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Separation of anti-angiogenic and cytotoxic activities of borrelidin by modification at the C17 side chain

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Abstract—A set of novel borrelidin analogues have been prepared by precursor-directed biosynthesis. Structure–activity relationship analysis suggests that steric structural arrangement within the C17 side chain is important for differentiating cytotoxic and anti-angiogenic activities. A C17-cyclobutyl analogue **3** was found to have markedly increased selectivity for in vitro angiogenesis inhibition over cytotoxicity and is therefore potentially useful as an anticancer agent.

Angiogenesis is crucial to tumour growth and metastasis. The development of solid tumors beyond 1–2 mm^3 in diameter has been established to require angiogenesis.[1,2] It is a multifaceted process and involves the coordination of proliferation, migration, adhesion and tubule formation of the endothelial cells. The therapeutic utilities of angiogenesis inhibitors in cancer treatment have been demonstrated by the clinical efficacies of bevacizumab and thalidomide. Anti-angiogenic activity is also an important component of some widely used chemotherapeutics such as paclitaxel, and the most recently marketed target-based therapeutics such as sunitinib and sorafenib. Therefore, angiogenesis inhibition is a validated approach to the development of anticancer therapeutics.

Borrelidin (**1**, Fig. 1) is a polyketide natural product and was identified as an inhibitor of angiogenesis using an in vitro rat thoracic aorta tubule formation (RATF) model.[3] In this model **1** inhibits tubule formation and disrupts newly formed capillary tubules, both in a dose-dependent manner. The latter activity has been shown to involve induction of apoptosis in the newly

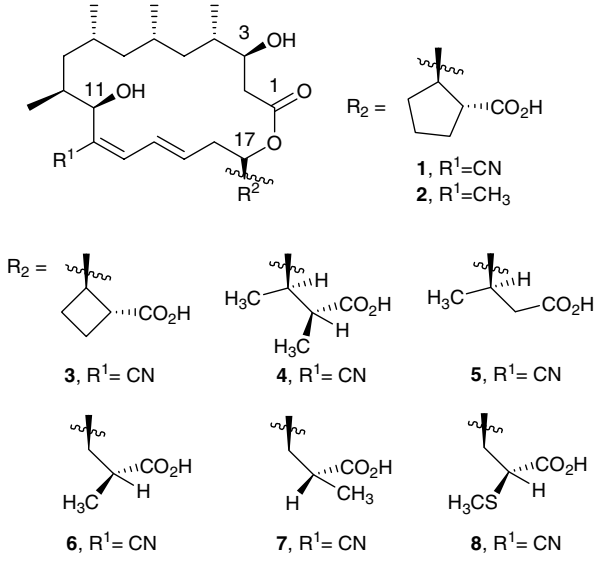

Figure 1. Structures of borrelidin **1** and analogues **2**–**8** generated by biosynthetic engineering.

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formed tubules. It inhibits angiogenesis in vivo, and is effective in the inhibition of spontaneous lung metastases of B16-BL6 melanoma cells at the same dosage that inhibits angiogenesis.[4] Borrelidin is also highly cytotoxic with a narrow therapeutic window and exhibits a wide spectrum of other biological activities.[5–7]

We have reported the cloning and sequencing of the gene cluster (*bor*) from *Streptomyces parvulus* Tü4055 that governs the biosynthesis of **1**, allowing us to propose a biosynthetic pathway, and have applied this knowledge to the rational biosynthetic engineering of the *bor* gene cluster in order to produce novel analogues of **1**.[8–11] We report here structure–activity relationship data within a series of novel borrelidin analogues with structural modifications at the C17 side chain and the discovery of a cyclobutyl analogue (**3**, Fig. 1) with significantly improved selectivity for in vitro angiogenesis inhibition over cytotoxicity. This may lead to an improved therapeutic index and potential as an anticancer agent.

The biosynthesis of **1** occurs in three distinct stages.[8] The first stage involves biosynthesis of (1*R*,2*R*)-cyclopentane-1,2-dicarboxylic acid which acts as the starter unit for polyketide chain assembly (the second stage). Polyketide assembly occurs on a modular polyketide synthase (PKS) and results in the release of pre-borrelidin (**2**). The final, post-PKS stage of biosynthesis involves oxidation of the methyl group at C12 of **2** and its conversion to a nitrile moiety. Disruption of the gene encoding the cytochrome P450 monooxygenase BorI involved in oxidation at C12 results in a mutant which accumulates **2** specifically.[10] To prepare analogues of **1** in which the cyclopentanecarboxylic acid moiety at C17 was replaced by cyclobutanecarboxylic acid (**3**) or by substituted propionic acid moieties (**4**–**8**), we utilized precursor-directed biosynthesis methods based on a non-producing mutant of the producing microorganism in which the supply of the PKS starter unit was disrupted, and to which analogues of this were fed.[11] Compounds **2**–**8** were thus produced as described previously.

The anti-proliferative activity of **1** and **3**–**8** was measured in vitro against 12 cancer cell lines using a modified propidium iodide assay[12] (Table 1). These 12 cell lines include generally sensitive cell lines as well as those with well-sdocumented characteristics including resistance to some anti-cancer compounds.[13,14] The data produced allowed a simple SAR analysis of this set of compounds. At the concentration studied (10 μM) **1**, **3** and **4** were equipotent as judged by mean growth inhibition of treated cells versus untreated (%T/C).

Among the acyclic analogues **4** was significantly more potent than **5**–**7**, indicating the importance of lipophilic substituents at the C18/C19 positions. Comparison between the enantiomers **6** and **7** demonstrates that the stereochemical arrangement at C19 corresponding to that of **1** is favoured, that is, the 19*R*-isomer **6** is more potent than the 19*S*-isomer **7**. The lack of activity for **8** indicates a limitation of the steric bulkiness which can be accommodated at C19. Interestingly the constraints put upon the dihedral angles of the *trans*-cyclobutyl moiety of **3** do not preclude its binding to target and subsequent activity.

Based on these data **3** and **4** were selected for further studies. In order to further evaluate their anti-proliferative activity the two compounds were examined in vitro in the NCI 60 cancer cell line assay which is run across a concentration range (5 log units) that allows calculation of IC_{50} values.[15] These data show that **1** and **4** have similar potency with mean IC_{50} values of 0.02 and 0.07 μM, respectively. Compound **3** was approximately 15-fold less potent than **1** with the mean IC_{50} of 0.30 μM against the 60 cell line panel. These data indicate that although the steric bulk of **3** and **4** are similar, the conformational freedom of the acyclic moiety of **4** in comparison to **3** allows for a *trans* disposition of the side chain alkyl and carboxylic acid moieties more similar to that

Table 1. In vitro growth inhibition of 12 cancer cell lines by compounds **1** and **3**–**8**

Cell lines	%T/C[a] for borrelidin analogues assayed at 10 μM							
	1	**3**	**4**	**5**	**6**	**7**	**8**	Adr[c]
SF268	3	4	3	13	14	57	54	9
251L	8	9	12	26	32	81	67	15
H460	4	7	3	38	49	84	79	3
MCF7	13	9	9	12	16	76	48	12
MDA231	12	8	7	20	26	90	81	11
MDA467	6	6	7	15	25	62	59	7
394NL	2	3	4	9	9	67	64	6
OVCAR3	11	16	6	32	29	91	102	10
DU145	5	16	7	55	68	74	75	5
LNCAP	7	9	7	24	34	92	59	10
A498	8	9	9	21	30	78	68	11
1138L	2	3	3	5	6	56	42	6
Mean[b]	7	8	6	23	28	76	67	9

[a] Mean percentage of viable cells in the treated group relative to the control group. Full details can be found in Ref. 11.
[b] Mean %T/C across the 12 cancer cell lines for each compound.
[c] Adriamycin control (run at 3 μM).

adopted by **1**. Compound **2** did not exhibit any anti-proliferative activity against cancer cell line nor any activity in the angiogenesis inhibition assays described below. Thus the nitrile group at C12 appears to be essential for biological activity. This may implicate covalent attachment via Michael addition at C13 of a nucleophilic residue of the (as yet unknown) molecular target. Experiments to determine the reversibility of inhibition and address this issue are planned.

We next examined the effectiveness of **1**, **3** and **4** in anti-angiogenesis assays in vitro using human umbilical endothelial cells (HUVEC). First, the growth inhibitory activity against HUVEC was measured across the concentration range 10^{-5} to 10^{-13} M using semi-log dilutions. As shown in Table 2 the IC_{50} values for **1** and **4** were about 0.5 and 1 nM, respectively. The IC_{50} value of 0.5 μM for **3** indicates a significantly reduced cytotoxicity (3 log units) against HUVEC compared to borrelidin **1** and the acyclic analogue **4**, consistent with the results of the NCI cancer cell screen described above.

From this experiment IC_{10} and IC_1 values were calculated for **3** and **4**. Borrelidin **1** showed a steep curve between active and inactive concentrations with a sudden drop of activity. At 1×10^{-10} M the anti-proliferative effect of **1** was lost. We therefore chose 1×10^{-10} and 1×10^{-11} M as the two non-toxic concentrations of **1**, together with the IC_{10} and IC_1 of **3** and **4**, for the pseudo-capillary formation assay using HUVEC monolayers on Matrigel.[16,17] This type of assay is commonly used to screen for inhibitors of angiogenesis[18] and is closely related to the RATF model.[3] Digitized pictures (computer-assisted video microscopy) were taken every 4 min over a 12-h period allowing a temporal analysis of cellular effects.[16,17] We were thus able to obtain data regarding tubule morphology, measuring both the length and number of tubules formed (see Fig. 2 and Table 3).

As seen in Figure 2, the effect of **3** upon the morphology of tubules was dramatic in comparison to the vehicle control with almost no branching or tubule formation occurring. Quantitatively, **3** caused more than 80% reduction in the number of branches formed at both the IC_{10} and IC_1. The dramatic effect of **3** at 25 pM shows that in this assay at least, **3** is an extremely potent inhibitor of angiogenesis in vitro. When compared to the vehicle control, **1** did not inhibit branch formation at either of the doses tested and **4** had no effect at the IC_{10} but showed significant reduction in the number of branches formed at the IC_1 (Table 3). None of the compounds affected the length to which any tubules grew once formed. This is unusual for the analysis of tubule formation in Matrigel monolayer assays,[16,17] but taken together these data indicate that borrelidin analogues act upon an upstream event within the process of angiogenesis.

Table 2. In vitro growth inhibition of HUVEC by **1**, **3** and **8**

Compound	IC_{50} (M)	IC_{10} (M)	IC_1 (M)
1	4.9×10^{-10}	1×10^{-10} [a]	1×10^{-11} [a]
3	4.5×10^{-7}	7.5×10^{-11}	2.5×10^{-11}
4	9.5×10^{-10}	7.5×10^{-13}	2.5×10^{-13}

[a] Selected non-toxic concentrations, not realistic IC_{10} and IC_1. Borrelidin showed a steep curve between active and inactive concentrations with a sudden drop of activity, that is, the anti-proliferative effect was lost at 1×10^{-10} M.

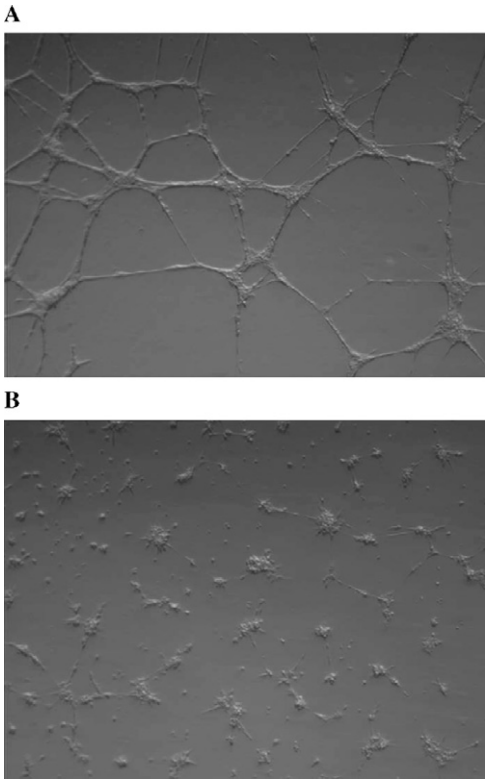

Figure 2. Representative pictures of HUVEC showing morphological differences between the control (A) and treatment with **3** (B). The number of branches and complete pseudo-capillaries were markedly reduced in the presence of a non-toxic concentration of **3** (25 pM). Similar inhibition was also observed in the presence of 75 pM of **3**.

Table 3. In vitro inhibition of HUVEC tubule formation by **1**, **3** and **8**

Compound	Number of branches at[a]		Mean branch length at[b] (μm)	
	IC_{10}	IC_1	IC_{10}	IC_1
1	230	233	64	65
3	40	38	62	65
4	246	116	63	62
Vehicle	223		63	

[a] Mean total number of completed pseudo-capillary branches per plate.
[b] Mean length of completed branches per node (partially completed branches were not counted).

The mechanism by which borrelidin exerts its angiogenesis inhibitory effect is unclear. Borrelidin is a potent inhibitor of the bacterial and mammalian threonyl-tRNA synthetases, a mechanism by which it exerts growth inhibitory effects.[19,20] This is supported by experiments with Chinese hamster ovary (CHO) cells where resistance to **1** correlates with up to a 20-fold increase in threonyl-tRNA synthetase activity.[21,22] The concentrations at which **1** effects protein and DNA synthesis in HUVEC however are similar to that for growth inhibition as measured in a specific HUVEC batch (12

and 20 nM, respectively),[3] a value which is significantly higher than that for tubule formation. Thus it appears that the growth inhibitory effect of **1** is related to the inhibition of threonyl-tRNA synthetase and may be different to that for angiogenesis inhibition. This idea that the two activities are mediated via different mechanisms is consistent with our data for **3**, which has an IC_{50} against HUVEC and cancer cells in the low μM range in contrast to **1** and **4** which have nM values, but retains potent, improved, anti-angiogenesis activity.

It has been reported that the inhibitory activity of **1** upon tubule formation in the RATF model is antagonized by threonine in a dose-dependent manner.[23] Threonine was however unable to antagonize the ability of **1** to collapse newly formed tubules in the same model. Similar data were obtained for HUVEC cell-based assays. The ability to collapse newly formed tubules was shown to involve caspases 3 and 8, consistent with the induction of apoptosis.

These data are puzzling in light of our observations for **3** reported herein. The next stage in our investigations will require an understanding of the effects that threonine has upon the activity of **3** against HUVEC proliferation and tubule formation, and, to determine whether or not **3** is an inhibitor of threonyl-tRNA synthetase.

Anti-angiogenic effects at subcytotoxic concentrations have been previously described for a subset of microtubule-targeting drugs, including the taxanes and vinca alkaloids.[24] These two activities, inhibition of cell motility and cell proliferation, seem to act through independent mechanisms,[25] although the molecular mechanism at the basis of this distinction is not yet clear. Borrelidin and its analogues would seem to have a similar, but distinct, pair of activities, which we have now shown are separable.

In conclusion, we have shown that biosynthetic engineering can be successfully applied to lead optimization. The products of targeted structural modifications at C17 of **1** by precursor-directed biosynthesis provided a productive route for the preparation of **3**, a compound which exhibits decreased toxicity and increased anti-angiogenic activity against HUVEC, demonstrating that the therapeutic index of these compounds can be significantly improved. The reduced toxicity of **3** was confirmed in an in vivo study in nude mice. The maximum tolerated dose (MTD) of **3** was >130 mg/kg, iv, whereas the MTD of **1** was found to be in the range of 5–15 mg/kg, iv (data not given).

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