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Synthesis of A83586C Analogs with Potent Anticancer and β-Catenin/TCF4/Osteopontin Inhibitory Effects and Insights Into How A83586C Modulates E2Fs and pRb

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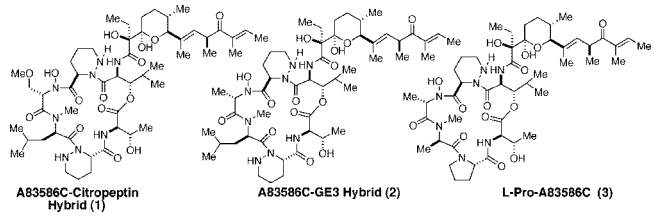
ABSTRACT



The synthesis of three potent new antitumor agents is described: the A83586C−citropeptin hybrid (1), the A83586C−GE3 hybrid (2), and L-Pro-A83586C (3). Significantly, compounds 1 and 2 function as highly potent inhibitors of β-catenin/TCF4 signaling within cancer cells, while simultaneously downregulating osteopontin (Opn) expression. A83586C antitumor cyclodepsipeptides also inhibit E2F-mediated transcription by downregulating E2F1 expression and inducing dephosphorylation of the oncogenic hyperphosphorylated retinoblastoma protein (pRb).

In the preceding paper, we reported the first total syntheses of (+)-azinothricin and (+)-kettapeptin via a new synthetic approach that gives greatly improved access to molecules of the A83586C class, as well as A83586C itself.[1] In this Letter, we now report on the application of this new strategy to the synthesis of three novel A83586C analogues: the A83586C−citropeptin hybrid (**1**), the A83586C−GE3 hybrid (**2**), and L-Pro-A83586C (**3**); molecules which, collectively, have provided powerful new insights into the functioning of this family as antitumor agents.

In this regard, we show here, *for the very first time*, that the hybrids **1** and **2**, and A83586C, are all highly potent inhibitors of β-catenin/TCF4-mediated signaling.[2] For **1** and **2** we also correlate these effects with a significant reduction

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(1) Hale, K. J.; Manaviazar, S.; George, J. H.; Walters, M. A.; Dalby, S. M. *Org. Lett.* **2009**, *11*, 733.

(2) For a previous Novartis report on less potent small molecule inhibitors of the oncogenic TCF4/β-catenin protein−protein interaction, see: Lepourcelet, M.; Chen, Y-N. P.; France, D. S.; Wang, H.; Crews, P.; Petersen, F.; Bruseo, C.; Wood, A. W.; Shivdasani, R. A. *Cancer Cell* **2004**, *5*, 91.

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in osteopontin (Opn) expression in metastatic rat Rama-37-Opn mammary epithelial cells. Opn is a metastasis-inducing protein whose transcription is instigated by the β-catenin/TCF4 interaction.[3]

We also clarify here the mechanism by which A83586C and its congeners potently repress E2F-mediated gene transcription within human cancer cells. Specifically, we show that E2F inhibition occurs directly as a result of the compounds downregulating E2F1 protein expression, while simultaneously promoting the dephosphorylation of oncogenic hyperphosphorylated pRb.

A83586C was the first member of the azinothricin family of depsipeptides to have its potent in vitro antitumor properties recorded (IC_{50} = 13.5 nM vs a CCRF-CEM human T-cell leukemia cell line).[4] Its sister molecule, (+)-citropeptin, was likewise reported to have pronounced antitumor effects in 1990. It inhibited the growth of murine P388 lymphocytic leukemia and B16 melanoma cell lines (IC_{50} = 0.1 and 0.02 μg/mL, respectively), and it conferred a 123% life extension on mice with the P388 leukemia tumor when administered intraperitoneally at doses of 2 mg/kg/day.[5] The third member of this family to have its antitumor characteristics profiled was GE3.[6] Its IC_{50} values ranged from 3.6−16 nM against three different human tumor cell lines, and remarkably, it produced a 47% reduction in tumor volume in mice xenografted with the incurable PSN-1 human pancreatic carcinoma when administered at 2 mg/kg/day.[6] As such, molecules of this genre (Figure 1) have now emerged as important new chemical genomics tools and medicinal chemistry leads.

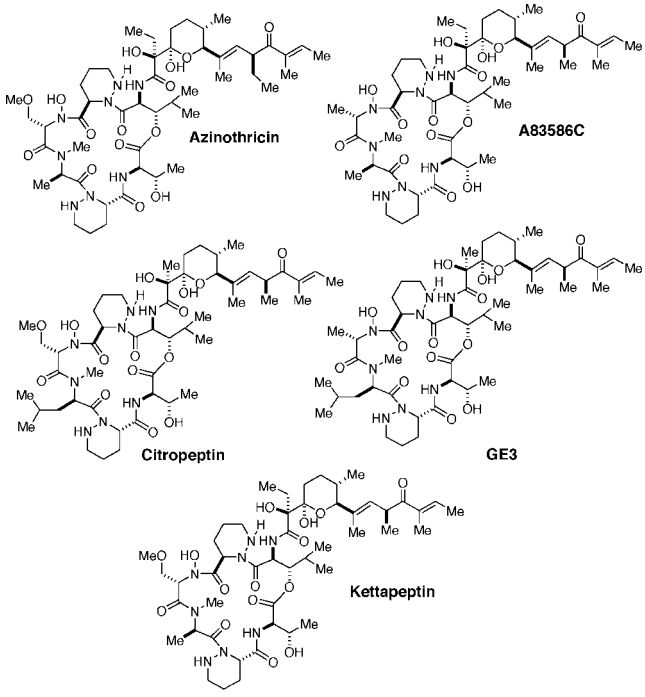
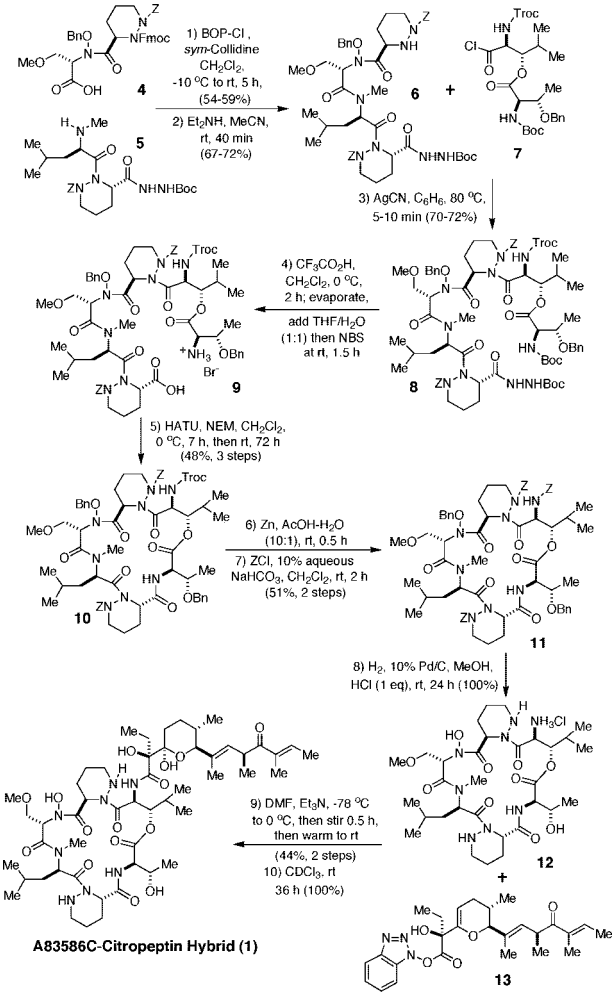


Figure 1. A83586C family of antitumor antibiotics.

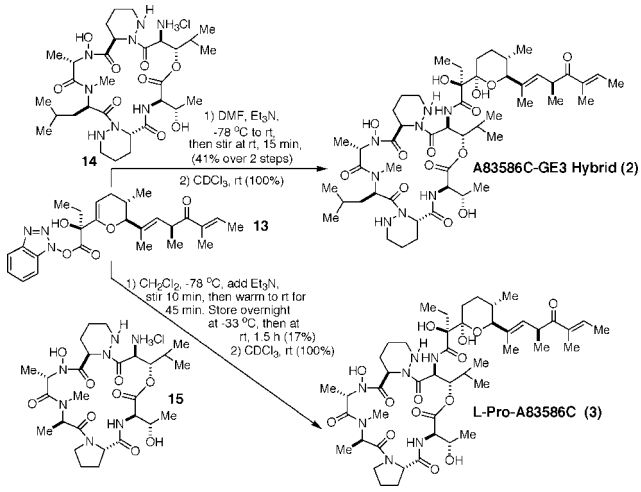
Foremost among the A83586C analogues that we wished to synthesize were the A83586C−citropeptin hybrid **1**, the A83586C−GE3 hybrid **2**, and L-Pro-A83586C (**3**). All were chosen in the hope that they might have enhanced antitumor effects and be more readily synthesizable than their natural congeners. While the construction of **1** would inevitably mean that a route to the previously unsynthesised citropeptin cyclodepsipeptide **12** would have to be developed, in the case of **2** and **3**, we had already devised an effective route to the GE3[7] and L-Pro-A83586C cyclodepsipeptides,[8] and so the main issue now centered around whether we could successfully perform the final chemoselective couplings of these peptides with **13**[1] (see Schemes 1 and 2) in decent yield and thereafter hydrate.

Scheme 1. Route Used to the A83586C−Citropeptin Hybrid (**1**)



Our route to **1** is presented in Scheme 1. It commenced with a BOP-Cl/*sym*-collidine mediated coupling[7] between **4**[1] and **5**[7] which proceeded in 54−58% yield. Following deprotection with diethylamine in MeCN, the requisite tetrapeptide **6** was isolated in 67−72% yield. The key union with acid chloride **7**[1] furnished hexapeptide **8** in 70−72% yield. The Boc-groups were next detached with trifluoroacetic

Scheme 2. Chemoselective Couplings Used for Construction of the A83586C−GE3 Hybrid (**2**) and L-Pro-A83586C (**3**)



acid in CH_2Cl_2, and the resulting acyl hydrazide was converted into the carboxylic acid by treatment with *N*-bromosuccinimide in aqueous THF.[1] The crude *seco*-amino acid **9** was then directly macrolactamized[1] under high-dilution conditions with HATU. The Troc-group was thereafter excised from **10**, and the crude amine temporarily blocked with a *Z*-group, to allow the zinc salts to be removed and the highly pure hexapeptide **11** obtained. The latter was then globally O-debenzylated by catalytic hydrogenolysis over 10% Pd/C in methanol containing 1 equiv of anhydrous HCl. The hydrochloride salt **12** was mixed with the activated ester **13**,[1] and DMF was added. The resulting solution was then cooled to −78 °C, and excess Et_3N (10 equiv) was introduced. After 5 min at −78 °C and gradual warming to rt, the coupled glycal was obtained in 44% yield from the *Z*-protected hexapeptide hydrogenation precursor. Prolonged storage in $CDCl_3$ cleanly hydrated this glycal to give the A83586C−citropeptin hybrid **1** quantitatively.

The coupling of **13**[1] with the GE3 cyclodepsipeptide **14**[7] was also performed in DMF, but in this instance, only 2 equiv of Et_3N was used (Scheme 2). Again, the desired A83586C−GE3 hybrid **2** was readily formed; it was isolated in 41% overall yield for the last three steps. To our great surprise, the coupling of **13** with **15**[8] only proceeded in low yield (17%). However, as we shall see, the incorporation of an L-Pro unit not only was detrimental to the chemistry but also greatly attenuated antitumor potency.

In the latter regard, the hybrids **1** and **2**, and A83586C itself, all potently inhibited the growth of a HCT-116 colon carcinoma cell line with IC_{50}'s of 47, 139, and 40 nM, respectively (Figure 2). However, compound **3** was a much less potent growth inhibitor (IC_{50} = 880 nM). Significantly, all four molecules also potently inhibited *β*-catenin mediated transactivation from the TCF4 promoter in HCT-116 cells after 24 h of drug exposure (IC_{50} for **1** = 5 nM, IC_{50} for **2** = 3 nM, IC_{50} for A83586C = 3 nM). Again, **3** was approximately a 100-fold less active inhibitor (IC_{50} = 430 ± 150 nM). The latter results were gathered via TOP-FLASH/FOP-FLASH assaying in HCT-116 colon cancer cells (see Supporting Information).[2] For **1** and **2**, we further validated our observations by examining the expression of a relevant *β*-catenin/TCF4 downstream target gene, osteopontin (Opn),[9] in Rama-37-Opn cells (Figure 3). Significantly, while Opn expression was markedly downregulated by **1** and **2**, *β*-catenin expression was left untouched suggesting that **1** and **2** are somehow blocking the *β*-catenin/TCF4 interaction.

compound	Colon RKO	Colon HCT116	Colon HT29	Lung A549	Breast MDA-MB435	Bone U2OS
1	18 ± 4	47 ± 4	50 ± 13	-	-	-
2	67± 5	139 ± 3	107 ± 9	-	-	-
3	-	880 ± 7[a]	-	-	-	-
A83586C	18	40	46	30 ± 10	90 ± 10	80 ± 10

a = 1 Day MTS Result

Figure 2. IC_{50} values (nM) of **1**, **2**, **3**, and A83586C on human tumor cell line growth (3 day MTS assay).

Cell Type	mRNA Expression OPN	mRNA Expression β-Catenin
Rama 37	38.0 ± 4.9	28.0 ± 1.2
Rama 37 + **1**	3.6 ± 0.43	29.0 ± 0.4
Rama 37 + **2**	6.2 ± 0.49	28.1 ± 0.5
Rama 37-OPN	89.2 ± 3.4	26.4 ± 0.9
Rama 37-OPN + **1**	15.2 ± 1.5	26.2 ± 0.4
Rama 37-OPN + **2**	21.7 ± 1.31	25.9 ± 0.2

Figure 3. Quantitative real-time PCR data for two rat breast cell lines treated with the A83586C−citropeptin hybrid **1** and the A83586C−GE3 hybrid **2** at a concentration of 10 nM in DMSO/DMEM.

Given Sakai's report that GE3 can prevent E2F/DP transcription factors from binding to target DNA,[6] we next examined whether synthetic A83586C could behave simi-

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larly. Active E2F transcription factors[10] are generally formed when one of four E2F proteins heterodimerize with one of two DP proteins, and the pRb lies in its hyperphosphorylated tumor-promoting state, due to upregulated cyclin/cdk growth signaling. Under such circumstances, the E2F transcription factor is free to switch on the transcription of a range of genes involved in cancer cell growth and proliferation. To probe whether A83586C could disrupt the E2F-DP interaction, we performed pull down experiments using GST-E2F.

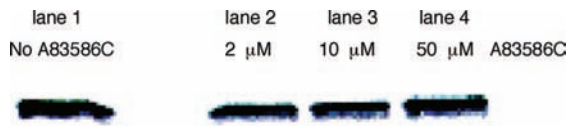

Figure 4. Results of GST-E2F1 (full length) pull down experiments with ^{35}S-labeled DP1 in the presence of A83586C dissolved in DMSO that had been diluted to the desired concentration in the pull down buffer.

The results (Figure 4) suggested that A83586C does not perturb this interaction.

We next evaluated whether A83586C in DMSO/ RPMI1640 culture medium could inhibit E2F-mediated transcription in HCT-116 colon carcinoma cells through an induction of pRb hypophosphorylation (Figure 5). Following

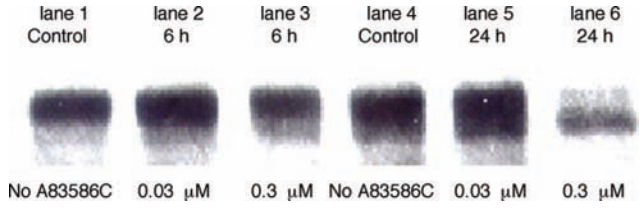

Figure 5. After 24 h, western blotting indicates that A83586C in DMSO/ RPMI1640 culture medium induces pRb-hypophosphorylation in HCT-116 human colon carcinoma cells at 0.3 μM.

exposure of such cells to a 0.3 μM solution of A83586C for 24 h, A83586C was indeed found to cause pRb hypophosphorylation.

Finally, the data in Figure 6 confirm that 0.3 μM solutions of synthetic A83586C in DMSO/RPMI1640 culture medium can strongly downregulate E2F1 protein expression in HCT116 cells over a 6−24 h period. However, cyclin A is only partially downregulated after 24 h of drug treatment, and p53 levels are left essentially undisturbed.

Significantly elevated Opn expression is associated with the most aggressive of metastatic human tumors, with high Opn levels correlating closely with poor prognostic outcome

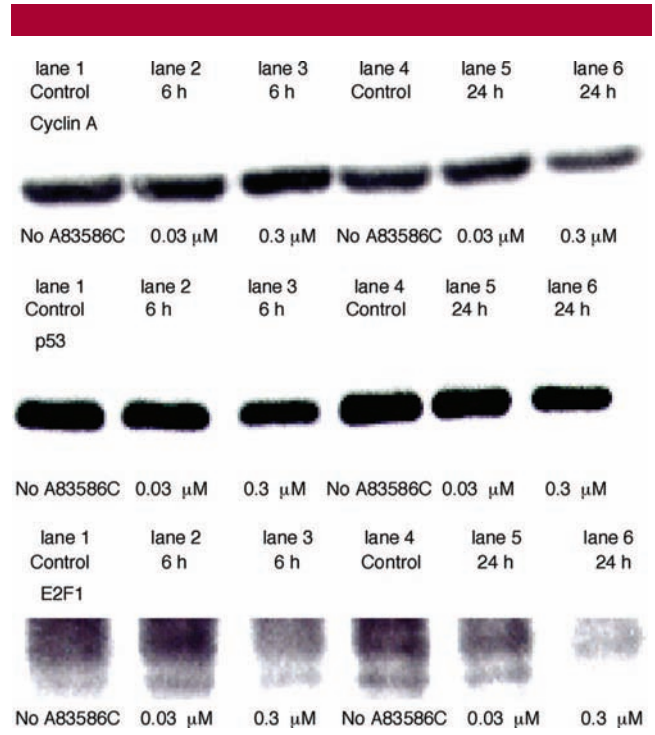

Figure 6. Western blotting indicates that, after 24 h, synthetic A83586C induces cyclin A and E2F-1 downregulation in HCT-116 human colon carcinoma cells at 0.3 μM. p53 levels remain unaffected.

in cancer patients.[3,9,11] Upregulated β-catenin/TCF4 signaling[2,3] and deregulated E2F/DP transcription factor activity[10] are also now strongly implicated in cancer onset and progression. The fact that A83586C and its hybridized analogues, **1** and **2**, can potently and concurrently operate on the β-catenin/TCF4/Opn and E2F/pRb oncological targets makes these molecules of outstanding interest for probing the functional interactions between these two signaling pathways.

In summary, our combined biological data, gathered on a range of A83586C analogues, and synthetic A83586C itself, all very strongly point to the antitumor properties of this class emanating, at least in part, from a beneficial modulation of the human β-catenin/TCF4/Opn and E2F/pRb oncological targets. With regards to the former, *molecules of the A83586C class are the most potent Wnt/β-catenin/TCF4 signaling antagonists so far discovered*.[2]

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Supporting Information Available: Full experimental procedures and detailed spectral data of all key compounds are reported. Copies of 500 MHz ^{1}H and 125 MHz ^{13}C NMR spectra are provided along with IR spectra and HRMS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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