause of its attenuated inhibitory activity, compared to compound **14**. While we believe that much of the emerging SAR from endothelial cell experiments can be explained by the kinase models proposed in Figure 2, additional experiments to assess the kinase affinity of active and inactive analogues will be necessary to further elucidate the role of kinase inhibition in the activity of cortistatin A analogues.

In conclusion, we report that cortistatin A is a ligand of a small group of kinases, with high-affinity binding to CDK8, CDK11, ROCK I, and ROCK II. Models of cortistatin A bound to ROCK I and CDK8 suggest that the isoquinoline binds to the kinase hinge and the steroid region of the molecule is complementary to the shape of the ATP-binding cleft, with the terminal polar A ring exposed to solvent, and a salt bridge between an aspartate sidechain (ROCK I only) and dimethylamino group of cortistatin A. ROCK I, ROCK II, CDK8, and CDK11 all contain an extended C-terminal domain which may place an aromatic sidechain in close proximity to cortistatin A and encapsulate the mouth of the ATP binding site. This may contribute to the high affinity of cortistatin A for these kinases relative to the rest of the testable kinome. The emerging SAR for cortistatin analogues can be understood through the kinase models presented here. However, it must be noted that the IC_{50} of cortistatin A for antiproliferative activity against HUVECs (1.8 nM) is almost one order of magnitude lower than the observed K_d for CDK8 (17 nM) and CDK11 (10 nM), and almost two orders of magnitude lower than its K_d for ROCK (I = 250 nM, II = 220 nM), and it is possible that other cellular targets may contribute to the observed antiproliferative activity, and/or cortistatin is metabolically converted within the cell to a more active ligand. Further work will be necessary to characterize the dose–response relationship of cortistatin A on ROCK/CDK8/CDK11 activity in HUVECs and establish the impact of ROCK/CDK8/CDK11 inhibition on HUVEC proliferation.

Experimental Section

Fasudil (**5**) and Y-27632 (**7**) were obtained from Sigma–Aldrich. The details of the high-throughput binding assay (KINOMEscan, Ambit Biosciences, San Diego, California) can be found in ref. [5]. A previously described hierarchical molecular modeling method[21] was used to dock cortistatin A and related analogues into both the crystallographic and homology model structures.

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