Klebsiella oxytoca enterotoxins tilimycin and tilivalline have distinct host DNA-damaging and microtubule-stabilizing activities

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Establishing causal links between bacterial metabolites and human intestinal disease is a significant challenge. This study reveals the molecular basis of antibiotic-associated hemorrhagic colitis (AAHC) caused by intestinal resident Klebsiella oxytoca. Colitogenic strains produce the nonribosomal peptides tilivalline and tilimycin. Here, we verify that these enterotoxins are present in the human intestine during active colitis and determine their concentrations in a murine disease model. Although both toxins share a pyrrolobenzodiazepine structure, they have distinct molecular targets. Tilimycin acts as a genotoxin. Its interaction with DNA activates damage repair mechanisms in cultured cells and causes DNA strand breakage and an increased lesion burden in cecal enterocytes of colonized mice. In contrast, tilivalline binds tubulin and stabilizes microtubules leading to mitotic arrest. To our knowledge, this activity is unique for microbiota-derived metabolites of the human intestine. The capacity of both toxins to induce apoptosis in intestinal epithelial cells—a hallmark feature of AAHC—by independent modes of action, strengthens our proposal that these metabolites act collectively in the pathogenicity of colitis.

Significance

Human gut microbes form a complex community with vast biosynthetic potential. Microbial products and metabolites released in the gut impact human health and disease. However, defining causative relationships between specific bacterial products and disease initiation and progression remains an immense challenge. This study advances understanding of the functional capacity of the gut microbiota by determining the presence, concentration, and spatial and temporal variability of two enterotoxic metabolites produced by the gut-resident Klebsiella oxytoca. We present a detailed mode of action for the cytotoxins and recapitulate their functionalities in disease models in vivo. The findings provide distinct molecular mechanisms for the enterotoxicity of the metabolites allowing them to act in tandem to damage the intestinal epithelium and cause colitis.

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These studies showed that the nonribosomal peptide synthetase (NRPS) platform does not yield TV directly, but instead an N-acylprolinol, which reacts spontaneously to form two further secondary metabolites we named tilimycin (TM) and culdesacin (19). The intrinsic reactivity of TM with indole yields TV. All three substances are stable in vitro, and importantly, although culdesacin has no obvious bioactivity, both TM and TV are cytotoxic to human cells (19, 20).

Insights into the cytotoxic functionalities of these substances in human cells are provided by their structures. TM and TV belong to the PBD family of natural products, which exhibit antibacterial and antitumor activity by alkylating DNA (22). This family of potent cytotoxic agents has been extensively investigated for use in systemic chemotherapy (23, 24). Structure–activity data for PBDs imply that TM will form a similar PBD–DNA adduct (25). By contrast, presence of an indole substituent on the diazepine ring of TV should block this activity. The molecular basis of TV cytotoxicity is thus an open question.

Here, we establish the causal links between K. oxytoca metabolites and disease. We first demonstrate that both cytotoxins are produced in the human body and use a murine model to determine their concentrations during an active phase of AAHC. We identify the different molecular targets of TM and TV and present a detailed mode of action study. Remarkably, the data show that the enterotoxin gene cluster produces distinct DNA-damaging (TM) and microtubule-stabilizing (TV) secondary metabolites. Although the functionalities of the enterotoxins differ, each substance triggered the apoptotic cell death characteristic for the colonic epithelium in AAHC (16, 17). These findings illustrate the versatility of bacterial host interactions mediated by a single secondary metabolite biosynthesis pathway and provide insights into the molecular mechanisms of pathobiont activity.

Results

K. oxytoca Enterotoxins Are Produced in the Human Intestine. The enterotoxin gene cluster (Fig. 1A) encodes two NRPS modules, NpsA and NpsB, and additional enzymes required to generate an anthranilate precursor (19). Genetic inactivation of the NRPS-operon eliminates cytotoxicity in vitro and pathology in vivo (17). Two end products of this secondary metabolite pathway are cytotoxic: TM and TV (19, 20). In the report of Tse et al. (20), the alternative name, “kleboxymycin,” was proposed for the substance we call TM. TV is formed by the intrinsic reactivity of an imine intermediate of TM with indole. Since stool of healthy humans typically contain millimolar concentrations of indole...
(26), concomitant production of both cytotoxins TV and TM in the gut is expected. To test this prediction, we analyzed colonic fluid and stool samples of AAHC patients. Presence of both enterotoxins in human samples taken during the active phase of disease was confirmed (Fig. 1 B and C and SI Appendix, Table S1). The triggering antibiotic therapy was terminated at diagnosis since patient conditions improve by halting selective growth of K. oxytoca (16). During the active phase of AAHC, stool of patient A contained both enterotoxins and 10⁷ colony-forming units (cfu) K. oxytoca g⁻¹ in marked contrast to the 10² cfu g⁻¹ feces typically cultured from asymptomatic intestinal carriers of K. oxytoca (27). Follow-up stool samples of this patient also contained TM and TV 3 d later, but after 5 d the metabolites were no longer detectable (Fig. 1C) consistent with reduced abundance of the pathobiont in stool (10² cfu g⁻¹). The incidence of AAHC is very low; thus, the number of patient samples available for analyses is quite limited. Nevertheless, presence of both enterotoxins in human disease as well as the temporal loss and elimination of the substances once therapy is started are compelling observations linking the microbial products to active colitis.

We then asked what concentrations of TM and TV are relevant to disease. Endoscopy performed on AAHC patients is typically preceded by acute diarrhea and colonic lavage; thus, physiologically relevant concentrations of the enterotoxins cannot be determined from the clinical samples. To address this key point quantitatively, we developed analytic methods and used murine disease models. Cecal contents and feces of mice colonized with K. oxytoca AHC-6 were collected during an active phase of AAHC (Fig. 1 D and E). Samples of diseased animals (n = 8) contained TM and TV, but both enterotoxins were absent in control mice (SI Appendix, Fig. S1). TM was more abundant in cecal content (24 ± 4 nmol g⁻¹) and feces (136 ± 40 nmol g⁻¹) of infected mice compared with TV (1 ± 0.4 and 19 ± 6 pmol g⁻¹, respectively) (Fig. 1F). Enterotoxin concentrations were also higher in feces compared with cecal contents. This finding might reflect the consistency of samples (liquid/solid ratio) or, possibly, continued production of toxins during intestinal passage. We conclude that TM and lower amounts of TV are produced in the human and murine intestine. The level of K. oxytoca colonization in experimental animals is much higher than in patients during active AAHC (10¹⁰ vs. 10⁷ cfu g⁻¹ stool); thus, we expect that the quantities of K. oxytoca enterotoxins sufficient to cause colitis in patients are lower than the concentrations determined in the murine model.

**TM and TV Disrupt Cell Cycle Progression.** Growth-inhibitory activities of TM and TV were determined in a variety of human immortalized cell lines and nontransformed vascular endothelial cells revealing 50% inhibitory concentrations (IC₅₀) in the (sub)micromolar range (SI Appendix, Table S2). In contrast to TV, TM also exhibited antibacterial activity (SI Appendix, Table S3). To gain insights into the cellular processes affected by these generally toxic compounds, we next tested their effects on cell cycle progression. Using flow cytometry, distinct profiles of cell cycle disruption were observed for populations of HeLa cells treated with TV, TM, or solvents. TV treatment of HeLa cells led to an accumulation of cells in the G2/M phase (Fig. 2). TM-treated cells were markedly arrested in G₁ or S phase.

**TM is a DNA-Damaging Agent.** Accumulation of a large fraction of cells at G1/S phase following exposure to TM is consistent with its predicted DNA-alkylating activity. We used biochemical and cellular assays to test this possibility. Structure–activity relationship data have shown that the diazepine ring system of PBDs interacts with the minor groove and stabilizes double-stranded DNA (dsDNA) to thermal denaturation in vitro (25). We determined a 0.5 °C higher melting temperature (ΔTₘ) for a dsDNA containing a putative PBD binding site relative to TM compared with solvent (SI Appendix, Fig. S2A). This value is in good agreement with the 0.7 °C ΔTₘ measured for the closest structurally related natural product analog DCS1 using calf thymus DNA (28) and less than GWL-78 (SI Appendix, Fig. S2B), a PBD-poly(N-methylpyrrole) conjugate engineered to strengthen minor groove contacts (29). We then asked whether the sequence selectivity predicted for TM (30) blocks site-specific endonuclease activity. Indeed, cleavage of a BamHI recognition site was inhibited in a concentration-dependent manner by TM and control GWL-78, but not by TV or buffer (Fig. 3A). By contrast, TM did not inhibit an endonuclease with an A-T–rich binding site (SI Appendix, Fig. S2C).

DNA alkylolation at guanine bases by a PBD or other agents should trigger a host cellular DNA damage response and activate multiple DNA repair enzymes including the base- and nucleotide-excision repair pathways. Incomplete excision removal of the PBD adduct may also lead to DNA single- and double-strand breaks (31). To test whether the K. oxytoca enterotoxins exert DNA damage, HeLa cells were treated with TM, TV, or the DNA-alkylating control GWL-78, and then subjected to comet analysis, a gel electrophoresis-based method to measure DNA damage in individual cells. Significantly increased DNA fragmentation was observed with HeLa cells after TM or GWL-78 treatment, compared with TV or solvents. Similar results were obtained with the colon cancer cell lines HT-29 and SW48 (Fig. 3B and SI Appendix, Fig. S2D). Lysates of the TM- and GWL-78–treated HT-29 cells also exhibited increased phosphorylation of the cell cycle checkpoint kinases CHK1 and CHK2 (Fig. 3C). Thus, the effects of TM on DNA alert master regulators of cellular responses to DNA damage and replication stress, and lead to accumulation of DNA single- and double-strand breaks.

To assess whether the intestinal epithelium exhibits genomic instability when exposed to TM, we chose to analyze tissues before day 5 of infection when apoptosis and exfoliation of the lining are excessive (Fig. 1E; see Fig. 6A). We used a pilot study to monitor the temporal increase in TM and TV concentrations in stool during the first 72 h of colonization (SI Appendix, Fig. S2E). TM was detected after 12 h and increased thereafter. TV production was comparatively delayed. To focus on the bioactivity of TM, we colonized additional animals with K. oxytoca AHC-6 or the toxin-deficient npsB mutant for 24 h when TV
levels in stool should still be low (SI Appendix, Fig. S2E). Upon killing, we determined cells per gram of stool and quantified TM and TV in cecal contents and feces (SI Appendix, Figs. S2 F and G). Trace amounts of TM were also detected in pooled blood samples and in some cases kidney of infected animals. Comet analysis revealed significant DNA fragmentation in enterocytes isolated from ceca of mice colonized by wild-type K. oxytoca, but not the toxin-deficient mutant (Fig. 3D).

To gain insights into how TM-induced DNA damage is sensed and repaired in host cells, we next asked whether mutation of genes encoding key repair factors would render cells hypersensitive to TM. Inactivating mutations were generated in the human haploid cell line HAP1 (32, 33). Equal numbers of cells for each mutant line were cultured with increasing concentrations of TM, TV, or controls, and cell viability was measured (Fig. 4A–C). We observed pronounced hypersensitivity to TM with mutant cells lacking the Cockayne syndrome group A or B (CSA or CSB) proteins compared with wild-type survival. These factors mediate transcription-coupled repair (TCR), a subpathway of nucleotide excision repair (NER) that targets DNA alterations blocking translocation of RNA polymerase through expressed genes. Cells lacking the NER factor xeroderma pigmentosum protein A (XPA), which functions downstream of CSA/B, also showed significantly increased susceptibility to multiple concentrations of TM. Lower viability was not detected for any mutant cell line tested with TV compared with wild type, but mutant cells exposed to control substance illudin S exhibited a response assay were confirmed with a short-term dose–response assay were confirmed with a long-term colony formation assay (Fig. 4C–D and SI Appendix, Figs. S3 A and D). The results of these experiments verify that HAP1 cells lacking CSA, CSB, and XPA were significantly more sensitive to TM, revealing that TCR proteins can recognize the DNA lesions caused by TM. If this mechanism of DNA damage recognition is also valid in vivo, we would expect a higher incidence of lesions to be detected in nontranscribed regions of the genome. To test this hypothesis, we performed a long amplicon analyses on genomic DNA isolated from cecal enterocytes of infected mice. Indeed, the lesion burden from cecal enterocytes of infected mice (24 h) showed tail DNA, tail length, and tail moment were significantly different when mice were colonized with K. oxytoca AHc6-6 (WT) compared with the ΔnpsB-mutant. Bars represent medians of each dataset (n = 9 mice, with ≥50 cells per mouse), and significance was determined with Mann–Whitney test (*P ≤ 0.05).
Tubulin Is the Molecular Target of TV. The results above indicate that TV has neither DNA-binding nor genotoxic activity, as expected. Exposure to TV blocked cell cycle progression at G2/M phase (Fig. 2). TV also impeded closure of a scratch created in a monolayer of HeLa cells (SI Appendix, Fig. S4). Considering that microtubules form the mitotic spindle, constitute the cytoskeleton, and facilitate cellular movement, we asked whether TV inhibits microtubule-dependent processes. To observe the effects of TV on the microtubule network, A549 lung carcinoma cells and HT-29 colon cancer cells were exposed to TV and stained with anti-tubulin or a spindle pole marker (Fig. 5A and SI Appendix, Figs. S5 A and B). Microscopy revealed aberrant spindle morphologies and micronucleation in A549 cells treated with TV, compared with solvent, in a manner resembling the effects of the microtubule-stabilizing drug paclitaxel (PTX) (Fig. 5A, 1–8). TV induced formation of abnormal type II bipolar spindles with atypically short distances between poles and poor DNA alignment at the metaphase plate (Inset 2). With increasing concentrations of TV, type III tri- and multipolar spindles (small star-shaped aggregates of microtubules and a ball of DNA) appeared (Insets 3–7) and the number of micronucleated cells increased (3–7, filled arrows). PTX-induced type III spindles are mostly monopolar and with more dense star-shaped microtubules (inset 8). Interphase microtubules of TV-treated cells acquired a straight, parallel orientation (4, 5) different from that of control cells. Loosely packed bundles appeared in some cells (4, 6, and 7, open arrows) in contrast to more abundant and compact bundles in cells treated with PTX (8, open arrow). Similar results were obtained for HT-29 cells (SI Appendix, Fig. S5 A and B). These results imply that TV is a microtubule-stabilizing agent.

To test whether TV binds tubulin directly, we assayed polymerization of purified αβ-tubulin heterodimers in vitro in the presence of TV, or control substances: PTX, the destabilizing drug nocodazole, and TM. TV but not TM stimulated the formation of polymers in a manner resembling PTX. This activity for TV was confirmed with independent protein preparations and different assay conditions (Fig. 5B and SI Appendix, Fig. S5C). Taken together, the data indicate that the TV effect on polymerization manifests at the nucleation phase, which occurs faster with TV than in buffer alone, and in the total amount of microtubules accumulated at the plateau phase. Transmission electron micrographs of reaction products verified that the increased absorbance we measured was due to formation of microtubules, not other polymers or aggregates (Fig. 5C). We conclude that TV increases polymerization of tubulin into microtubules.

To explore the mechanism underlying this activity, we investigated the stoichiometry of TV in the polymer. Polymerization products were fractionated by centrifugation, and the proportion of TV cosedimenting with microtubules vs. the fraction remaining in the supernatant was determined by HPLC-UV/VIS analysis. In this experiment, we also varied the nucleotide content of tubulin. The protein is active for microtubule assembly in the GTP-bound state, but hydrolysis to GDP results in an inactive conformation; thus, the GTP-like state of tubulin is stabilized by PTX by preferential binding of PTX to assembled tubulin with a 1:1 stoichiometry. The properties of TV-tubulin interactions observed here provide strong evidence that TV and PTX stabilize microtubules by
different mechanisms. If so, we would also expect tumor cells that have acquired resistance to PTX, due to mutations in β-tubulin affecting drug binding, to remain susceptible to TV. Indeed, two PTX-resistant ovarian cancer lines (35), 1A9PTX10 and 1A9PTX22, were equally sensitive to TV (IC_{50} = 4.5 and 1.9 μM, respectively) as the parental line 1A9 (IC_{50} = 3.6 μM), whereas higher doses of PTX were required to inhibit growth of 1A9PTX10 (IC_{50} = 29.9 nM) and 1A9PTX22 (IC_{50} = 30.9 nM) compared with 1A9 (IC_{50} = 2.7 nM).

**Cytotoxicity and AAHC.** The predominant histopathological feature of AAHC in humans and animal models is epithelial alterations characterized by increased apotosis (Fig. 6A) and mucosal hemorrhage. Our earlier work with TV showed that cellular exposure induces apotosis (17). Relative abundances of the enterotoxins measured in patient and mouse samples in this study imply a predominant role for TM in pathogenicity. We therefore measured caspase 3/7 activity in SW48 cells treated with TM (Fig. 6B), confirming that the metabolites act independently to induce apoptotic cell death. Induction of cellular apoptosis involved loss of the prosurvival Mcl-1 protein (Fig. 6C). Degradation of Mcl-1 was observed following exposure to each enterotoxin as well as the apoptosis inducer PTX. Nonetheless, the molecular targets we identified for the enterotoxins support the prediction that the mechanisms leading to apotosis will differ.

To assess whether tumor suppressor p53 (TP53) gene expression was up-regulated upon exposure to the cytotoxins, we measured total p53 protein with SW48 cells, since these carry the wild-type TP53 gene. Total p53 protein was elevated through treatment with DNA-reacting agents TM and GWL-78 (Fig. 6D). In a cascade of downstream reactions, p53 can activate a large number of genes including genes encoding DNA damage recognition components of NER. Activation of p53 for a DNA damage response involves phosphorylation of Ser15. Activation at this specific residue was indeed detected in response to both DNA-reactive substances TM and GWL-78, but not by the tubulin inhibitor TV (Fig. 6D). This result strengthens the notion that the cellular responses to the *K. oxytoca* enterotoxins trigger distinct pathways leading to cell death.

**Discussion**

Functional characterization of intestinal metabolites and signals in host-microbiota-pathogen interactions is a difficult challenge, but also indispensable in understanding host-microbe interactions relevant for the development of intestinal disease. This study shows that the distinct chemistries of the *K. oxytoca* enterotoxins alter their molecular targets and confer different functionalities. The PBD ring system of TM mediates interactions with DNA, which are blocked by the indole substituent in TV. TM showed activities in vitro characteristic for the DNA-reactive PBD family: duplex stabilization and endonuclease protection, induced DNA-reactive substances TM and GWL-78, but not by the tubulin inhibitor TV (Fig. 6D). This result strengthens the notion that the cellular responses to the *K. oxytoca* enterotoxins trigger distinct pathways leading to cell death.

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Despite low $\beta\leq$ during homeostasis. In vitro effects of TM and TV match features of AAHC. (4). Kruskal www.pnas.org/cgi/doi/10.1073/pnas.1819154116

In the microtubules (42). Notably, the bound fraction of TV in- the polymer. This quantitative relationship differs strikingly from presence of TV contain a small fraction of the compound bound to taxane or peloruside site (41). Microtubules assembled in the within one of two distinct nonoverlapping sites on tubulin: the microtubule-stabilizing compounds, bacteria are less common pro-

That, although plants and marine sponges are a rich source of produce TV is an unusual feature of this system. Conversion of that TM-mediated antagonism of microbial competitors may prolong bacterial persistence. Unlike colibactin, TM release does not require host cell contact. (50) proposed that colonizing microbiota-derived small-molecule genotoxins produced by select members of the Enterobacteriaceae (46–49). In contrast to TM's DNA-damaging activity, alkylation of DNA by colibactin induces acute DNA double-strand breaks and megalocytosis in eukaryotic cells (50). Incomplete repair of host DNA damage following infec-

As a result, assembly would be favored, as we observed in the presence of TV. This finding points to a mechanistic hypothesis by which TV could be an interfacial ligand of the longitudinal interface which TV could be an interfacial ligand of the longitudinal interface for total Mcl-1 normalized to $\mu$M staurosporin (ST), and mice colonized with $K. oxytoca$ (AHC-6). Bars indicate medians ($n = 8$). Kruskal–Wallis test followed by Dunn's multiple comparison ($P \leq 0.05$). (B) Percent caspase 3/7-positive SW48 cells without (−) or after 24-h treat-

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Our findings showing that TM and TV are produced in the hu-

Fig. 6. In vitro effects of TM and TV match features of AAHC. (A) histo-

Third, given the potential harm done to the host, we also wonder why gut residents produce genotoxins. Nougayrède et al. (50) proposed that colonizing $E. coli$ may use colibactin to slow the renewal of enterocytes by blocking the cell cycle, thereby prolonging bacterial persistence. Unlike colibactin, TM release does not require host cell contact. $K. oxytoca$ that secrete TM did not show a colonization advantage over the mutant strain during antibiotic induced dysbiosis (17). However, the antibacterial activity toward other gut residents shown in this study implies that TM-mediated antagonism of microbial competitors may confer the real advantage to $K. oxytoca$ during homeostasis.

Finally, the intrinsic reactivity of TM with available indole to produce TV is an unusual feature of this system. Conversion of TM to TV repurposes the molecule, alters its cellular effects on the host, and presumably mediates a different spectrum of microbe–microbe interactions within this niche. In conclusion, the $K. oxytoca$ enterotoxin system contributes remarkable functional versatility to this organism's activities as a pathobiont of the human intestine.
Methods

Human Colonic Luminal Fluid and Stool Samples. A 29-y-old female patient (A) suffering from systemic lupus erythematosus was treated with ampicillin/sublactam for pneumonia and desibuprofen for arthralgia. After 12 d of antibiotic therapy, she developed severe abdominal pain and hematochezia. Intestinal ultrasound and colonoscopy showed severe segmental hemorrhagic colitis starting in the descending colon. Symptoms improved 2 d after stopping the antibiotic therapy, and the colitis subsided completely after 10 d without additional specific therapy. Stool culture was negative for Clostridium difficile, Yersinia, EHEC, Shigella, Campylobacter, and Salmonella species. Patient B, a 32-y-old female, and patient C, a 35-y-old female, were treated with amoxicillin/clavulanate in addition to nonsteroidal antiinflammatory drugs for sinusitis and as antibiotic prophylaxis after orthognathic surgery, respectively. Both patients developed bloody diarrhea with abdominal cramps and were diagnosed with acute AAHC by colonoscopy. Colitis subsided after discontinuation of the triggering antibiotic therapy. K. oxytoca isolates from patients A, B, and C carry the enterotoxin gene cluster as determined by PCR and were positive for cytotoxicity. Colonic luminal fluids aspirated during diagnostic colonoscopy and stool samples were stored at −20 °C. The protocol for sample acquisition was approved by the ethics committee of the Medical University of Graz (17-199 ex 05/06) and patients’ informed consent was obtained.

Mouse Infection Models. Animal experiments were performed as previously described (17). Adult female C57BL/6NCrl mice with SOPF status (Charles River; Janvier Labs) were housed in individually ventilated cages. Studies were performed in accordance with the Commission for Animal Experiments of the Austrian Ministry of Science (GZ 66.007/0006-II/3b/2011 and GZ: BMVFW-66.007/0002-WVF3/3b/2017) and the local ethics committee.

For the AAHC model, 8-wk-old mice of treatment groups received Curam (amoxicillin/clavulanate, 2,000/200 mg; Sandoz) 100 mg/kg/treatment intraperitoneally twice daily at t = 0, 8, 24, 32, 48, 56, and 72 h. Mice were infected intragastrically three times (t = 0, 24, and 48 h) with 1 × 10^7 cfu of K. oxytoca AHC-6 (with chronosomally integrated kanamycin resistance marker) resuspended in 100 μL of LB broth. At day 5, the mice were killed by cervical dislocation, and the entire intestinal tract was removed.

For short-term colonization experiments, 8- to 13-wk-old mice were administered amoxicillin (0.4 mg mL⁻¹; Genaxxon Bioscience) in drinking water and mice of treatment groups were infected intragastrically once with 1 × 10^7 cfu of K. oxytoca AHC-6 or ΔnpsB.

Cell Cycle and Test Substances. The HAP1 cell line is a derivative of near-haploid leukemia cell line KBM7 that was reprogrammed to adherent growth. The HAP1 and mutant derivatives were grown in Iscove’s modified Dulbecco’s medium containing l-glutamine and 25 mM Hepes, pH 7. HeLa cells were cultured in DMEM and T84, HT-29 (CLS Cell Lines Service), and A549 cells in a 1:1 mixture of Ham’s F-12 and DMEM supplemented with 2 mM glutamine and 1 mM sodium pyruvate. Endothelial cell growth medium was cultured in minimum essential Eagle medium supplemented with 2 mM glutamine and 1 mM sodium pyruvate. HeLa cells were cultured in DMEM and T84, HT-29 (CLS Cell Lines Service), and A549 cells in a 1:1 mixture of Ham’s F-12 and DMEM supplemented with 2 mM glutamine and 1 mM sodium pyruvate. Endothelial cell growth medium was used for the cultivation of human umbilical vein endothelial cells (PromoCell). SW48 were grown in McCoy’s 5A (modified) medium. All media were supplemented with 10% FBS and 100 μg mL⁻¹ penicillin/streptomycin (Gibco). Cell lines were obtained from ATCC (if not stated otherwise) and incubated at 37 °C with 5% CO₂ in 95% humidity.

Butanol solutions of synthetic TM, and DMSO solutions of synthetic TV, GWL-78, PTX, nocardazole, staurosporin, and illudin S were stored at −20 °C.

Metabolite Analysis. Human stool samples and cecal contents and feces of mice and control mice spiked with TM and TV were mixed 1:1 (w/v) with n-butanol for 5 min, centrifuged [16,000 × g, 15 min, room temperature (RT)] and filtered (nylon, 0.2 μm). Butanol extracts of mouse cecal contents from the AAHC model were mixed with “Silica Gel 60” (particle size, 40–63 μm; Merck) and loaded into a Pasteur pipette containing glass wool. All extracts were prepared with MeOH (HPLC grade) and CHCl₃ (99.2%, stabilized with 0.6% EtOH). The crude extracts were treated with 30 μL of MeOH, mixed thoroughly by vortexing (20 s), and ultrasonicated (3 min, 22 °C) until homogeneity. The mixtures were applied on prepared silica columns (height, 2 cm; conditioned with CHCl₃/MeOH, 1:1 (vol/vol)) and allowed to enter the pad via gravity. The pad was carefully eluted with portions of CHCl₃/MeOH (5:1, (vol/vol) 5 × 50 μL): the combined extracts were concentrated by reversing the solvents (22 °C, 60 min) and dried on the rotary evaporator (8 mbar, 40 °C, 8 min). The samples were stored at −18 °C.

Human colonic luminal fluid from patient A was centrifuged (4,000 × g, 30 min, RT). Supernatant was filtered (0.45 and 0.22 μm), mixed 1:1 (vol/vol) with n-butanol by vortexing (30 s), and centrifuged (10,000 × g, 5 min, RT). Colonic luminal fluids of patients B and C were mixed 1:1 (vol/vol) with n-butanol, vortexed for 5 min, and centrifuged (16,000 × g, 15 min, RT). Supernatants were filtered (0.22 μm). The organic phase was concentrated to dryness via vacuum centrifugation at 45 °C. Dried extracts were stored at −20 °C. Colonic fluid extract of patient A was purified before measurement. The extract was dissolved in 200 μL of MeOH and applied to a preparative TLC plate (silica gel; #2513040; 1,500 μm; 20 × 20 cm). The plate was used evolving using CHCl₃/MeOH (=20:1) (vol/vol), and then air-dried. The silica gel in the region Rᵣ = 0.1–0.5 was scraped into 150 mL of MeOH. The mixture was stirred for 20 min at 22 °C and filtered through a cellulose filter. The pad was rinsed with 100 mL of MeOH, and the solvent was removed under reduced pressure.

HPLC–High-Resolution–ESMS. For HPLC–high-resolution (HR)-ESMS analysis, samples were re-extracted in MeOH (300 μL), transferred to sterile autosampler vials (polypropylene, 250 μL), and held at 20 °C in the autosampler before measurement. HR-ESMS applies an atmospheric pressure electrospray ionization source (ES) using nitrogen as nebulizer and drying gas. Measurements were performed in positive ionization mode, with a drying gas temperature of 440 °C, spray voltage of 3.5 kV, and a resolution of 70,000 (FWHM). The HPLC system was equipped with a Shodex Axashpak A or a ODP-50 column (4 × 125 mm; 5 μm particle size) using mobile phases A [water including 0.1% formic acid (vol/vol) and B (acetonitrile)] under gradient elution conditions: 0–10 min, 10–12.5% B; 10–10.5 min, 12.5–30% B; 10.5–15 min, 30% B; 15–25 min, 30–50% B; 25–30 min, 50% B; 30–30.5 min, 50–10% B; and 30.5–35 min, 10% B. Lons were recorded as protonated [M+H⁺] form in single-ion monitoring mode and masses set to m/z: 225.1077 (TM) and 334.1550 (TV). Column temperature was set to 30 °C; flow rate, 0.5 mL min⁻¹; and injection volume, 10 μL.

Cytotoxicity Assay. Cell survival was measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining after 48-h incubation with TM and TV, as previously described (17).

Agar Diffusion Assay. Cell suspensions (0.5 McFarland) were spread on Columbia Blood Agar and dried for 15 min. Antimicrobial discs were soaked with 10 μL (40 μg) of TM, TV, or solvents, dried, and transferred to the inoculated agar plates. The inoculated plates were incubated at 37 °C for 24–48 h under aerobic or anaerobic conditions.

Cell Cycle Analysis. Cells were harvested and washed twice with ice-cold PBS. The pellet was resuspended in 500 μL of PBS, and 5 mL of 70% ethanol (~20 °C) was added dropwise under constant shaking. Fixed and permeable cells were centrifuged (720 × g, 4 min), washed with PBS containing 0.5% FBS, and then resuspended in 200 μL of Pi-hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100, 100 μg mL⁻¹ RNase A (Fermentas), 50 μg mL⁻¹ PI (Sigma Aldrich)) and incubated for 20 min at RT in the dark. Equivalent numbers of cells (events) were then sorted according to the PI signal strength via FAC (BD LSR II Flow Cytometer; BD Biosciences). BD FACSDIVA software (version 8) and ModFit LT software (version 4 and 5) were used to analyze and plot the data.

Thermal Denaturation. To generate a DNA template, complementary primers (5′-CGATAAACATTCGTTTGGGACCAAGGTCATTGGGAATACTGCGCAAAATG-TGATAG-3′) were heated 10 min at 90 °C, and then slowly cooled (1 h) to RT. Ten micromolar dsDNA in 10 mM Tris HCl, pH 7.5, 10 mM NaCl, and 1 mM MgCl₂ was incubated with 10 μM TM, TV, GWL-78, and solvents at 37 °C for 18 h. One hour before analysis, DNA was incubated with SYBR Green at 37 °C. Thermal denaturation was performed from 20 to 90 °C with a ramping of 0.5 °C. Absorbance was measured at 260 nm on a CFX Real-Time PCR Detection System (Bio-Rad) in quadruplicates in three independent experiments.

Endonuclease Inhibition. DNA substrate (1,740 bp) containing a single BamHI and SspI recognition sequence was generated by PCR. Five hundred nanograms of
DNA were incubated with test substances or solvents for 16 h at 37 °C in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Treated DNA was incubated with 20 U of endo-
nuclease for 3 h at 37 °C, and then resolved electrophoretically. Nucleolytic activity was calculated as intensity of the cut DNA fragment normalized to the intensity of all bands visible and expressed as inhibition percentage.

Isolation of Enterocytes. Mice were colonized with K. oxytoca for 24 h, and ceca were extracted, washed with ice-cold PBS, and inverted. Epithelial cell suspension was obtained after 30-min incubation in PBS with 5 mM EDTA. Cells were dissociated by incubation at 37 °C for 20 min in TrypLE Express Enzyme (Thermo Fisher Scientific). In total, 1 × 10^6 cells were pelleted.

Single-Cell Electrophoresis—Comet Assay. After 4-h treatment with TM, TV, or control substances, cells were harvested by gentle scraping, then pelleted (700 × g, 2 min, 4 °C), washed, and resuspended in ice-cold PBS to obtain 1 × 10^6 cells/mL. Cells were carefully mixed with agarose at a 1:10 ratio (vol/vol) and 75 μL/well were immediately transferred onto the comet slide and analyzed following the manufacturer’s instructions (OxiSelect comet assay kit; Cell Biols). Tail length, tail DNA, and tail moment, defined as tail length times tail DNA, were calculated with OpenComet software.

Sensitivity of DNA Repair-Deficient Mutants. Cells were treated with TM, TV, ilinu5, or solvent controls. For dose–response curves, cell viability after 48 h was determined using the CellTiter-Blue (Promega). All wash buffer used was replaced with 50 μL of reagent, plates were shaken for 30–45 min in darkness, and luminescence was detected on a plate reader. Values were plotted by sigmoidal curve fitting.

Long Amplicom PCR. gDNA was isolated from 1 × 10^6 cecum cells with the Qiagen Genomic-tip 20/G Kit using the Qiagen Genomic DNA buffer set according to the supplier instructions (OxiSelect comet assay kit; Cell Biols). Tail length, tail DNA, and tail moment, defined as tail length times tail DNA, were calculated with OpenComet software.

Transmission Electron Microscopy. HT-29 cells grown on coverslips were fixed with methanol-free 4% paraformaldehyde, postfixed with 1% osmium tetroxide, and stained with 0.1% uranyl acetate in 30% ethanol. The samples were dehydrated in an ascending ethanol series and embedded in Epon-araldite. Ultrathin sections were stained with lead citrate and examined under a transmission electron microscope at 80 kV.

Signaling Technology, cells were washed 3 × 5 min in PBS, and then incubated with anti-rabbit IgG (H+L), (Fab')2 fragment (Alexa Fluor 488 conjugate) for 2 h in the dark. After washing 2 × 5 min in PBS, DAPI staining followed (15 min). The washed coverslips were embedded in Mounting Medium (Roth). A Nikon Inverted Microscope Eclipse Ti-E/B with confocal laser scanning was used to analyze the samples at different magnifications, and images were edited with the Fiji software (ImageJ).
Caspase 3/7 Flow Cytometry. The CellEvent Caspase-3/7 Green Flow Cytometry Assay Kit containing the SYTOX AAADVanced dead cell stain (Thermo Fisher Scientific) was used to determine Caspase-3– or -7–positive cells and exclude necrotic cells. Cells were grown and harvested as described in Methods. Cell Cycle Analysis, washed with ice-cold PBS, and incubated with CellEvent Caspase-3/7 Green Detection Reagent for 25 min at 37 °C. Staining with SYTOX AADvanced dead cell stain solution for 5 min at 37 °C followed. Cells were dead and used using 488-nm excitation and applied standard fluorescence compensation on a BD LSR II Flow Cytometer. Fluorescence emission with 530/30 BP (Caspase-3/7) and 690/50 BP (dead cell stain) filters or their equivalents was used.

Quantification and Statistical Analysis. Protein and DNA signals were quantified with the Image Lab Software (Bio-Rad). Significance (*p ≤ 0.05) was determined with statistical tests (in GraphPad Prism) specified in figure legends. Values for substances were compared with respective solvents, and n represents the number of independent experiments performed, if not stated otherwise.

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