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Nannocystin A: an Elongation Factor 1 Inhibitor from Myxobacteria with Differential Anti-Cancer Properties**

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In memory of Hans-Ulrich Nägeli

Abstract: *Cultivation of myxobacteria of the Nannocystis genus led to the isolation and structure elucidation of a class of novel cyclic lactone inhibitors of elongation factor 1. Whole genome sequence analysis and annotation enabled identification of the putative biosynthetic cluster and synthesis process. In biological assays the compounds displayed anti-fungal and cytotoxic activity. Combined genetic and proteomic approaches identified the eukaryotic translation elongation factor 1α (EF-1α) as the primary target for this compound class. Nannocystin A (**1**) displayed differential activity across various cancer cell lines and EEF1A1 expression levels appear to be the main differentiating factor. Biochemical and genetic evidence support an overlapping binding site of **1** with the anti-cancer compound didemnin B on EF-1α. This myxobacterial chemotype thus offers an interesting starting point for further investigations of the potential of therapeutics targeting elongation factor 1.*

The use of diverse microorganisms for the discovery of bioactive natural products is still an underexploited area of chemical biology. In the past, many antibiotics and natural products with diverse biological activities have been isolated from classical sources like actinobacteria and common filamentous fungi.[2] Recently, cultivation-independent approaches have started to uncover the enormous potential of the uncultivated bacterial diversity.[3] A great example is the emerging field of bacterial symbionts of animals, unambiguously proving that the true producers of the majority of sponge metabolites are uncultured bacteria.[4] The discovery of the antibiotic teixobactin from a previously unknown bacterial genus of Gram-negative bacteria highlights the powerful combination of targeted cultivation techniques and natural product research.[5] As in the case of the teixobactin producer, many bacteria require specialized protocols for their isolation from environmental samples. An intriguing class are the myxobacteria, a group of highly differentiated, motile, Gram-negative bacteria, which have demonstrated a huge potential for natural product biosynthesis.[6] Here we have investigated the compound production potential of myxobacteria of the *Nannocystis* genus and describe the isolation and structure elucidation of a novel compound class from *Nannocystis sp.* MB1016. Comparison of the analytical data showed that the main product from MB106 is identical to Nannocystin A (**1**) as recently published by Hoffmann et al.[7] Here we report the identification of the biosynthetic gene cluster in the genome of the *Nannocystis* producer strain, the antiproliferative activity against a panel of 472 cell lines, and the target identification of this compound class. Using a suite of genetic and affinity chromatography techniques we demonstrate that nannocystin A (**1**) targets eukaryotic translation elongation factor 1 and show potential use for such chemical agents as cancer therapeutics.

The myxobacterium *Nannocystis sp.* MB1016 was cultivated at 500 L scale for 5 days followed by extraction of the culture broth with ethyl acetate. Reversed-phase chromatography of the extract yielded 2200 mg of **1** as main product of the cultivation (Tables S2 and S3, Supporting Information (SI)). The structure and absolute configuration was determined by single-crystal X-ray analysis (Figure 1A and Figure S1, SI). The identified compound consisted of an aliphatic polyketide chain and three amino acids forming an unusual 21-membered macrocyclic lactone. Five additional derivatives (**2**–**6**) could be identified in the extract and their structures elucidated based on comparison of the NMR data with the NMR data of **1** (Tables S4 and S5, SI). Nannocystin A1 (**2**) and A0 (**3**) differed from **1** in the number of chlorine atoms in

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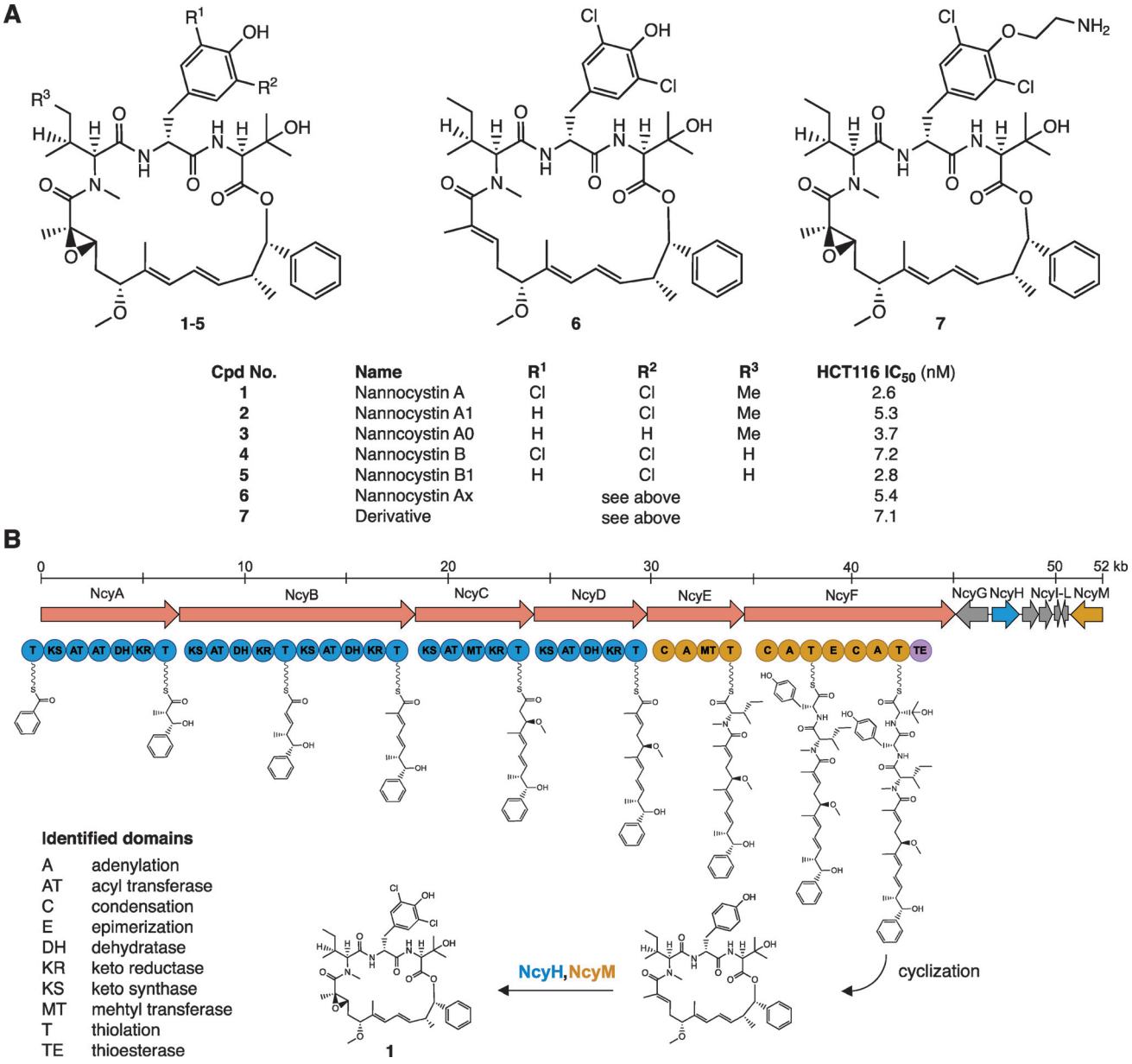

Cpd No.	Name	R^1	R^2	R^3	HCT116 IC_{50} (nM)
1	Nannocystin A	Cl	Cl	Me	2.6
2	Nannocystin A1	H	Cl	Me	5.3
3	Nanncoystin A0	H	H	Me	3.7
4	Nannocystin B	Cl	Cl	H	7.2
5	Nannocystin B1	H	Cl	H	2.8
6	Nannocystin Ax		see above		5.4
7	Derivative		see above		7.1

Figure 1. A) Chemical structure and biological activity of nannocystin A (**1**) and its analogs. A semi-synthetic derivative (**7**) was synthesized for affinity purification of the target protein. B) Domain representation of the identified biosynthetic gene cluster in the *Nannocystis sp.* MB1016 strain.

the tyrosine building block. In nannocystin B (**4**) and B1 (**5**) valine is incorporated instead of isoleucine. In nannocystin Ax (**6**) the epoxide is missing and replaced by a double bond (Figure 1 A, for details on the isolation and characterization procedures, see the Supporting Information).

The draft genome sequence of the producer was determined using 454 sequencing technology. The genome could be assembled into 12 contigs with a total size of approximately 12 Mbps. Biosynthetic gene clusters (BGC) were predicted using AntiSMASH.[8] Guided by the structure of the nannocystins, we focused on hybrid cluster with non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) modules. Out of the 25 BGCs, five revealed PKS/NRPS hybrids, but only one of these candidate clusters harbored the expected number of adenylation and ketosynthase/acetyltransferase domains. The biosynthetic genes for the uncommon benzyl starter unit appeared not to be co-localized with the BGC. Two genes encoding putative tailoring enzymes, a tryptophan halogenase and a P450 were identified downstream of the synthase genes (Figure 1 B, Tables S6 and S7, for details and accession of the sequence, see the Supporting Information). Based on the isolated analogs and the biosynthetic cluster we postulate that a cyclic intermediate is formed first through the activity of polyketide synthases encoded by NcyA, NcyB, NcyC and NcyD and the two NRP synthases NcyE, NcyF. The product of these chain elongation reactions is modified through the P450 NcyH introducing the epoxide at positions 9 and 10. The halogenase NcyM incorporates two

chlorine atoms on the tyrosine yielding the main fermentation product (**1**).

Testing of all compounds identified potent activity against human HCT116 cells (Figure 1 A) demonstrating that the epoxide and the halogenation were not required for activity. Testing of **1** revealed antifungal activity against *S. cerevisiae* (IC_{50} 75 μM) and allowed for haploinsufficiency profiling (HIP). The HIP target discovery assay is based on a genome-wide, collection of heterozygous knockout yeast strains, each of which contains a marked gene deletion.[9,10] It has been shown that heterozygous diploid strains that bear a deletion in one copy of a small molecule's target gene show increased sensitivity to that small molecule relative to those strains that have two copies of the gene.[11] This is a likely consequence of decreased expression of the target in the heterozygous diploid strain. Testing of **1** at sublethal doses yielded reproducible HIP profiles with hits in both α (*TEF2*) and β (*EFB1*) subunits of the translation elongation factor 1 (EF-1) complex (Figure 2 A and Figure S3, SI). *GUF1* encoding a mitochondrial GTPase[12] and *YCL007c* encoding a dubious ORF linked to vacuolar ATPase function[13] scored as additional, reproducible hits. It is noteworthy that the EF-1α subunit in many eukaryotic species is encoded by two genes, including *S. cerevisiae* with the paralogs *TEF1* and *TEF2*. *S.c.* Tef1p and Tef2p share 100% identical protein sequences, are functionally redundant and only *TEF2* is represented in the HIP collection.[14] Consequently, in the heterozygous *TEF2/tef2* strain three functional genes encoding the EF-1α subunit persist. Thus the observed hypersensitivity of this strain in the context of the hypersensitivity of the β subunit led us to hypothesize that nannocystin A (**1**) targets the fungal EF-1 complex.

To validate this hypothesis and to test for target conservation in a mammalian context we subjected 100 million

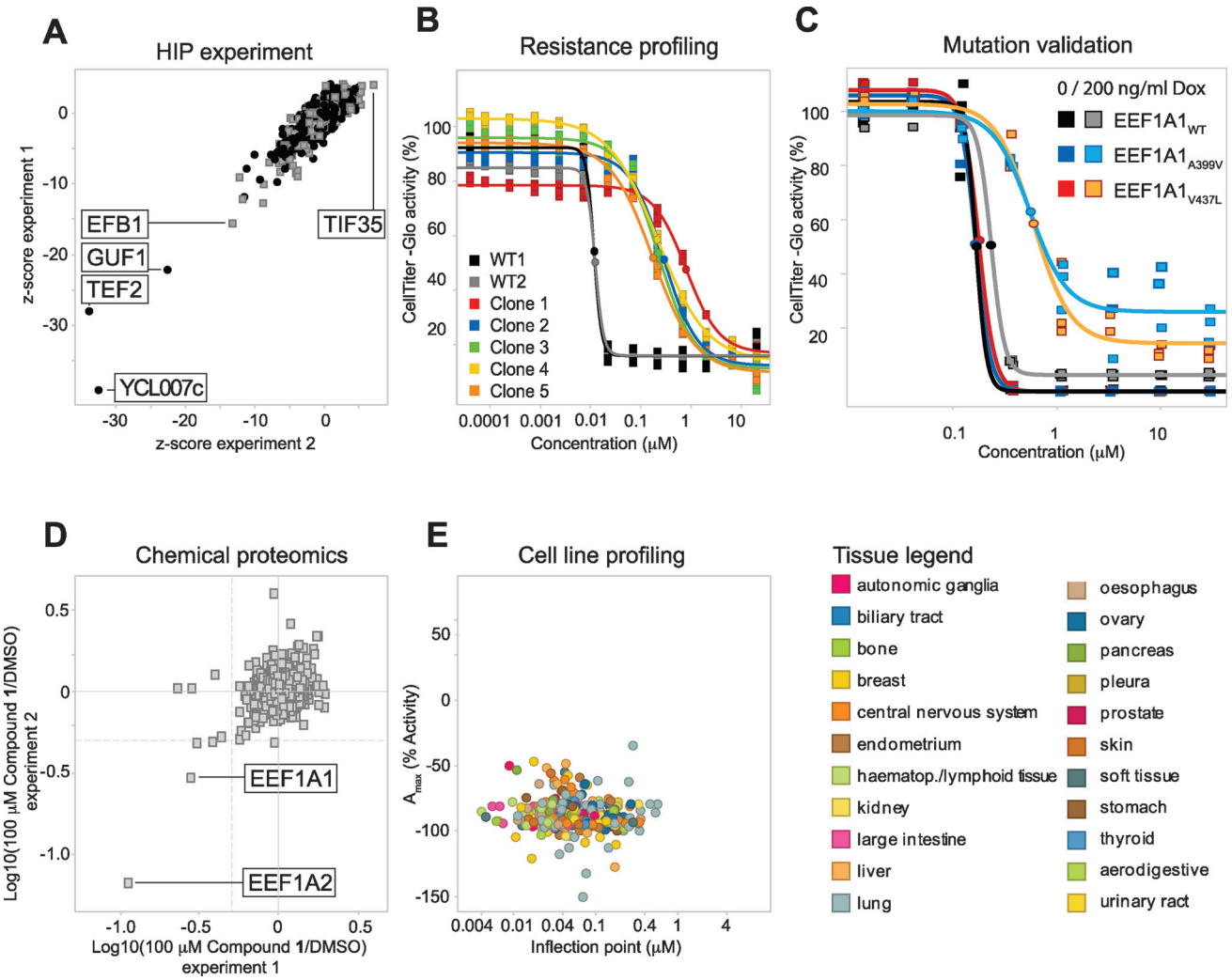

Figure 2. A) Haploinsufficiency profiling of **1** in yeast reveals genetic hits linked to the EF-1 complex. Gray boxes and black dots indicate essential and non-essential genes, respectively, plots displays z-scores[1] for two replicate experiments. B) Induced mutagenesis followed by selection identifies five clones with increased resistance to **1** as depicted by dose-response testing. C) Validation of the identified, resistance-conferring mutations in wild-type cells. D) Unbiased, competition-based chemoproteomics identifies EEF1A1 and EEF1A2 as protein interactors of **1**. Scatter plot showing competition of binding to affinity matrix by 100 μM compound **1** for two replicate experiments plotted as Log10 fold change over DMSO control for 3644 human proteins. Dashed lines denote 50% competition. E) IC_{50} determination against 472 cancer cell lines suggests differential activity.

HCT116 cells to random mutagenesis using *N*-ethyl-*N*-nitrosourea and selected for resistant clones at doses that killed wild-type cells. Incubation for 10 days identified five colonies that appeared to be resistant to 100 nM nannocystin A (**1**) treatment. These clones were isolated and subjected to dose-response testing against **1** and the structurally unrelated microtubule toxin taxol. All five colonies showed IC_{50} shifts between 17- and 79-fold against **1** but no obvious IC_{50} shift against taxol suggesting that the resistance mechanism was specific (Figure 2B and Figure S4, SI). Guided by the HIP target hypothesis we directly sequenced both genes coding for EF-1α in mammalian cells: EEF1A1 and EEF1A2. Sequence analysis identified no mutations in EEF1A2 but all clones harbored mutations in EEFA1 (Table 1). Whereas four clones were identified with two mutations resulting in amino acid changes, one clone carried one mutation only. In all five resistant clones the same amino acid position (A_{399}) was mutated either to valine or threonine. Resistance appeared to be dominant as with the exception of one clone all others were heterozygous. The genetic constitution of three of the five clones shown in Table 1 indicate that they represent independent clones, adding strong evidence that the A_{399} mutation was at least one of the major determinants for nannocystin A resistance. These three clones were tested against twelve additional, chemically diverse compounds and all isolated nannocystins but resistance remained specific against the nannocystin chemotype (Figures S5 and S6, SI). To further validate this finding we cloned the EEF1A1 wild-type and $A_{399}V$ cDNA under control of the doxycyclin-inducible promoter and stably transduced it into HCT116 wild-type cells. Induction of the transgenes lead to notable resistance in the clones expressing the mutant form of EF-1α adding further proof that nannocystin A activity is genetically linked to EF-1α (Figure 2C and S8, SI).

Table 1: Analysis of resistant, mammalian cell clones.

Strain	Mutation1 DNA/Prot	Mutation2 DNA/Prot	Cpd. **1** IC_{50}[a]	Taxol IC_{50}[a]
WT1	–	–	0.01	0.01
WT1	–	–	0.01	0.01
Clone1	$C_{1196}T/A_{399}V$[b]	–	0.79	0.01
Clone2	$C_{1196}T/A_{399}V$	$A_{1357}G/K_{453}Q$	0.29	0.01
Clone3	$C_{1196}T/A_{399}V$	$A_{1357}G/K_{453}Q$	0.22	0.01
Clone4	$C_{1195}A/A_{399}T$	$A_{1357}G/K_{453}Q$	0.22	0.01
Clone5	$C_{1196}T/A_{399}V$	$A_{1357}G/K_{453}Q$	0.17	0.01

[a] Curve fit parameters are listed in the Supporting Information.
[b] Homozygous mutation.

Genetic linkage of EF-1α to nannocystin A activity does not provide final evidence that the compound directly interacts with this target thus we aimed at performing proteomic interaction studies.

Compound **1** was subjected to chemical derivatization adding an ethylamino group to the hydroxy group of the tyrosine moiety (Figure S2, Table S8, SI). This derivative (**7**) displayed similar activity against yeast and HCT116 cells as the parental compound **1** and HIP profiling resulted in the same set of hits supporting that it inhibited the same target as the parental compound (Figure 1A and S3C, S3D, SI). An affinity matrix was generated by immobilization on NHS-activated sepharose beads and used for affinity enrichment of putative interacting proteins of nannocystin A from HCT116 cell lysate in presence of varying concentrations of free compound **1** or DMSO (dimethylsulfoxide). Quantitative mass spectrometry-based analysis of two replicate experiments and using isobaric stable isotope tags allowed the identification and quantitation of 3644 proteins with only EEF1A1 and EEF1A2 showing reproducible, significant, and dose-responsive competition (Figure 2D, Table S9, SI). In a follow-up experiment, the known EEF1A inhibitor didemnin B was tested as competitor compound against the nannocystin-based affinity matrix.[15] Didemnin B showed competition similar to **1** of EEF1A1 as detected by Western blot, suggesting an overlapping binding site of these two distinct cyclic peptide scaffolds (Figure S9, SI). This observation was additionally supported when testing the identified resistant HCT116 clones against didemnin. All tested clones showed cross-resistance against didemnin B (Figure S7, SI).

Both genetic as well as proteomic experiments supported that nannocystin A directly interacts with EF-1α. We thus pursued an in silico docking approach to investigate potential binding sites of **1** on EF-1α. We first mapped the identified resistance conferring residue A_{399} into the published crystal structure of EF-1α of *S. cerevisiae*.[16] *H.s.* $eEF1A1A_{399}$ corresponds to A_{397} in *S.c.* Tef2p and localizes to a shallow cavity on the surface of domain III. Using the Glide docking tool within the Schrödinger software package (Glide, version 6.5, Schrödinger, LLC, New York, NY, 2014) it was possible to dock **1** into the cavity on domain III (Figure 3). The starting conformation of nannocystin A used for the docking procedure was the unbound X-ray structure (Figure S1, SI). A docking grid delimiting the area of possible binding for nannocystin A was built around A_{397}. The resulting docking pose indicated that the compound interacted with the protein structure mainly through hydrophobic interactions. Ala_{397}, for example, created a sub-pocket to accommodate a dimethyl group belonging to the ligand, whereas another methyl group belonging to the nannocystin A was positioned in a small hydrophobic pocket created by Val_{435} interacting with this amino acid residue. In both cases, a hydrophobic amino acid residue with a bulkier side chain would fill these sub-pockets and collide with the functional groups of **1** illustrated in the suggested docking pose. Validity of the model was experimentally tested by introducing the $V_{435}L$ mutation (corresponding to $V_{437}L$ in EEF1A) in the doxycyclin-inducible system described above and dose-response testing of the cells (Figure 2C). As predicted, the mutation mediated resistance to **1** causing a similar IC_{50} shift as observed for the $A_{399}V$ mutation identified in the genome-wide resistance screen. The notion that the A_{399} mutations generated cross resistance to didemnin B led us to test if didemnin B could be docked into the same cavity as **1**. Pursuing a similar strategy like for **1** we found this to be the case. The didemnin B docking solution suggested many compound–protein interactions similar to those observed for **1** (Figure S10, SI).

EF-1 levels and mutations are implicated to play significant roles in several cancers.[17] We investigated the thera-

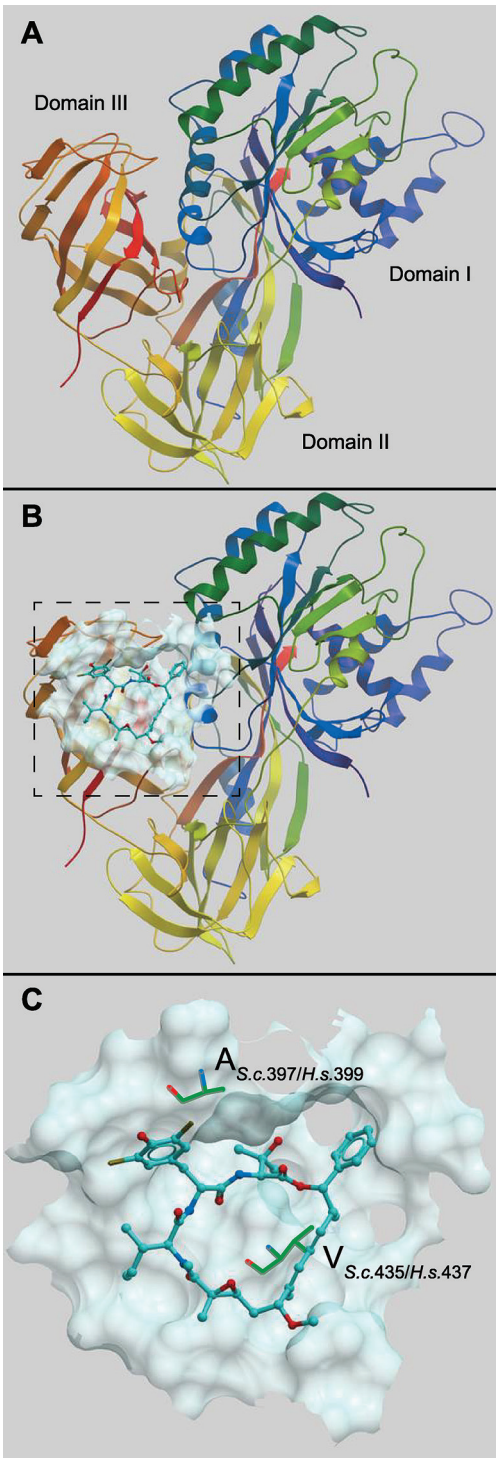

Figure 3. In silico docking of **1** into the EF-1α crystal structure. A) Ribbon model of the *S.c.* EF-1α crystal structure, domains are labeled. B) The *S.c.* EF-1α protein with the in silico docked nannocystin A (**1**) compound. The area enlarged in panel (C) is highlighted by a dashed box. C) Close up of **1** in the proposed binding pocked. Residues identified to give resistance are labeled.

peutic potential of nannocystin A and EF-1 modulation by testing the compound in 8-point dilution series against a panel of 472 cell lines from the cancer cell line encyclopedia (CCLE).[18] **1** showed IC_{50} values ranging from 0.5 μM to 5 nM across the tested cell lines (Figure 2E). When cancer cell line features (gene expression, mutations, copy number) were correlated to compound activity, the most statistically significant expression feature amongst solid tumor lines was low expression of EF1A1 (Tables S10 and S11, SI).

In summary, we describe the characterization of a novel cyclic lactone compound isolated from a myxobacterium *Nannocystis* sp. followed by identification of the biosynthetic cluster. Our discovery was paralleled by the study by Hoffman et al. confirming correct determination of the compound structure.[7] We show that the nannocystins were active against both yeast and mammalian cells and using a yeast-based innovative genome-wide profiling approach we could hypothesize the EF-1 complex to be the likely target. Raising of resistant mutants and affinity proteomics approaches allowed us to confirm EF-1α as the primary target of nannocystin A in human cells. The mapping of the compound binding site was achieved by sequencing of resistant mutants.

Dose-response testing of all identified derivatives revealed potent activity in a range of 2.5–10 nM against mammalian HCT116 cells, notably including compound **6**, indicating that the epoxide moiety is not an essential feature of the pharmacophore. This is supported by the in silico docking model where the epoxide sticks out of the pocket and is not involved in obvious compound–protein interactions. EF-1α is the identified binding protein of the cyclic depsipeptide didemnin B, which was originally isolated from a marine tunicate and recently discovered to be produced by the marine α-proteobacterium *Tistrella mobilis*.[15,,19b,20] Derivatives of didemnin B were the first marine natural products to reach phase II clinical trials, they have a broad spectrum of anticancer, anti-angiogenesis and pro-apoptotic activities reported.[21] Our proteomic and resistance profiling data support an overlapping binding site of nannocystin A with didemnin B on EF-1α. (Figures S7 and S9, SI). The nannocystins thus provide an exciting new chemotype to modulate EF-1 biology and may serve as an alternative starting point to develop new medicines.

Keywords: didemnin B · elongation factor 1α · myxobacteria · nannocystin A · natural products

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