

Gatorbulin-1, a distinct cyclodepsipeptide chemotype, targets a seventh tubulin pharmacological site.

Susan Matthew^{1,7}, Qi-Yin Chen^{1,7}, Ranjala Ratnayake^{1,7}, Charles S. Fermaintt², Daniel Lucena-Agell³, Francesca Bonato³, Andrea E. Prota⁴, Seok Ting Lim⁵, Xiaomeng Wang⁵, J. Fernando Díaz³, April L. Risinger², Valerie J. Paul⁶, Maria Ángela Oliva^{3*}, Hendrik Luesch^{1,5*}

¹Department of Medicinal Chemistry and Center for Natural Products, Drug Discovery and Development (CNPD3), 1345 Center Drive, Gainesville, Florida 32610, USA

²Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229, USA

³Centro de Investigaciones Biológicas Margarita Salas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

⁴Laboratory of Biomolecular Research, Division of Biology and Chemistry, Paul Scherrer Institut, CH-5232 Villigen, Switzerland

⁵Lee Kong Chian School of Medicine, Nanyang Technological University, 59 Nanyang Drive, 636921, Singapore

⁶Smithsonian Marine Station, 701 Seaway Drive, Fort Pierce, Florida 34949, USA

⁷These authors contributed equally.

*email: luesch@cop.ufl.edu; marian@cib.csic.es

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Abstract

Tubulin-targeted chemotherapy has proven to be a successful and wide spectrum strategy against solid and liquid malignancies. Therefore, new ways to modulate this essential protein could lead to new antitumoral pharmacological approaches. Currently known tubulin agents bind to six distinct sites at α/β -tubulin either promoting microtubule stabilization or depolymerization. We have discovered a new binding site at the tubulin intradimer interface where a novel microtubule-destabilizing cyclodepsipeptide, termed gatorbulin-1 (GB1) binds. GB1 has a unique chemotype and was isolated from a marine cyanobacterium. We have elucidated this dual, chemical and mechanistic, novelty through multidimensional characterization, including compound isolation, multi-nuclei NMR-based structure determination, revealing the modified pentapeptide with a functionally-critical hydroxamate group, and validation by total synthesis. We have investigated the pharmacology using isogenic cancer cell screening, cellular profiling, and complementary phenotypic assays, and unveiled the underlying molecular mechanism by *in vitro* biochemical studies and high-resolution structural determination of the α/β -tubulin-GB1 complex.

Significance:

Natural products provide the inspiration for most drugs, and marine natural products in particular are emerging as promising new therapeutics with new targets or mechanisms of action. Pharmacological targeting of tubulin dynamics has been a validated strategy for cancer therapy for decades, yielding structurally-diverse natural products and derivatives, including paclitaxel, vincristine, maytansine, and eribulin, targeting six different binding sites. We discovered a new chemical scaffold from marine cyanobacteria that targets a seventh novel tubulin binding site. We report the entire spectrum of the discovered chemical and biological novelties, including the isolation, structure determination and chemical synthesis of the new natural product, and the investigation of its mechanism of action, target identification, and binding mode elucidation at the atomic level.

Microtubules are polarized polymers consisting of α/β -tubulin heterodimers involved in cellular structure, motility, proliferation, and intracellular trafficking¹. Pharmacological targeting of tubulin dynamics at different sites (**Fig. 1a**) has been a validated strategy for cancer therapy for decades and has mostly been linked to the antimitotic effects of these compounds, although increasing evidence has emerged for the importance of non-mitotic effects¹. Natural products targeting tubulin in particular have yielded a wealth of chemically diverse agents and provided the basis for several FDA-approved drugs, both for cancer and other pathologies, and either alone or as antibody-drug conjugate (ADC), including paclitaxel, vincristine, maytansine, eribulin, and colchicine (**Fig. 1b,c**). Compounds can be classified based on their binding to one of the six known binding sites, and even though they are all targeting tubulin, they have shown distinct pharmacological effects. Therefore, there is a persistent interest in the identification of novel microtubule-targeting agents. Two α/β -tubulin binding sites are associated with microtubule stabilization (taxane and laulimalide/peloruside sites, **Fig. 1a,b**), while binding to four other sites causes microtubule destabilization (vinca, maytansine, colchicine, and pironetin sites, **Fig. 1a,c**)¹.

Our investigation of marine cyanobacteria as a source of potential anticancer agents has previously yielded the modified peptides dolastatins 10 and 15²⁻⁴, targeting the “peptide site” within the vinca domain^{5,6}. Three ADCs with a dolastatin 10 analogue (monomethyl auristatin E, MMAE) as the cytotoxic payload are approved for the treatment of various lymphomas and refractory bladder cancer, while dolastatin 15-based ADCs have advanced to clinical trials⁴. We identified both dolastatins 10 and 15 as indirect hypoxia-inducible factor (HIF) inhibitors based on differential cytotoxicity against a panel of isogenic HCT116 colorectal cancer cells^{4,7}, which indicated that HIF inhibition is functionally relevant for the mechanisms of action of these compounds. HIF is activated in solid tumors, promotes metastasis, and targeted screening early in the drug discovery process could provide a rapid indication for requisite selectivity for cancer treatment⁸⁻¹⁰. Using the same isogenic screening system, we now identified a new antiproliferative agent that also possessed preferential activity for oncogenic KRAS-

and HIF-1 α -containing HCT116 cells and is a microtubule-destabilizing cyclodepsipeptide. We named the compound gatorbulin-1 (GB1, **1a**, **Fig. 1c**), in analogy to eribulin (Eisai Research Institute), to symbolically represent the discovery of its unique chemical structure and pharmacological potential at the University of Florida and global Gator Nation partners. Here we report the bioassay-guided isolation, structure determination, synthesis, preliminary structure-activity-relationships, mechanism of action, target identification, and binding mode elucidation. Our studies revealed that GB1 represents a new chemical scaffold targeting a novel binding site near the colchicine site and displays distinct pharmacology (**Fig. 1a**).

Results

Isolation and structure determination of gatorbulin-1. Various collections of the cyanobacterium *Lyngbya cf. confervoides* during blooms off the coast near Ft. Lauderdale¹¹ (Broward County) were extracted with EtOAc–MeOH (1:1). The extracts, previously proven to be rich in secondary metabolites and possessing antifeedant activity^{12,13}, were either solvent-solvent partitioned first or directly applied onto a Diaion HP-20 column and then fractions subjected to reversed-phase HPLC to afford GB1 (**1a**) as an optically active, colorless amorphous solid ($[\alpha]_D^{20}$ –84.0 (*c* 0.10, MeOH)) and as the most antiproliferative extract component by bioassay-guided isolation using colon cancer cells, along with a minor analogue, termed gatorbulin-2 (GB2, **1b**, **Fig 2a**). Doubling of virtually every signal in the ¹H NMR spectrum of **1a** recorded in DMF-*d*₇ suggested the presence of an asymmetric dimer or the presence of conformers in a ratio of 1:1. This observation coupled with the [M + H]⁺ ion peak at *m/z* 484.2043 obtained by HRESI/APCIMS and ¹³C NMR data suggested a molecular formula of C₂₀H₂₉N₅O₉ (calcd for C₂₀H₃₀N₅O₉, 484.2044) and consequently the presence of conformers in the NMR solvent. NMR analysis using ¹H NMR, ¹³C NMR, COSY, HMQC, and ¹H–¹³C HMBC data was carried out for both conformers, revealing two sets of five spin-coupled systems as part of a pentapeptide

structure; one signal set appeared slightly broader (**Table 1, Fig. 2b**, Supporting Information **Figs. S1–S7**).

For both signal sets, one putative NH singlet each (δ_{H} 8.28 and 8.60) showed COSY correlations to sp^2 -methylene protons (δ_{H} 6.46/5.22 for conformer 1, 6.22/5.11 for conformer 2), which also appeared as singlets in the ^1H NMR spectrum. Correlations from the NH to the corresponding olefinic methylene carbon (δ_{C} 101.8 and 103.0), to a quaternary sp^2 hybridized carbon (δ_{C} 136.3 and 136.2) and to two carbonyl carbons (δ_{C} 165.9/170.5 for conformer 1 and 166.8/170.2 for conformer 2) defined the first residue (unit A, **Fig. 2a**) as a dehydro-alanine (DhAla) residue (**Table 1, Fig. 2b**).

The second multi-proton spin system consisted of two methylenes (δ_{H} 4.31/3.25, 2.58/1.58 for conformer 1), two methines (δ_{H} 4.42, 2.53) and one methyl group (δ_{H} 1.12 d). COSY analysis established their arrangement supported by HMBC data (**Table 1, Fig. 2b**). The terminal methine and methylene carbons of this spin system appeared to be nitrogenated (δ_{C} 62.6, 56.9) and the HMBC correlation of one of the methylene protons (δ_{H} 4.31) to one of the methine carbons (δ_{C} 62.6) clarified that the carbons were joined in a 3-methyl pyrrolidine structure (**Fig. 2b**), which upon further analysis of HMBC correlations to a carbonyl carbon (δ_{H} 170.8) identified the second residue (unit B, **Fig. 2a**) as a 4-methylproline unit (4-MePro). In a similar fashion, a signal set corresponding to the second conformer for this unit was unambiguously identified (**Table 1**).

Analysis of the third spin system was straightforward and this unit consisted of only one methine and a methyl group and NMR data (**Table 1, Fig. 2b**), consistent with an acylated lactic acid (Lac) moiety (unit C, **Fig. 2a**).

Two singlets of another signal set for heteroatom-bound protons (δ_{H} 7.26/7.09 for conformer 1 and δ_{H} 7.39/7.21 for conformer 2) showed cross-peaks in the COSY spectrum, suggesting a primary amide for the fourth moiety. Another set of singlets at δ_{H} 3.09 and 3.14 was indicative of an *N*-methyl tertiary

amide group; expectedly these signals exhibited HMBC correlations with a carbonyl carbon of the adjacent residue (δ_C 169.9 and 169.7) and for conformer 1 also to the α -carbon of the N-methylated amino acid (δ_C 58.0). Rigorous 2D NMR analysis (**Table 1**, **Fig. 2b**) established the fourth residue (unit D, **Fig. 2a**) as an *N*(α)-methyl- β -hydroxy-asparagine (*N*(α)-Me- β -OH-Asn). Even though the significant broadening of all signals for this unit for the second conformer resulted in fewer HMBC correlations, all ^1H and ^{13}C NMR resonances could be assigned except for the α -carbon since its NMR signal was too broad to be observed (**Table 1**).

The fifth and last moiety (unit E, **Fig. 2a**) exhibited similarity to a lactic acid or alanine moiety, yet the α -carbon resonated at higher field than the corresponding carbon for lactic acid (δ_C 64.7 and 60.0) and thus more likely bore a nitrogen atom, which then in turn had to bear a substituent that was not accounted for yet. This NMR analysis so far led to the assignment of all atoms except one oxygen and hydrogen based on HRMS analysis. In the ^1H NMR spectrum, the only unassigned signal at this point was a signal for an exchangeable proton at δ_H 11.35 (br s) for conformer 1 and at δ_H 10.58 (br) putatively for conformer 2, which could not be rationalized by a secondary amide since it did not show a COSY correlation to the nitrogen-bearing methine while also resonating too far downfield. The chemical shifts were consistent with carboxylic acid protons which could exist in a linear structure; however, it would not leave a substituent for the nitrogen atom in the alanine-like moiety. Therefore, a bond between two heteroatoms, nitrogen and oxygen, had to be invoked which led us to propose a *N*-hydroxy group in a cyclic hydroxamate (*N*-OH-Ala); its hydroxy proton was also expected to resonate between δ_H 10–12 as observed (**Table 1**).

The doubling and overlap of signals for several carbonyl carbons for different conformers slightly complicated the sequencing of the individual units. To ultimately prove the existence of the hydroxamate and to validate the nature of the nitrogen atoms we carried out a ^1H - ^{15}N HMBC and ^1H - ^{15}N HSQC analysis (**Table 1**, Supporting Information **Figs. S8, S9**). HMBC correlations of the H-3a/b

methylene protons of the DhAla unit to a nitrogen atom resonating at $\delta_N -258.2$ (relative to external MeNO₂, δ_N 0.0) supported the earlier assignment of a secondary amide (**Fig. 2b,c**). The *N*-Me protons of the *N*-Me-3-OH-Asn showed HMBC correlations to a nitrogen possessing a chemical shift of $\delta_N -273.8$, an expected value for a tertiary amide (**Fig. 2b,c**). Most importantly and confirmatory for the hydroxamate moiety were two- and three-bond correlations from the α -methine and the β -methyl protons to a signal at $\delta_N -202.6$ (**Fig. 2b,c**) which is in agreement with literature values for hydroxamate nitrogens ($\delta_N -199.2$ for polyoxypeptin A¹⁴), further validating the proposed structure for GB1 (**1a**). The ¹H-¹⁵N HSQC spectrum, in addition to the secondary amide proton for DhAla ($\delta_N -258.2$), also showed one-bond correlations for both protons of the primary amide functionality for both conformers ($\delta_N -280.5$ and -279.6 , **Fig. 2c**). The presence of the hydroxamate functionality was supported analytically by ferric hydroxamate complex formation¹⁵ (Supporting Information).

Further support for the proposed structure was found with the isolation of its *N*-deoxy-derivative, gatorbulin-2 (GB2), or *N*-deoxy-GB1 (**1b**, **Fig. 2a**). The ¹H NMR spectrum was strikingly similar to the one of **1a**, including the presence of conformers in a 1:1 ratio in DMF-*d*₇ (Supporting Information, **Table S1**, **Figs S10–S15**). The most significant difference appeared to be the lack of the N-OH protons at δ_H 10–12; instead a new set of doublets appeared in the range for amide protons (δ_H 8.21 and 8.38 for conformers 1 and 2).

To establish the absolute configuration, we performed acid hydrolysis to liberate the individual units and synthesized all isomers of the amino acid standards for comparative chiral HPLC analysis and advanced Marfey's analysis. The 4-MePro standards were prepared as described previously¹⁶ and the *N*(α)-Me- β -OH-aspartic acid stereoisomers as described in the Supporting Information. We detected *L*-erythro-*N*(α)-Me- β -OH-Asp, (2*S*,4*S*)-4-Me-Pro, and L-Lac in the hydrolyzate of **1a**. The structures and identical absolute configurations were confirmed by conversion of **1a** into **1b** via TiCl₃-mediated reduction (**Fig.**

2a)¹⁷. Upon acid hydrolysis, **1b** yielded L-Ala as detected by chiral HPLC analysis, establishing the remaining stereogenic center.

Structure validation by the total synthesis of gatorbulin-1. To prove the structure and overcome the supply issue, we embarked on the total synthesis. The retrosynthetic analysis of total synthesis of GB1 (**1a**) is shown in **Figure 2d**. The final product **1a** could be obtained from the fully masked cyclized precursor **2** by sequential deprotection. The site between 4-MePro and (Se)-phenylselenocysteine (Sec(Ph)) was chosen for macrolactamization. In linear precursor **3**, Fmoc-Fm pair was designed as the protection groups of amino and carboxy termini, respectively, which could be cleaved simultaneously with base to provide the precursor of macrocyclization¹⁸. Sec(Ph) was proposed as the pro-unit of DhAla¹⁹. Linear compound **3** was disconnected into four building blocks **4–7**, which could be constructed from commercially available reagents (e.g., **6** from **8** and **9**) using established or modified protocols.

Figure 2e depicts the synthetic route to GB1 (**1a**). The synthesis of acid **4** was adopted from published procedures²⁰. (2*R*,3*R*)-epoxysuccinic acid (**10**) was converted to *erythro*-*N*(α)-methyl-3-hydroxy-L-aspartic acid (**11**) by treatment with methylamine-water under reflux. Then **11** was selectively esterified with acidic methanol under refluxing²¹ to provide monoester **12**. Without purification, aminolysis of **12** with ammonia (gas) in MeOH provided *N*(α)-methyl- β -hydroxy-asparagine (**13**)^{20,21}, which had poor solubility in MeOH, so that pure product could be obtained by simple filtration. Sequential protections of the groups of **13** using standard methods²² provided full masked compound **17**. Finally, acid **4** was obtained from **17** by hydrogenation with palladium catalyst.

The synthesis of building block of allyloxamine **5** adopted the triflate method²³. Allyl was chosen as NOH protecting group as it could be selectively removed by Pd(Ph₃P)₄ in the presence of

dehydropeptide. Acid **6** was synthesized from (*4S*)-*N*-Boc-4-methyl-Pro (**9**) and benzyl-L-lactate (**8**) by standard protocols of esterification, protection and deprotection. Following established procedures¹⁹, *N*-Boc-serine (**22**) was converted to BocSec(Ph) (**24**) via β -lactone **23**, which was esterified with FmOH to provide building block **7**. Dehydro-alanine could be obtained from phenylselenocysteine by oxidative β -elimination.

The fusion of building blocks was initiated by coupling of acid **4** with allyloxamine **5**. Acid **4** was activated to the acid chloride, which was then coupled with **5** in presence of AgCN^{24,25} to provide **25** in 50% isolated yield and 85% yield based on recovered starting materials (BRSM). This acylation was not successful when other common coupling reagents were used because of poor nucleophilicity of the nitrogen and high steric hindrance²⁶. Compound **26** was obtained in 78% yield by BEP-mediated coupling²⁷ of acid **6** with the free methyl amine generated by selective deprotection of **25**. Selective removal of *t*-butyl group of **26** by TMSOTf/2,6-lutidine²⁸ afforded acid **27**, which was coupled with free amine from **7** using BOP as coupling reagent to yield the linear compound **3** in 86% yield. The use of the *t*-butyl protecting group prevented diketopiperazine²⁹ formation upon coupling of **6** and **25**, and ensured that trityl and TBS groups were intact for the generation of acid **27**. Both Fmoc and Fm protection groups were removed simultaneously when compound **3** was exposed to Et₂NH in MeCN. The macrocyclization was mediated by PyBOP/HOAt to give macrocycle **2** in 60% yield for two steps. Removal of TBS of **2** with the TBAF/HOAc buffer^{22,30} provided **28**, and subsequent oxidation of SePh with NaIO₄¹⁹ yielded dehydropeptide **29**. Trityl was removed with TFA in CH₂Cl₂³¹ to yield primary amide **30**. The removal of allyl group by Pd(PPh₃)₄/PhSiH₃^{30,32} provided final product **1a**. The removal sequence for trityl and allyl groups are interchangeable; however, the yield would drop from 66% to 35%. GB1 (**1a**) was purified by reversed-phase TLC plate (C18). The overall yield over 20 steps from the epoxy acid **10** was 4% (see Supporting Information for details and **Figs. S16–S61** for all NMR

spectra). The synthetic sample was identical to the isolated natural product, which was verified by NMR, HRMS and optical rotation (Supporting Information, **Figs. S62, S63**).

Gatorbulin-1 cellular profiling identifies the mechanism of action. GB1 (**1a**) was identified as the extract's active component against colon cancer cells and showed an IC_{50} of 0.80 μ M (MTT assay) against HCT116 colorectal cancer cells (**Fig. 3a**), while **1b** was inactive at the highest concentration tested ($IC_{50} > 10 \mu$ M), indicating that the hydroxamate moiety is indispensable to the antiproliferative activity. Isogenic cell line selectivity screening indicated preferential activity against parental HCT116 compared with the oncogenic KRAS knockout or double knockout of both HIF-1 α and HIF-2 α transcription factors (**Fig. 3a**)⁷. Deconvolution using single knockouts of HIF-1 α and HIF-2 α clearly demonstrated that only cells depleted in HIF-1 α had reduced susceptibility to GB1, which is consistent with HIF-1 α being in the same pathway and activated by oncogenic KRAS. Furthermore, normal epithelial colon cells (CCD841-CoN) were less inhibited, indicating an additional promising level of selectivity (**Fig. 3a**). The preference for HIF-1 α expressing cells parallels the selectivity profiles for microtubule agents we previously discovered (dolastatins 10 and 15)^{4,7}. DNA content analysis revealed the concentration-dependent G₂/M cell cycle accumulation characteristic for antimetabolic agents (**Fig. 3b**). A concentration of GB1 (4 x IC_{50}) that results in a complete antiproliferative response, initiated a concomitant downregulation of the HIF-1 α target gene *VEGFA* in parental HCT116 cells (**Fig. 3c**) that was even more pronounced than for dolastatin 15⁴, which also elevated GB1's potential as antiangiogenic agent. Consistent with the role of microtubule dynamics in angiogenesis³³, GB1 also inhibited endothelial tube formation without cytotoxicity (**Fig. 3d-f**). Endothelial cell tube formation in Matrigel mimics the process of blood vessel formation *in vivo*.³³ Our study suggested that 10 μ M GB1 was necessary to block the ability of human umbilical vein endothelial cells (HUVECs) to form tube-like structures within 3 h (**Fig. 3d**). Quantification of the structural network revealed a significant

reduction in the number of nodes, number of junctions, number of meshes and total length of tubes (**Fig. 3e**). Importantly, GB1 did not affect HUVEC cell viability, even after 24 h (**Fig. 3f**). Taken together, this reveals an antiangiogenic potential of the compound in inhibiting the formation and stabilization of a three-dimensional vascular network. The additional *VEGF* downregulation in growth factor secreting cells, which is a driver of angiogenesis, is expected to result in a pronounced antiangiogenic action of GB1 *in vivo*.

The NCI-60 cell line screen data (**Fig. 3g**), analyzed by the COMPARE algorithm³⁴, were indicative of a cytotoxicity profile most related to antimetabolic/tubulin agents, including paclitaxel, eribulin, colchicine and vinca bis-indole alkaloid derivatives (P 0.75–0.85), suggesting that the biochemical mechanisms of action are related. While GB1 displayed an $IC_{50} > 10 \mu\text{M}$ against normal mucosal colon cells, it had submicromolar activity against HCT116 cells (GI_{50} 306 nM) and was even more potent against COLO205 cells (GI_{50} 92 nM) based on the NCI-60 data (sulforhodamine B assay). Other susceptible cell types included certain melanoma (SK-MEL-5), ovarian (OVCAR-3) and prostate (DU-145) cancer cells, which correspond to certain cancers where microtubule agents have been successful. Furthermore, cervical and breast cancers are relevant indications, prompting additional studies in these cell types (see below)³⁵.

Target identification and gatorbulin-1 underlying molecular mechanism. First, using a biochemical assay, we demonstrated that GB1 directly inhibits tubulin polymerization *in vitro* in an iron-independent manner (**Fig. 4a,b**). Second, we tested GB1 binding to commonly targeted sites for microtubule-destabilizing agents, including the vinblastine, maytansine and colchicine sites (**Fig. 1a**), by employing fluorescent bona fide probes³⁶⁻³⁸. GB1 was unable to displace the fluorescent derivatives of eribulin and maytansine indicating that it did not bind to these sites. However, GB1 successfully competed with a

fluorescent probe of the colchicine site (MTC) (**Fig. 4c,d**) with an apparent binding affinity of $1.01 \pm 0.08 \times 10^6 \text{ M}^{-1}$, which is below colchicine apparent binding affinity ($1.5 \times 10^7 \text{ M}^{-1}$;³⁹).

We performed subsequent cell-based studies with GB1 in comparison with combretastatin A4 (CA-4), a known colchicine site binder. We demonstrated that GB1 promotes microtubule depolymerization in A-10 (rat smooth muscle) interphase cells, similar to CA-4, although with lower potency (**Fig. 4e**). GB1 promoted aberrant mitotic spindle formation at submicromolar concentrations (**Fig. 4f**). HeLa cervical cancer cells were highly susceptible to GB1; the compound induced accumulation of cells in the G₂/M cell cycle phases after 18 h, with low levels of apoptosis as suggested by an emerging sub-G₁ population (**Fig. 4g**). We then measured the caspase-3/7 activity in a concentration- and time-dependent manner, validating that treatment with GB1 induces apoptosis at concentrations that affect tubulin dynamics in cells, especially after 24 h and beyond, similar to CA-4 (**Fig. 4h**). We then compared the antiproliferative effects of GB1 and CA-4 in a panel of triple negative breast cancer (TNBC) cells and in drug resistant models, including parental and Pgp-overexpressing SK-OV-3 ovarian adenocarcinoma cells (48 and 96 h treatment, respectively) and parental and β III expressing HeLa cells (48 h)⁴⁰. The compounds showed a different fingerprint. GB1 does not circumvent Pgp or β III-tubulin mediated drug resistance (**Fig. 4i**), indicating that it was mechanistically distinct from CA-4. Additionally, it has a distinct profile of relative efficacy against a panel of TNBC cell lines as compared to CA-4 (48 h, **Fig. 4i,j**). MDA-MB-231 cells pre-treated with GB1 for 24 h demonstrated a significant concentration-dependent inhibition of cancer cell migration and invasion abilities across a transwell membrane as compared to DMSO-treated controls, without significantly affecting cell viability (**Fig. 4k**). This phenotype is also consistent with the microtubule-targeting mechanism⁴¹. Taken together, the mechanistic biochemical and pharmacological data suggested that GB1 can block ligand binding to the colchicine site but may not fit in the classic pocket, resulting in a distinct profile of cellular activity.

To further probe our hypothesis and identify the specific binding mechanism, we aimed at high-resolution macromolecular crystallography. T₂R-TTL system (two α/β -tubulin dimers in complex with the stathmin-like protein RB3 and tubulin tyrosine ligase) has successfully produced several structures of ligand bound to tubulin using X-ray crystallography⁴². However, GB1 soaking experiments did not reveal any difference density related to the ligand. We finally succeeded using the T₁-DARPin (T1D) complex that was previously used to solve the structure of colchicine-bound tubulin by Serial Millisecond Crystallography⁴³. We solved the structure of T1D-GB1 at 1.94 Å resolution (**Table S2**), and we unequivocally found ligand density at a new site that is close but clearly different from the colchicine binding site (**Fig. 5a**). This binding site sits at the intra-dimer interface between α - and β -tubulins. It is equivalent to the vinca site but the defined pocket boundaries are reversed (**Fig. 5b**), meaning that contacts with loop T7, helix H10 and strand S9 are provided by β -tubulin (whereas these are α -tubulin in the vinca site) and α -tubulin supplies interactions by loops T5 and H6-H7 (which correspond to β -tubulin in the vinca site).

The structure superimposes very well with the T1D apo-structure (PDB 4drx) with an overall r.m.s.d. of 0.39 Å over 771 C α -atoms, suggesting that the binding of GB1 does not affect the conformation of tubulin. Indeed, there are no major changes on the secondary structural elements surrounding the compound. GB1 is a cyclic peptide that shares a slightly greater buried interface area with α -tubulin (304 Å²) than with β -tubulin (278 Å²) displaying interactions with both tubulin molecules. GB1 methyl-proline group (4-MePro) is sandwiched at the bottom of the pocket within the α -tubulin molecule between α Y210 (helix H6) and α Y224 (helix H7) (**Fig. 5c**), while the carbonyl moiety of this residue points toward the lateral chain of β Q247 (loop T7). The next clockwise residue is the dehydro-alanine (DhAla) that interacts with α -tubulin through hydrogen bond to the α R221 (loop H6-H7) side chain amide. The same carbonyl additionally co-coordinates a crystallographic water molecule that is shared with other carbonyl and hydroxy groups of the compound. The following residue is an alanine that

interacts with β -tubulin through a coordinated crystallographic water molecule to the β V355 (strand S9) amide nitrogen. The hydroxamate of this alanine (*N*-OH-Ala) hydrogen bonds to β D329 (helix H10) side chain O δ 2 and O δ 1 (**Fig. 5c**), rationalizing the critical function of the hydroxamate for binding. The hydroxy propanamide residue (*N*(α)-Me- β -OH-Asn) makes extensive contacts with α - and β -tubulins through loop T5, including the carbonyl moiety of α P175 and α Q176 and the carbonyl and the amide of α S178, and strand S9, with β K352 main chain and β V353 amide nitrogen (**Fig. 5d**). Additionally, the *N*-Me group of this residue further hydrogen bonds to β V353 carbonyl moiety. The carbonyl of the lactic acid (Lac) that closes the ring with 4-MePro further stabilizes contacts with the α -tubulin loop T5 through hydrogen bond to the carbonyl of α Q176.

The tubulin-colchicine (PDB 5nm5) and tubulin-GB1 structures superimpose with an overall r.m.s.d of 0.497 Å over 756 C $_{\alpha}$ -atoms with the main differences located at β -tubulin loop T7 and α -tubulin loop T5 (**Fig. 5e**). These loops imperatively change their conformations upon colchicine binding because, otherwise, the trimethoxy phenyl ring of colchicine (**Fig. 1a**) would clash with β L247 and β N248 (loop T7) and the amide of ring B would clash with the α T179 (loop T5). The presence of GB1 at the new site precludes the simultaneous binding of a second ligand in the colchicine binding site due to GB1 interactions with those loops, which correlates with the results of our competition assays that increasing concentrations of GB1 prevents the MTC fluorescence probe binding to tubulin (**Fig. 4c,d**).

Discussion

We took an integrated approach towards natural products drug discovery by targeting minor, highly bioactive compounds from a chemically prolific cyanobacterium, combining innovative screening and rigorous bioassay-guided isolation and structure determination with chemical synthesis to overcome the supply problem, and achieving in depth-mechanistic studies as well as direct target and binding site

identification. We advocated for such an approach to fully exploit the proven potential of natural products and increase the value of bioactive natural products⁴⁴. The ultimate key for a successful natural product drug discovery campaign is the choice of the source organism. We have been focusing on marine cyanobacteria, which are prolific yet underexplored marine prokaryotes with a tremendous biosynthetic potential. The gatorbulin-yielding sample was derived from a blooming “superproducer” of secondary (specialized) metabolites (natural products) that previously yielded lyngbyastatins 4-6, pompanopeptins A and B, tiglicamides, largamides/largamide D oxazolidine, most of which are noncytotoxic serine protease inhibitors^{12,13,45-47}. Beyond showcasing the biosynthetic capacity of marine cyanobacteria, our discovery of GB1 exemplifies that marine cyanobacterial natural products occupy therapeutically relevant chemical space that could lead to the discovery of new biology, chemical tools or even drug leads.

GB1 is a small (MW < 500 g/mol) cyclodepsipeptide, unique from most cyanobacterial modified peptides or peptide-polyketide hybrids, which dominate the landscape of bioactive natural products produced by marine cyanobacteria⁴⁸. GB1 is densely functionalized with all amino acids being modified and the presence of one hydroxy acid. Natural products possessing all these unusual structural features of GB1 (the hydroxamate, C-hydroxylated and dehydro-amino acids, modified proline) have not been reported. The 4-methylproline residue is a rare amino acid but has been previously found in cyanobacterial natural products¹⁶. Interestingly, the hydroxamate group that is typical for metal chelators (especially iron siderophores) and present in other antiproliferative compounds⁴⁹, plays an iron-independent functional role in GB1’s binding to tubulin as the major mechanism of antiproliferative action. The additional metal binding ability potentially increases the pharmacological complexity of GB1 and remains to be investigated. However, the iron complexing ability due to the hydroxamate functionality was proven but appeared not to play a predominant role with respect to the antiproliferative activity, based on the NCI-60 profile and cell cycle arrest characteristic for tubulin targeting agents. We

neither found the metal was necessary for GB1 binding to tubulin and the inhibition of tubulin assembly into microtubules in our in vitro assays.

Tubulin targeting agents strongly affect microtubule dynamics, which is a key feature for the functioning of these filaments in a wide range of biological processes. In fact, many of these compounds have been originally discovered within the extracts from living sources that often have mobility restrictions and hence, use them as a protection mechanism against consumers. Some compounds promote microtubule stabilization through the interaction with either the taxane site (in the lumen of the microtubule) or the pelo/lau site (wall of the microtubule). The underlying mechanism is under discussion but these compounds prevent microtubule depolymerization by locking lateral and/or longitudinal contacts. Microtubule-destabilizing compounds are more common and frequently more toxic. These induce rapid microtubule disassembly and prevent tubulin polymerization by two different molecular mechanisms: blockage^{50,51} and wedging^{52,53}. Compounds that bind to the tip (maytansine) site (top surface of β -tubulin) or pironetin site (bottom surface of α -tubulin) prevent the incorporation of a new tubulin heterodimer to the microtubule-growing end due to the inactivation of the contact surfaces. Hence, these follow a blockage mechanism. Meanwhile, compounds interacting with the colchicine or the vinca site block the essential curve-to-straight conformational change in tubulin upon assembly, which necessarily comprise microtubule formation. Our GB1 high-resolution structure revealed a new site and contributed to understanding the molecular mechanism underlying the compound's destabilization effect. GB1 binds to a pocket localized between α - and β -tubulin, close to the colchicine site and equivalent to the vinca site (that sits between two tubulin heterodimers), denoting a common underlying mechanism to destabilize microtubule formation: wedging. The colchicine site is an elongated pocket, where some compounds (colchicine, podophyllotoxin or noscapine) bind to the first third, interacting with β -tubulin S9 and H7 (some also interact with the α -tubulin T5 loop), whereas

other compounds (e.g., nocodazole) bind deeper and also interact with the β -tubulin beta sheet of the N-terminal domain^{52,54,55}. None have the extensive interactions with α -tubulin that are observed with GB1.

Most tubulin-targeting agents bind to β -tubulin, which is the active molecule within the heterodimer upon GTP hydrolysis, with the exception of the pironetin site that is localized in α -tubulin. Tubulin has been revealed as a complex molecule since it includes two similar targeting sites that comprise interfaces of the two proteins either, within the heterodimer (intradimer interface, gatorbulin site) or between two heterodimers (interdimer interface, vinca site). Interestingly, these sites are equivalent but contact surfaces are swapped between tubulin subunits. The top surface contains α -tubulin (vinca) or β -tubulin (gatorbulin) residues, whereas the bottom surface contributes with β -tubulin (vinca) or α -tubulin (gatorbulin) amino acids. Since tubulin is a key target for the treatment of cancer, this increases our options for the screening and development of new, safer and more effective drugs. GB1 is a cyclic depsipeptide that represents a new chemotype that differs from other peptides targeting tubulin such as dolastatin 10 and possesses low toxicity and molecular weight, adding to its promising small-molecule, drug-like properties and translational potential.

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Author contributions

S.M., isolation, structure determination and preliminary bioactivity studies, data interpretation

Q.Y.C., total synthesis, analytical compound characterization

R.R., mechanistic studies, cellular profiling, cell cycle, COMPARE and gene expression analysis, preliminary tubulin polymerization assays, apoptosis assays, data interpretation, final figure preparation

C.S.F., immunofluorescence, cell cycle, tubulin polymerization assays, TNBC and drug resistant panel profiling

D.L.A., ligand displacement assays

F.B., crystallization experiments, data collection

A.E.P., assistance and troubleshooting with the production and expression of DarPin and the design of the TD1-GA-1 crystallization system

S.T.L., angiogenesis, migration and invasion assays

X.W., supervision of angiogenesis, migration and invasion assays, data interpretation

J.F.D., supervision of ligand displacement assays, data interpretation, manuscript editing

A.L.R., supervision of immunofluorescence, HeLa cell cycle, tubulin polymerization assays, TNBC and drug resistant panel profiling, data interpretation

V.J.P., collection and identification of cyanobacterium, provided cyanobacterial extracts, manuscript editing

M.Á.O., supervision of crystallization, binding site analysis and data interpretation and discussion, manuscript editing

H.L., overall project supervision, study design and integration, data interpretation, manuscript preparation

All authors have contributed to the writing of the manuscript.

Competing Interest Statement

The University of Florida has filed a patent application relating to the content of this article.

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Figures

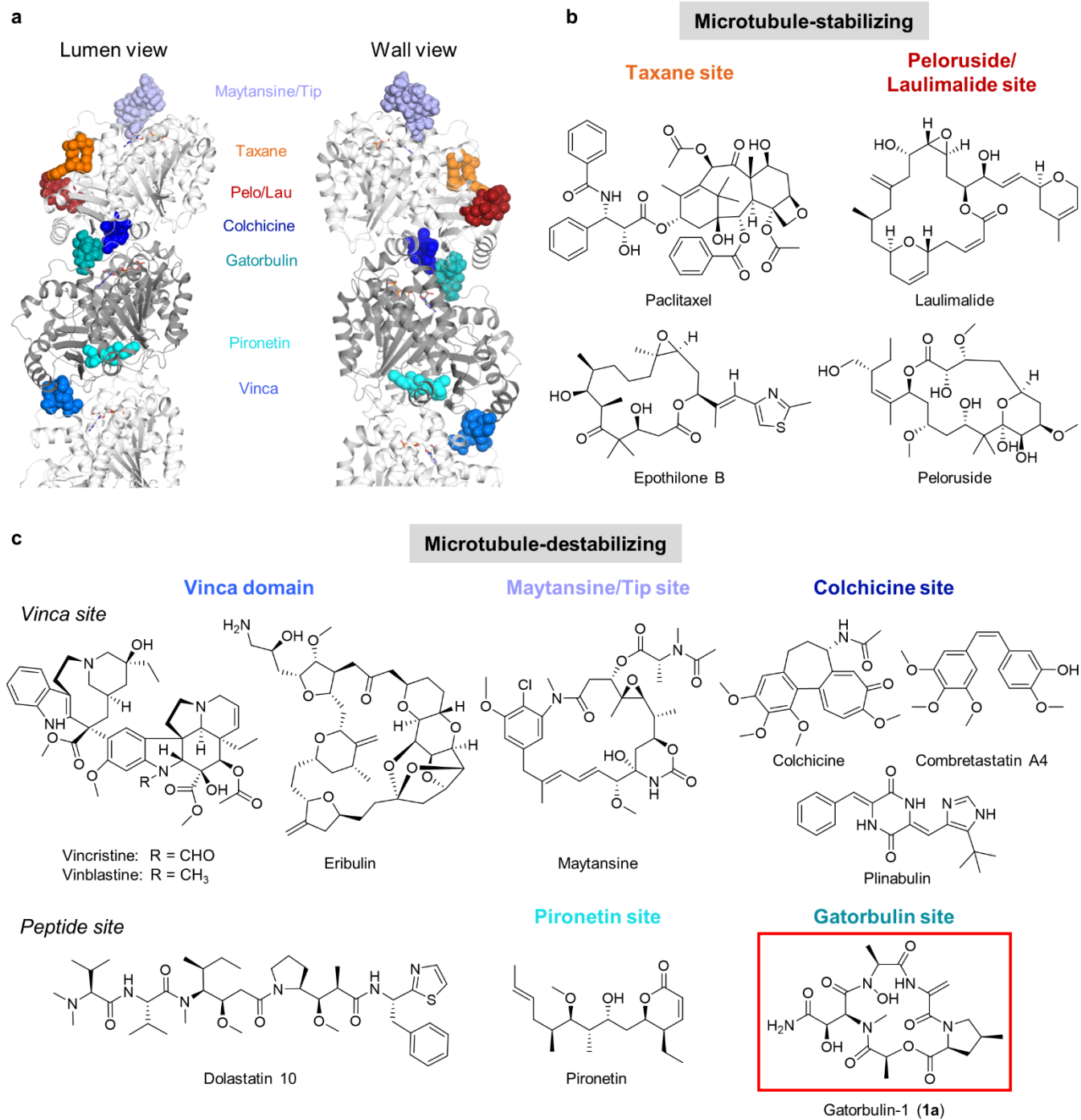


Figure 1. Binding sites and structures of microtubule-targeting agents. a, Tubulin heterodimer (β -tubulin grey and α -tubulin white) in ribbon representation, where six known binding sites have been

highlighted showing representative ligands in sphere representation: maytansine (PDB 4tv8, violet); epothilone (PDB 4o4i, orange); peloruside (PBD 4o4j, red); colchicine (PDB 4o2b, dark blue); pironetin (PDB 5fnv, cyan) and, vinblastine (PDB 4eb6, light blue). New gatorbulin binding site has been also included (PDB 7alr, teal). **b,c** Representative compounds targeting tubulin binding sites. **b**, Microtubule-stabilizing agents. **c**, Microtubule-destabilizing agents, including the structure of gatorbulin-1 (**1a**).

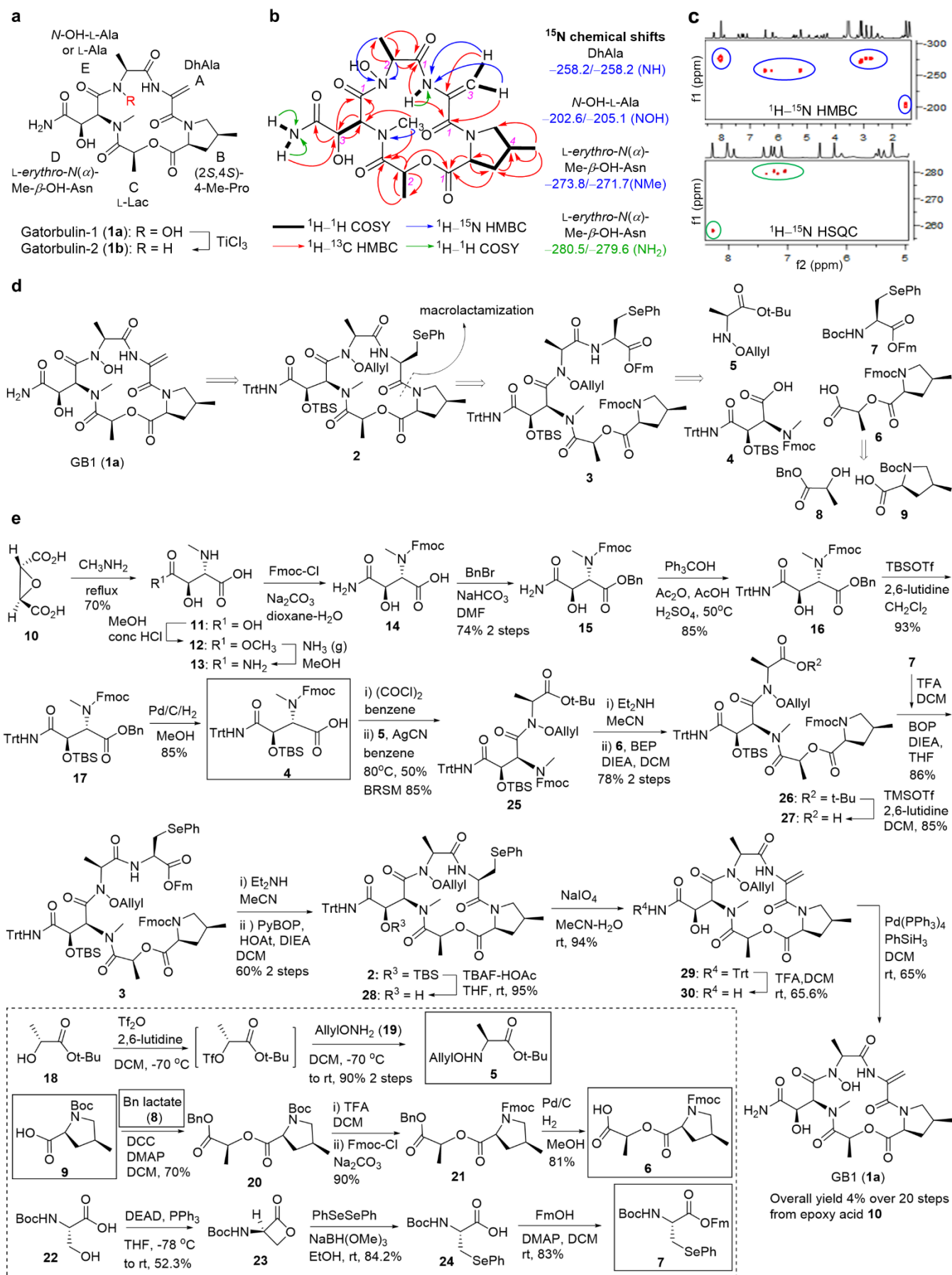


Figure 2. Structure determination and total synthesis of gatorbulin-1, Structures of the isolated natural products, gatorbulin-1 (GB1, **1a**) and its *N*-deoxy-derivative, gatorbulin-2 (GB2, **1b**). GB1 units are labeled A–E. **b**, Homo- and heteronuclear 2D NMR correlations for GB1. **c**, Selected regions of the ^1H – ^{15}N HMBC and ^1H – ^{15}N HSQC spectra of GB1. **d**, Retrosynthetic analysis for the total synthesis of gatorbulin-1 (GB1, **1a**). **e**, Forward synthetic route. Longest linear sequence is shown in the main scheme. Building blocks **4–9** from the retrosynthesis (panel **d**) are indicated by boxes with solid lines. The synthesis of the building blocks **5–7** is provided in the box with the dashed line.

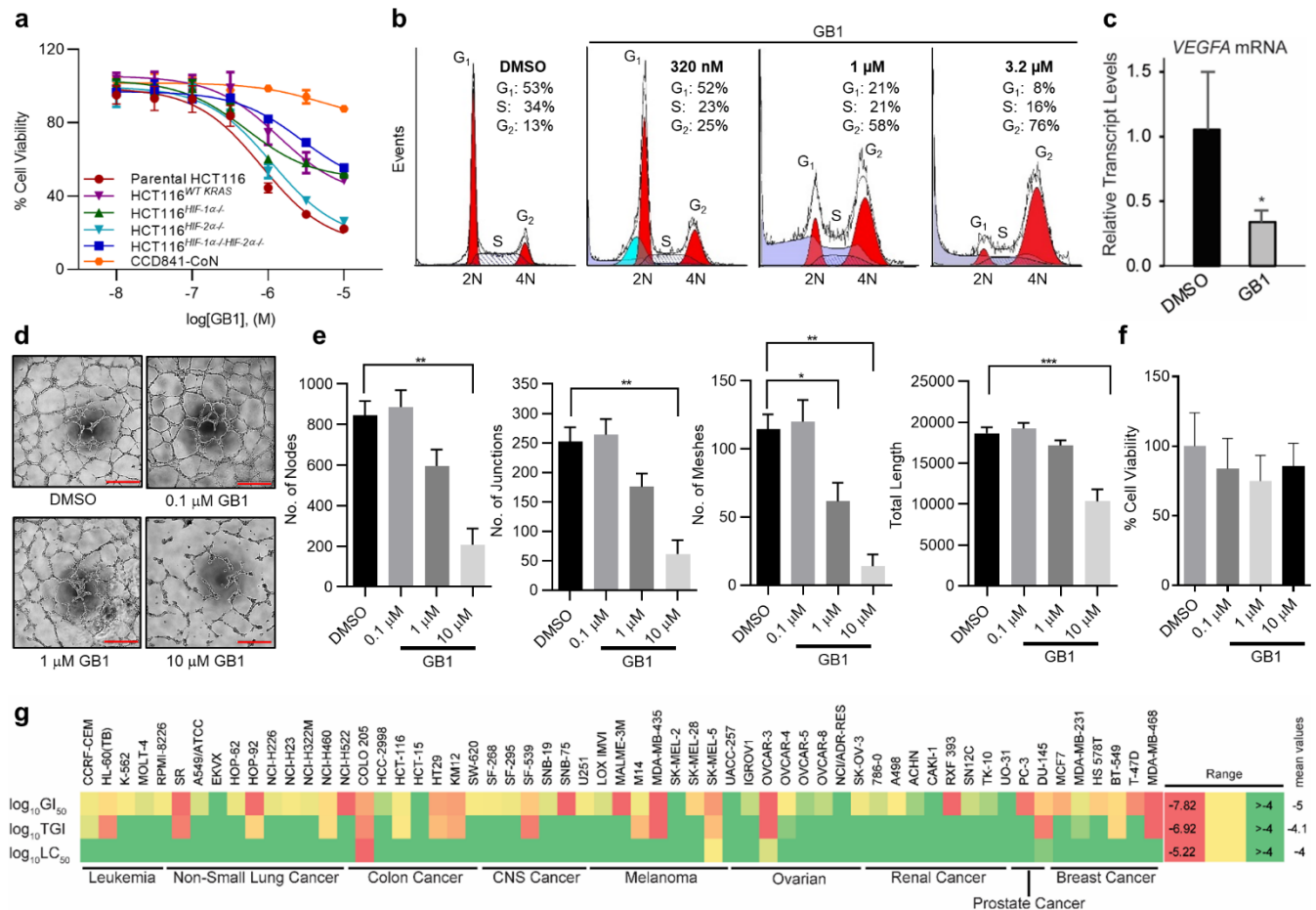


Figure 3. Mechanism of action of gatorbulin-1 through cellular profiling. **a**, Antiproliferative activity of GB1 in parental HCT116 colon cells, isogenic HCT116 knockout cells, and CCD-841CoN normal epithelial colon cells (48 h treatment). GB1 showed a marginal effect on the viability of CCD-841CoN normal epithelial colon cells (0.5% DMSO vehicle). Parental HCT116 cells and HCT116^{HIF-2α/-} were most susceptible, while the potency and efficacy of GB1 were reduced against HCT116^{HIF-1α/-HIF-2α/-}, HCT116^{HIF-1α/-}, and oncogenic KRAS knockout (HCT116^{WT KRAS}). Cell viability was measured by MTT assay (n = 3). Data are represented as average ± SD. **b**, Cell cycle analysis. HCT116 cells were treated with GB1 (320 nM, 1 μM, 3.2 μM) or vehicle control (0.25% DMSO) for 24 h and DNA content assessed by flow cytometry of propidium iodide stained cells. GB1 induced G₂/M accumulation. **c**, HIF target gene (*VEGFA*) expression after 16 h exposure of parental HCT116 cells to GB1 (3.2 μM) or vehicle (0.25% DMSO). RNA was isolated, reverse transcribed and subjected to qPCR using TaqMan analysis. *β-actin* served as endogenous control. Error bars indicate mean ± SD of three replicates (student *t* test, *p < 0.05). **d,e,f**, GB1 inhibits HUVEC tube formation *in vitro* without toxicity. **d**, Representative images of HUVEC tube formation in growth factor-reduced Matrigel upon treatment with DMSO (0.1%) or varying concentrations (10 μM, 1 μM or 0.1 μM) of GB1 (9 h). *Scale bar*: 200 μm. All images shown are representative and data are represented as mean ± SEM; One-way ANOVA followed by Tukey's multiple comparisons test; *p < 0.05, **p < 0.01 and ***p < 0.001. **e**, Quantification of number of nodes, number of junctions, number of meshes and total length of tubes (n = 3). **f**, GB1 does not affect HUVEC cell viability. HUVEC cell viability was quantified by absorbance at 490 nm using MTS assay (24 h). GB1 treatment did not affect total number of viable HUVEC cells compared to DMSO-treated control (n = 3). For panels **e** and **f**, data are represented as mean ± SEM; One-way ANOVA followed by Tukey's multiple comparisons test. **g**, Heatmap for the performance of GB1 across

cell lines in the NCI-60 screen using three different values (growth-inhibitory effect, GI_{50} ; cytostatic effect, TGI; cytotoxic effect, LC_{50} ; concentration in M).

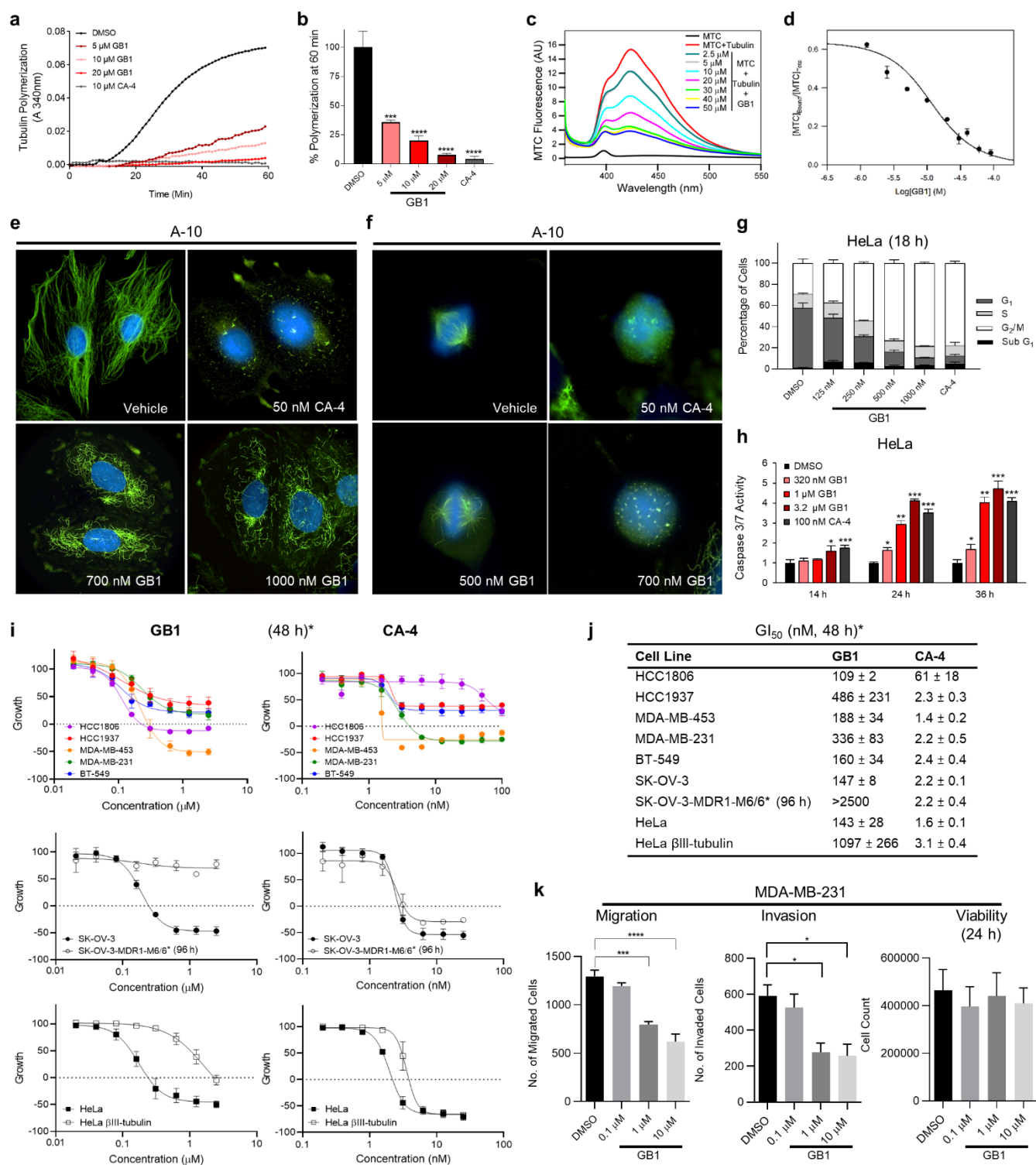


Figure 4. Target identification and selectivity profiling of GB1 in comparison with colchicine binder combretastatin A-4 (CA-4) in different cell types and resistance models. a,b, GB1 directly inhibits the polymerization of purified tubulin. **a**, Polymerization of purified porcine brain tubulin (20 μM) over time as monitored by light scattering at 340 nm with vehicle (DMSO; 1%), CA-4 (10 μM) or 5–20 μM GB1 (representative experiment). **b**, Quantification of tubulin polymerization at 60 min. Error bars represent \pm SEM from three independent experiments. One-way ANOVA with Dunnett's post-hoc test, *** $p < 0.001$, **** $p < 0.0001$. **c,d**, Displacement of 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-

cycloheptatrien-1-one (MTC) from the colchicine site. **c**, Fluorescence emission spectra of 10 μ M MTC in 10 mM phosphate-0.1 mM GTP buffer pH 7.0 in the absence (black line) and the presence of 10 μ M tubulin (red line) plus varying concentrations (5–50 μ M) of GB1 or vehicle (DMSO; 1%). Representative set from three experiments shown. **d**, Displacement isotherm at 25 °C of MTC by GB1. The data points were fit to the best value of the binding equilibrium constant of GB1, assuming 0.8 sites per tubulin dimer ($n = 3$). **e**, GB1 promotes microtubule depolymerization in interphase cells. **e**, A-10 cells were treated with vehicle (DMSO; 0.1%), 50 nM CA-4, 700 nM GB1, or 1000 nM GB1 for 18 h. Microtubules were visualized using a β -tubulin antibody (green) and DNA was visualized using DAPI (blue). Images are representative of three independent experiments. **f**, GB1 promotes formation of aberrant mitotic spindles. A-10 cells were treated with vehicle (DMSO), 25 nM CA-4, 500 nM GB1, or 700 nM GB1 for 18 h. Mitotic spindles were visualized using a β -tubulin antibody (green) and DNA with DAPI staining (blue). Images are representative of three independent experiments. **g**, GB1 promotes concentration-dependent accumulation of cells in the G_2/M phases of the cell cycle in HeLa cells. HeLa cells were treated with vehicle (DMSO; 0.1%), 125–1000 nM GB1, or 100 nM CA-4. Cell cycle distribution was evaluated by quantification of propidium iodide staining by flow cytometry. Error bars represent \pm SEM from three independent experiments. **h**, GB1 causes apoptosis in HeLa cells with increased caspase activity. HeLa cells were treated with increasing concentrations of GB1 and CA-4 (100 nM) using 0.5% DMSO as solvent control. Caspase-3/7 activity was measured using Caspase-Glo 3/7 reagent kit at 14 h, 24 h and 36 h. Data represents average of three replicates with \pm SD. The p -values were calculated using two-tailed Student t -test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. **i,j** Antiproliferative and cytotoxic effects of GB1 and CA-4 in a panel of TNBC cell lines, in parental and Pgp- expressing SK-OV-3 cells, or in parental and β III expressing HeLa cells (sulforhodamine B assay, vehicle: 0.5% DMSO). *Treatment time was 48 h, except for SK-OV-3-MDR1-M6/6 (96 h). **i**, Dashed line at $y = 0$ denotes cell density at the time of compound addition with negative y -values indicating cytotoxicity. **j**, Concentrations that caused a 50% decrease in growth inhibition (GI_{50}) were determined by non-linear regression. Results represent three independent experiments \pm SEM. **k**, Effects of GB1 on transwell migration and invasion of serum-starved MDA-MB-231 breast cancer cells pre-treated with GB1 for 24 h. Pre-treated cells were allowed to migrate or invade for 5 h, and both processes were inhibited in a dose-dependent manner. Quantitative analysis of migrated or invaded DMSO (0.1%) or GB1 pre-treated MDA-MB-231 cells across a Matrigel layer ($n = 3$). GB1 treatment does not affect overall MDA-MB-231 cancer cell count (24 h) as determined by Trypan Blue exclusion assay ($n = 3$). Data are represented as mean \pm SEM; One-way ANOVA followed by Tukey's multiple comparisons test; * $p < 0.05$, *** $p < 0.001$.

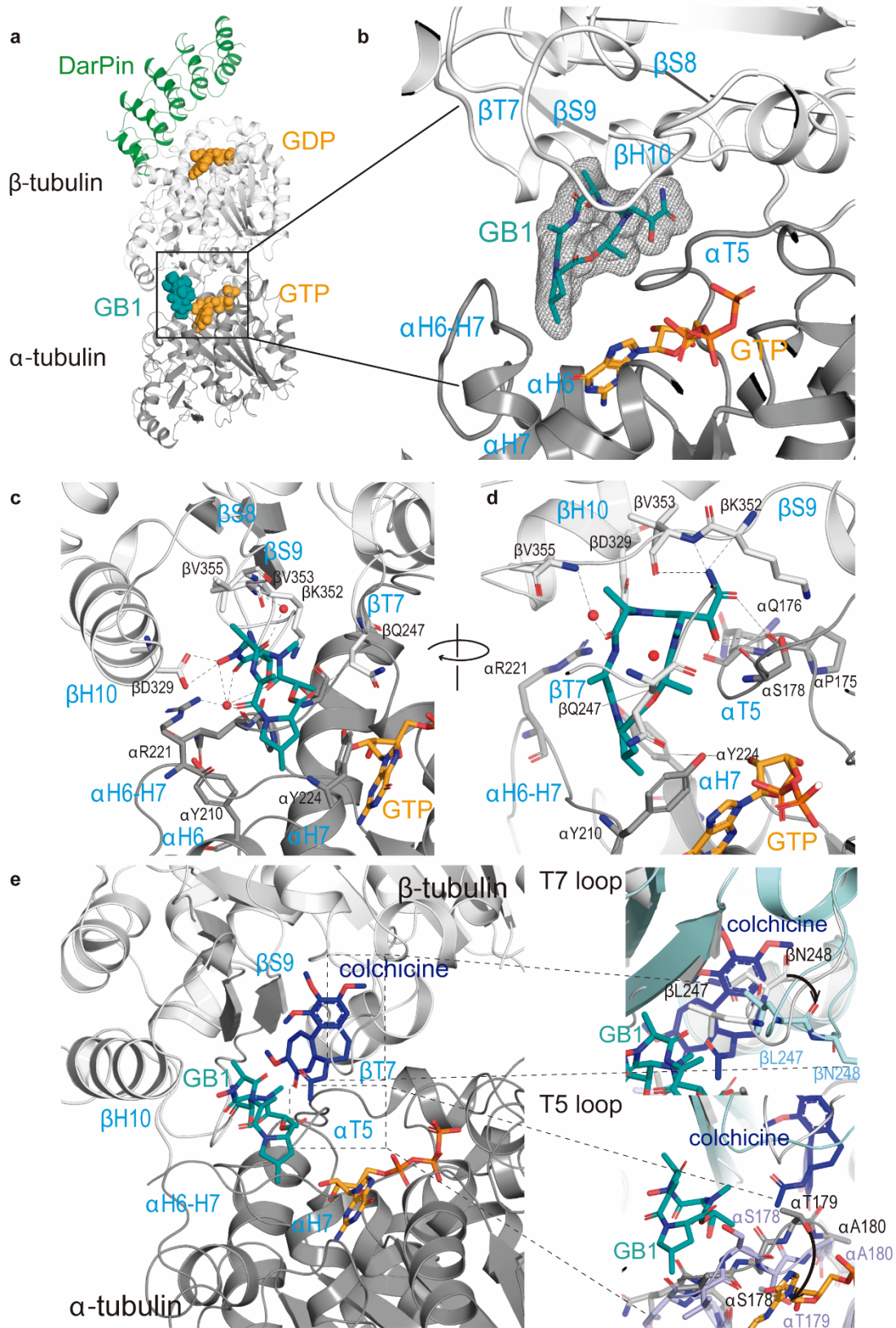


Figure 5. Crystal structure of TD1-gatorbulin-1 complex. **a**, Overall view of the TD1-GB1 complex (PDB 7alr). Tubulin (α -tubulin grey and β -tubulin light grey) and DarPin (green) are in ribbon, and tubulin bound nucleotides (orange) and the ligand GB1 (teal) are in sphere representation, respectively. **b**, Zoom into the composite Gatorbulin-1 site. Simulated annealing omit map of GB1 in the corresponding TD1 complex structure. The mFo-DFc electron density map (grey mesh) is contoured at 3.0σ . The GB1 molecule is shown in stick representation and α - and β -tubulin in ribbon labeling the secondary structural elements involve in protein-compound interaction in blue. **c,d**, Close-up view of the interaction observed between GB1 (teal, sticks) and tubulin (α -tubulin grey and β -tubulin light grey, ribbon). Interacting residues are shown in stick representation and are labeled. Panel **d** is a rotated view of panel **c**. **e**, Comparison of GB1 (teal) and colchicine (blue) binding sites, where tubulin in ribbon and ligands in stick representation. Zoom in panels to show α -tubulin loop T5 and β -tubulin loop T7 conformational changes required for colchicine accommodation when compared colchicine (PDB 5nm5) and GB1 (PDB 7alr) structures. Ligands in stick representation (GB1, teal; colchicine, blue) and tubulin in ribbon representation (PDB 5nm5, blue and, PDB 7alr, grey). Main residues involved are in stick representation and labeled.

Table 1. NMR data for gatorbulin-1 (GB1, **1a**) for both conformers (1:1) in DMF-*d*₇

unit	C/H no.	δ_{H} (<i>J</i> in Hz) ^a	conformer 1					conformer 2		
			δ_{C} , mult ^b	$\delta_{\text{N}}^{\text{c}}$	COSY ^a	¹ H- ¹³ C HMBC ^a	¹ H- ¹⁵ N HMBC ^d	δ_{H} (<i>J</i> in Hz) ^a	δ_{C} , mult ^b	$\delta_{\text{N}}^{\text{c}}$
DhAla (A)	1		165.9, qC						166.8, qC	
	2		136.3, qC						136.2, qC	
	3a	6.46, s	101.8, CH ₂		H-3b	1, 2		6.22, s	103.0, CH ₂	
	3b	5.22, s			H-3 ^a	1		5.11, s		
	NH	8.28, s		-258.2		1, 1 (Ala)	H-3a, H-3b	8.60, s		-258.2
4-Me-Pro (B)	1		170.8, qC						170.4, qC	
	2	4.42, t (7.0)	62.6, CH		H-3a, H-3b	1, 3		4.94, dd (9, 8)	63.8, CH	
	3a (<i>proS</i>)	2.58, m	37.0, CH ₂		H-2, H-3b, H-4	1, 2, 4, 5, 6		2.53, m	40.8, CH ₂	
	3b (<i>proR</i>)	1.58, m			H-2, H-3a, H-4	1, 2, 4, 5, 6		1.65, m		
	4	2.53, m	34.7, CH		H-3a, H-3b, H-5a, H-5b, H ₃ -6	3, 5, 6		2.38, m	32.4, CH	
	5a (<i>proR</i>)	4.31, dd (-10.0, 6.0)	56.9, CH ₂		H-4, H-5b	2, 3, 4, 6		3.71, dd (-11.0, 7.0)	55.6, CH ₂	
	5b (<i>proS</i>)	3.25, dd (-10.0, 8.5)			H-4, H-5 ^a	3, 6		3.11, dd (-11.0, 11)		
6	1.12, d (7.0)	18.0, CH ₃		H-4	3, 4, 5		1.11, d (7.0)	16.9, CH ₃		
Lac (C)	1		169.9, qC						169.7, qC	
	2	5.47, q (7.0)	72.9, CH		H ₃ -3	1, 3, 1 (MePro)		5.39, q (7.0)	72.1, CH	
	3	1.42, d (7.0)	17.6, CH ₃		H-2	1, 2		1.41, d (7.0)	17.3, CH ₃	
<i>N</i> (α)-Me- β -OH-Asn (D)	1		170.4, qC						169.4, qC	
	2	5.51, br d (9.5)	58.0 (br), CH		H-3,	1, 2		5.85	not observed	
	3	4.51, dd (9.5, 4.5)	68.8, CH		H-2, 3-OH	1, 2, 4		4.65, br dd (6, 6)	69.7, CH	
	3-OH	6.05, br d			H-3			5.06, br		
	4		175.1, qC						174.8	
	<i>N</i> -Me	3.09, s	34.0, CH ₃	-273.8		2, 1 (Lac)	<i>N</i> -Me	3.14, s	34.5 (br), CH ₃	-271.7
	4-NH _a	7.26, s		-280.5	4-NH _b			7.39, s		-279.6
4-NH _b	7.09, s			4-NH _a	3		7.21, s			
<i>N</i> -OH-Ala (E)	1		170.5, qC						170.2, qC	
	2	4.29, q (6)	64.7, CH		H ₃ -3, NH	1, 3		4.71, br q (6)	60.0, CH	
	3	1.54, d (6)	13.8, CH ₃		H-2	1, 2		1.42, d (6)	15.6, CH ₃	
N-OH	11.35, br s		-202.6			H-2, H ₃ -3	10.58, br		-205.1	

^aRecorded at 600 MHz. ^bRecorded at 150 MHz. ^cDeduced from ¹H-¹⁵N HSQC and ¹H-¹⁵N HMBC (500 MHz). ^dRecorded at 500 MHz.