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Macrocyclic colibactin induces DNA double-strand breaks via copper-mediated oxidative cleavage

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Abstract

Colibactin is an assumed human gut bacterial genotoxin, whose biosynthesis is linked to clb genomic island that distributes widespread in pathogenic and commensal human enterobacteria. Colibactin-producing gut microbes promote colon tumor formation and enhance progression of colorectal cancer via DNA double-strand breaks-induced cellular senescence and death; however, the chemical basis contributing to the pathogenesis at the molecular level has not been fully characterized. Here we report the discovery of colibactin-645 a macrocyclic colibactin metabolite that recapitulates the previously assumed genotoxicity and cytotoxicity. Colibactin-645 shows strong DNA DSBs activity in vitro and in human cell cultures via a unique copper-mediated oxidative mechanism. We also delineate a complete biosynthetic model for colibactin-645, highlighting a unique fate of the aminomalonate building monomer in forming the C-terminal 5-hydroxy 4-oxazolecarboxylic acid moiety through the activities of both the polyketide synthase
ClbO and the amidase ClbL. This work thus provides a molecular basis for colibactin’s DNA DSBs activity and facilitates further mechanistic study of colibactin-related CRC incidence and prevention.

Graphical Abstract

Human microbiota is a massive consortium of all microbes that reside in and on human bodies. These microbes are increasingly being correlated to human health and disease, but the underlying molecular mechanisms of human-microbe interactions often remain elusive\textsuperscript{1,2}. Interrogating the specialized metabolites produced by human microbiota allows a thorough study of chemical regulatory and signaling processes, and improves our understanding of the interplay between microbiota and host at a molecular level. Despite the importance of these small molecules in human health and disease, it is often challenging to characterize them because of the difficulty in the culture and genetics of producing microbes and the low titers of these metabolites\textsuperscript{3–5}.

A well-known example of such specialized metabolite is colibactin, a cryptic human gut bacterial genotoxin that has captured the attention of both biologists and chemists due to its significant effects on human health and intriguing biosynthetic logic\textsuperscript{6–8}. The biosynthesis of colibactin is linked to a 54-kilobase nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) hybrid gene cluster\textsuperscript{9} (clb pathogenicity island, Supplementary Fig. 1), which has been phenotypically associated with the pathogenesis of colorectal cancer (CRC)\textsuperscript{9–15}. In particular, \textit{in vitro} infection with \textit{Escherichia coli} strains harboring \textit{clb} induced DNA double-strand breaks (DSBs) in cultivated human cells, leading to cell cycle arrest and eventually cell death\textsuperscript{9}. Subsequent physiological studies showed that \textit{clb} bacteria induced \textit{in vivo} DNA damage and genomic instability in enterocytes\textsuperscript{10}, caused cellular senescence\textsuperscript{11,12}, increased intestinal permeability\textsuperscript{13}, and promoted colon tumor formation in mouse models of chronic intestinal inflammation\textsuperscript{12,14,15}, suggesting that these bacteria could promote human CRC development on a broader level\textsuperscript{8}. Consistently, \textit{clb} \textit{E. coli} was over-represented in biopsies isolated from CRC patients compared to non-CRC controls (~60\% vs. ~20\%, respectively)\textsuperscript{14,16}. In addition to its remarkable association with human health, the \textit{clb} island was also identified in the genomes of other proteobacteria, including coral and honeybee symbionts, suggesting an even more comprehensive role that colibactin might play in mediating evolutionarily conserved or consistent interactions between bacteria and hosts\textsuperscript{17,18}.
Given the physiological importance of intestinal pathology induced by human body’s microscopic residents, it is urgent to reveal the molecular identity of genotoxic colibactin as the missing link between certain gut microbes and DNA DSBs and decode the mechanism underlying colibactin-induced DNA damage. Despite tremendous efforts, colibactin’s structural elucidation remains a formidable challenge due to its instability, low titer, and the elusive and complex biosynthetic logic of clb pathway. This knowledge gap has prevented comprehensive studies of colibactin-related CRC incidence and prevention, and limited mechanistic investigations of even more extensive influence of clb island on microbe-host interactions.

In order to investigate the corresponding genotoxic colibactin that possesses intrinsic DNA DSBs activity and causes chromosome aberrations, the following three issues need to be addressed. 1) The mutation of individual clb genes revealed that all genes encoding NRPS-PKS and associated biosynthetic enzymes were indispensable to the genotoxicity of clb island, however, the colibactin metabolite that requires all of the clb genes for its biogenesis has not been identified. 2) The precise role of ClbP, a membrane-bound peptidase that was proposed to be important for colibactin maturation, remains unknown. 3) The induction of DNA DSBs has been defined as a signature feature of clb island, yet the conclusive evidence for colibactin directly mediating DNA breakage is still lacking, despite that precolibactin-546 (5) showed a weak DNA crosslinking activity in vitro in the presence of reducing agents (Fig. 1a). Of the many types of DNA damage that exist within cells, the DNA DSBs are considered to be the most hazardous lesions, suggesting the remarkable cytotoxicity of the yet-to-be-identified colibactin metabolite that exerts a direct DNA DSBs activity. Here we report the structural elucidation of a new mature macrocyclic colibactin metabolite, and further show that the macrocyclic colibactin induces DNA DSBs in vitro and in various human cell cultures via a unique copper-mediated oxidative mechanism.

Results

Discovery of complete colibactin precursor

Our previous efforts to identify colibactin biosynthetic intermediates resulted in the structural elucidation of precolibactin-886 (10), which was isolated from a clb+ heterologous expression strain E. coli DH10B/pCAP01-clb with disrupted clbP and clbQ that encode a peptidase and a type II thioesterase mediating the off-loading of clb pathway intermediates, respectively. The double mutation of ΔclbPΔclbQ increased the titer of downstream metabolites from the NRPS-PKS assembly line, enabling the structural characterization of 10 whose biogenesis requires all components of the megasynthase assembly line except the PKS ClbO. We then searched for a more complete colibactin derivative that could account for the activity of ClbO. The initial examination of the ΔclbPΔclbO mutants for the selective loss of metabolites identified a precolibactin metabolite with m/z 970 (named precolibactin-969, 11) in a trace amount (Fig. 1, Fig. 2a). To facilitate the structural elucidation of 11, additional regulatory/resistance clb genes including clbR and clbS were explored to probe their effects on the production of 11. ClbR is a known positive transcriptional regulator and its overexpression
previously led to a five-fold increase in the prodrug motif accumulation\textsuperscript{22}, and ClbS is a colibactin resistance protein that was proposed to sequester or modify colibactin and thereby prevent self-inflicted DNA damage\textsuperscript{32,33}. While overexpression of \textit{clbR} had no obvious effect on the titer of \textbf{11}, inactivation of \textit{clbS} resulted in a notable four-fold increase in the titer of \textbf{11} along with other precolibactins (Fig. 2a, Supplementary Fig. 2). The observed eliciting phenomenon in \textit{ΔclbS} is consistent with the proposed function of ClbS, and we thus used the \textit{ΔclbPΔclbQΔclbS} mutant strain for the subsequent precolibactin production and purification.

From a 2,000-L fermentation culture of \textit{ΔclbPΔclbQΔclbS}, 50 μg of \textbf{11} was obtained after extraction with organic solvent followed by multiple rounds of reversed-phase liquid chromatography purification. \textbf{11} was isolated as white and amorphous powder, and its molecular formula was determined as C\textsubscript{44}H\textsubscript{59}N\textsubscript{9}O\textsubscript{12}S\textsubscript{2} by high-resolution mass spectrometry (HRMS) (m/z 970.3799, calculated: 970.3797) (Supplementary Fig. 3), which has an additional C\textsubscript{3}HNO\textsubscript{2} compared to the formula of \textbf{10}. The presence of an extra nitrogen atom in \textbf{11} is consistent with the known aminomalonate substrate utilization by ClbO\textsuperscript{26,27}, which was also supported by the isotope-labeled precursor feeding experiments, suggesting the incorporation of an additional aminomalonate compared to \textbf{10} (Supplementary Fig. 4). Similar to \textbf{10}, \textbf{11} was isolated as an approximately equal mixture of two diastereomers (Supplementary Fig. 5). Analysis of extensive nuclear magnetic resonance (NMR) spectra and high-resolution tandem mass spectrometry (HRMS\textsuperscript{n}) fragmentation data demonstrated that \textbf{11} and \textbf{10} share the same macrocyclic scaffold from C-1 to C-40 (Fig. 1, Supplementary Text, Supplementary Figs. 6 and 7, and Supplementary Table 1), indicating that ClbO functions towards the end of the NRPS-PKS assembly line to incorporate the last building monomer of aminomalonate. However, we were not able to assign the structure of this extra region (C-41 to C-44) based on the NMR spectra due to the apparent proton deficiency feature and the extremely low titer of \textbf{11} at this stage.

We then turned to the PKS activity of ClbO to predict the fate of the corresponding aminomalonate unit. In the \textit{clb} locus, two PKS modules, Clb\textsubscript{KPKS} and ClbO, were enzymatically established to incorporate an aminomalonate extender unit\textsuperscript{26,27}. Both PKS modules have domains organized into KS-AT*-ACP (Fig. 1b). A maximum likelihood tree revealed a close phylogenetic relationship between these two KS domains (Supplementary Fig. 8), suggesting a similar activity of Clb\textsubscript{KPKS} and ClbO. While Clb\textsubscript{KPKS} was shown to incorporate aminomalonate through a decarboxylative Claisen condensation in forming \textbf{10} (Fig. 2b), this reactivity does not account for the addition of three carbon atoms promoted by ClbO in forming \textbf{11}. Considering the typical observation that the titers of upstream colibactin metabolites were significantly higher than those of downstream metabolites\textsuperscript{25,28}, we searched for a possible intermediate that is stalled at Clb\textsubscript{KPKS} with an additional of C\textsubscript{3}HNO\textsubscript{2} in its molecular formula compared to precolibactin-712 (\textbf{7}) to facilitate the total structural determination of \textbf{11} (Fig. 1). Careful analysis of the culture extracts of \textit{ΔclbPΔclbQΔclbSΔclbO} revealed a new metabolite (named precolibactin-795a, \textbf{8}) with the molecular formula of C\textsubscript{39}H\textsubscript{53}N\textsubscript{7}O\textsubscript{9}S\textsubscript{1} (m/z 796.3697, calculated 796.3698) (Fig. 2c, Supplementary Fig. 9). A total of 1.1 mg of \textbf{8} from a 500-L fermentation culture were obtained and extensive analysis of the NMR spectra and HRMS\textsuperscript{n} fragmentation data.
indicated that in comparison with 7 and precolibactin-795b (9), 8 contains a unique 5-hydroxy oxazole moiety next to the terminal carboxyl group (Fig. 1, Supplementary Text, Supplementary Figs. 10–12, and Supplementary Tables 2 and 3). We propose that to assemble 8, the aminomalonate unit is incorporated into the assembly line through nucleophilic attack of the amine in the aminomalonate extender unit on the upstream peptidyl-S-T thioester of ClbJ, followed by synchronous cyclization and release (Fig. 1, Fig. 2d). This novel biosynthetic logic of accommodating a rare aminomalonate building block was further supported by the gene inactivation and isotope labeled precursor feeding experiments (Fig. 2c, Supplementary Fig. 4). We thus deduce that 11 contains the same 5-hydroxy oxazole moiety next to its terminal carboxyl group, which is derived from the aminomalonate extender unit of ClbO and formed through the same chemical logic as in 8 (Fig. 1, Fig. 2e). Furthermore, a precolibactin metabolite (precolibactin-943, 12) with m/z 944 corresponding to the decarboxylative condensation activity of ClbO was also observed (Supplementary Fig. 13), but its titer was only approximately 10% of that of 11. Additional gene inactivation experiments showed that ClbL, a putative amidase, was required for the biosynthesis of 8 and 11, but not 9, 10 and 12, suggesting that ClbL promotes the nucleophilic attack of the amine in the aminomalonate extender unit (Fig. 1, Fig. 2).

**Maturation of colibactin**

Precolibactin-969 (11) is hitherto the largest colibactin derivative that requires all components of the NRPS-PKS assembly line for its biosynthesis. We next examined whether ClbP, the dedicated peptidase for colibactin maturation, is capable of hydrolyzing this precursor in the bacterial periplasm and releasing the mature colibactin (Fig. 3a). Incubation of 11 with the culture of E. coli expressing ClbP resulted in the complete loss of 11 and the production of both the prodrug motif N-myristoyl-D-asparagine (14) and a new metabolite (named colibactin-645, 13) with the molecular formula of C_{26}H_{27}N_{7}O_{9}S_{2} (m/z 646.1394, calculated 646.1384) (Fig. 3, Supplementary Fig. 14). 13 was confirmed to be the mature compound of 11 with a free N-terminus after cleavage and release of the prodrug motif based on the comparative HR-MS/MS analysis (Supplementary Fig. 14). It is notable that different from 11 and 14, 13 is a very water-soluble compound which could not be extracted by typical organic solvents such as ethyl acetate\(^{21,24}\). Additionally, we observed a significantly increased recovery yield of 13 from the ClbP-expressing E. coli culture upon treatment of metal chelators, such as ethylenediaminetetraacetic acid (EDTA) and Chelex-100 (Fig. 3b). The positive effect of metal chelators on metabolite yields from E. coli cultures was also observed for 11, but not for other precolibactins such as 2, 5, and 7 (Supplementary Fig. 15). These results suggested the susceptibility of colibactin-645 (13) and its precursor (11) to trace metals for possible degradation.

**Colibactin production by a native strain**

We next investigated whether the native clb\(^{+}\) E. coli strain could produce the same colibactin-645 to probe if 13 was a native metabolite or an artifact arising from a non-natural biosynthetic pathway in a heterologous host. LC–MS analysis of cell-free culture extracts of the wild-type clb\(^{+}\) E. coli CFT073 and its clb\(^{-}\) mutant revealed a peak identical to 13 only in the wild-type clb\(^{+}\) strain, confirming that 13 is the native product of the clb pathogenicity

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island (Fig. 3b). It is notable that after enrichment from a 2-L of fermentation culture, only a trace amount of 13 was detected by HRMS analysis, indicating the low titer of 13 or its chemical lability. Since previous work showed that direct contact between bacterial and eukaryotic cells was required for full toxicity of colibactin, we further examined whether a majority of 13 are associated with the producing cells. 13 was not detected in the cellular extract of clb+ E. coli CFT073 (Fig. 3b), suggesting that the mature colibactin was secreted after production and highly unstable after secretion.

**DSBs activity of colibactin in vitro**

After obtaining the highly sought mature colibactin (13), we examined its DNA DSBs in vitro using the pBR322 plasmid DNA strand scission assay, a surrogate test for DNA damage. Although 13 showed sparse DNA damage activity upon incubation with DNA, in the presence of Cu(II), but not other metals such as Fe(III) and Fe(II), both 13 and its precursor precolibactin-969 (11) caused significant DNA breakage with the formation of both nicked (Form II) and linearized (Form III) DNA from the supercoiled plasmid DNA (Form I) (Fig. 4a, Supplementary Fig. 16). Since 13 and 11 demonstrated comparable DNA damage activities in initial tests, we used 11 as an appropriate substitute for 13 in the following in vitro assays because 11 was more readily available. A time-course experiment of DNA cleavage was then performed to determine if the colibactin-induced linearized DNA arose from coupled strand-cleavage events (DSBs), or from an accumulation of unrelated single-strand breaks (SSBs). All three forms of DNA were visible on the gel, showing classical evidence of DSBs (Fig. 4b). A Freifelder–Trumbo analysis was further performed to calculate the number of SSBs ($n_1$) and DSBs ($n_2$) per molecule of DNA after treatment with 11 at various time points, which resulted in a constant ratio of SSBs to DSBs (5.35:1) (Supplementary Fig. 17). This number is significantly lower than 120:1 that was expected if DSBs were to arise from an accumulation of unrelated SSBs, and is comparable to some of the well-known DNA DSBs inducers including (-)-lomaiviticin A (5.3:1) and bleomycin (9:1), supporting the coupled strand-cleavage activity of colibactin. It is notable that under the same reaction condition, 11 displayed a stronger DNA DSBs activity than bleomycin which also requires the presence of a redox-active metal ion for DNA cleavage (Supplementary Fig. 18).

The observed Cu(II)-mediated DSBs activity of colibactin is reminiscent of the oxidative mechanism of DNA cleavage involving a metal center reduction. The addition of neocuproine, a specific Cu(I) chelator, completely sequestered the DSBs activity of 11, suggesting that Cu(I) is an essential component for 11-induced DNA cleavage (Fig. 4c). Surprisingly, the presence of a reducing agent, such as β-mercaptoethanol (β-ME) or dithiothreitol (DTT), had no obvious effect on the DSBs activity of 11 (Fig. 4c). We thus propose that the reduction of Cu(II) to Cu(I) may be mediated by the DNA or by 11 itself, and the latter was supported by the free Cu(I) determination assays upon incubation of 11 and Cu(II) (Supplementary Fig. 19). In addition, 10 demonstrated a comparable copper reduction activity as 11, suggesting that the same macrocyclic scaffold in both compounds could be the active center for Cu(II) binding and reduction (Supplementary Figs. 19 and 20). The parallel monitor of the mixture of 10 and Cu(II) by HRMS further showed a loss of the mass signal for 10 over time which was accompanied by an approximately stoichiometric
formation of Cu(I) (Supplementary Fig. 21), and also a presence of a new mass signal with an isotopic pattern of copper-bound complex \(^{40}\) (Supplementary Fig. 22). Although this new mass signal was weak and transient which prevented its further characterization, this data supported the direct binding of \(10\) to copper and the instability of \(10\) in the presence of copper. We further determined the binding constant of copper with \(10\) to be \(\sim 4120 \text{ M}^{-1}\) (Supplementary Fig. 23).

The oxidative mechanism of DNA cleavage was further probed by adding various reactive oxygen species (ROS) scavengers. Plasmid DNA damage by \(11\) was not measurably influenced by the hydroxyl radical scavengers mannitol and dimethyl sulfoxide (DMSO) (Fig. 4d), which argues against participation of the freely diffusible hydroxyl radical in the observed cleavage and distinguishes the mechanism by which colibactin incises DNA from a sole Fenton-like one\(^{41}\). The addition of superoxide dismutase (SOD), which catalyzes the conversion of the superoxide radical into hydrogen peroxide (H\(_2\)O\(_2\)), did not measurably influence DNA cleavage by \(11\) (Fig. 4d). In contrast, potassium iodide (KI), a H\(_2\)O\(_2\) scavenger, and catalase, which mediates the decomposition of H\(_2\)O\(_2\), significantly inhibited the cleavage reaction (Fig. 4d). These results suggested that H\(_2\)O\(_2\) was involved in mediating DNA cleavage \emph{in vitro}, consistent with the observation of a significant increase in H2-DCFDA fluorescence (a sensor of hydroxyl and peroxyl radicals, and hydrogen peroxide production) in non-transformed human lung fibroblast cells infected by colibactin-producing \emph{E. coli}\(^{31}\).

The DNA DSBs activity of \(11\) was next compared to other precolibactins for a preliminary structure–activity relationship study. Under the same reaction condition, \(10\) displayed a significantly weaker DSBs activity than \(11\) (Supplementary Fig. 18), demonstrating that the extra 5-hydroxy oxazole moiety in \(11\) was important for augmenting the DSBs activity. The DSBs activity of \(5\), a precolibactin that has previously demonstrated DNA-crosslinking activity due to its aza-spirocyclopropane warhead, was also tested\(^{24}\). \(5\) did not display DNA-damaging activity even at concentrations as high as 5 mM (Supplementary Fig. 18).

**DSBs activity of colibactin in cells**

We next examined the DNA damaging activity of colibactin in various human cell lines. Production of phosphorylated histone H2AX (\(\gamma\)H2AX) and translocation of the p53 binding protein 1 (53BP1) are early events in the cellular response to DNA DSBs\(^{42,43}\). Four hours after exposure to 50 nM of \(13\), HeLa cells showed formation and colocalization of foci derived from \(\gamma\)H2AX and 53BP1 (Fig. 5a). By comparison, the \(\gamma\)H2AX and 53BP1 foci were undetectable in cells treated with 50 nM of \(11\) (Fig. 5a), in contrast to the comparable activity of \(13\) and \(11\) in the pBR322 plasmid DNA strand scission assay. This result supported that maturation was a prerequisite for colibactin’s genotoxicity \emph{in vivo}\(^{15}\). In addition, \(15\), the mature product of \(10\) after ClbP cleavage (Supplementary Fig. 24), also demonstrated a significantly lower activity than that of \(13\) (Fig. 5a), consistent with the lower DSBs activity of \(10\) than \(11\) \emph{in vitro} (Supplementary Fig. 18). The similar foci formation and colocalization were also observed in other cell lines such as human normal colon epithelial FHC cells, human normal colon fibroblast CCD-112 CoN cells, and colorectal cancer HCT-116 cells treated with 50 nM of \(13\) (Supplementary Fig. 25), which
established that the cellular response to 13 was not cell-line specific, consistent with previously reported cytopathic effect in various cell lines that were infected by clb+ E. coli strains. A neutral comet unwinding assay was also conducted as an effective and independent method to evaluate the occurrence of DNA DSBs in cells treated with 13. Consistent with the results of γH2AX and 53BP1 induction, a four-hour exposure of Hela cells to 13 caused accrued DNA lesions in a concentration-dependent manner, demonstrated by the migration of cleaved DNA fragments (comet tail) from the nucleoid (comet head) under the influence of an electric field (Fig. 5b). Furthermore, the treatment of either EDTA or bathocuproinedisulfonic acid (BCS), an extracellular Cu-sequestering agent, significantly alleviated the levels of DNA damage caused by the purified compound 13 or the infection of clb+ E. coli CFT073 (Fig. 5c, d, Supplementary Fig. 26), which is in agreement with the observed dependence of copper for colibactin-induced DNA DSBs in vitro.

Discussion

Despite extensive studies on the biology of the clb pathogenicity island and the chemistry of the clb encoding enzymes, the genotoxic colibactin metabolite with intrinsic DNA DSBs activity had escaped all screening surveillance in the past decade. For the first time, through strain engineering, large-scale fermentation and metabolite comparison, we have identified and characterized the highly sought genotoxic colibactin metabolite, colibactin-645 (13). The biosynthesis of 13 requires all predicted biosynthetic enzymes encoded on the clb pathogenicity island; more importantly, 13 recapitulates its pre-assumed DNA DSBs activity both in vitro and in cell cultures, distinguishing 13 from all previously identified metabolites associated with this pathogenicity island. Interestingly, although macrocyclic colibactins, including 10, 11, 13, and 15, required copper for their bioactivity, they quickly degraded in the presence of copper, which prevented direct characterization of any colibactin-Cu complex. This is akin to the instability of the activated bleomycin that was suggested to have a half-life of only several minutes at 4 °C after binding to a reduced transition metal. In addition to the low abundance and chemical lability, the macrocyclic mature colibactin appeared to be polar compound that stayed in the aqueous phase during organic solvent extraction, which could further contribute to the difficulty in the genotoxic metabolite detection.

The biosynthesis of 13, as well as 8 and 11, features a new fate for the atypical aminomalonyl extender unit utilized by PKSs. The incorporation of this aminomalonyl extender unit has been previously elucidated through a traditional decarboxylative Claisen condensation in zwittermicin, guadinomine and colibactin biosynthesis. In particular, ClbKPKS has been shown to promote the decarboxylative condensation of the aminomalonyl unit that contributes for thiazole and 2,5-dihydro-5-hydroxyoxazole formation in 10 biosynthesis. In this study, we showed that an amidase, ClbL, was required for the biosynthesis of 8 and 11, and further proposed that ClbL promotes the amide bond formation through nucleophilic attack of the amine in the aminomalonate extender unit attached to the ClbK or ClbO, leading to the generation of the terminal 5-hydroxy 4-oxazolecarboxylic acid moiety in 8 or 11 (and thus 13), respectively. Furthermore, identification of the precolibactin
metabolite 12 suggests that similar to ClbK, ClbO is capable of catalyzing the decarboxylative condensation of the aminomalonyl unit, although this activity is outcompeted by the activity of ClbL, resulting in a much higher titer of 11 than 12. This finding is consistent with the recent independent discovery\(^4^8\), showing that ClbL is a promiscuous amide bond-forming enzyme that links aminoketone and \(\beta\)-keto thioester substrates. The relaxed substrate specificity of ClbL is likely one of the main contributing factors to the production of several complex colibactin metabolites associated with the \(clb\) island, including the markedly different colibactin metabolites with DNA cross-linking activities that were recently identified in independent studies\(^4^9\).

Based on the DNA damage assays both \textit{in vitro} and in cells, we propose the following mechanism for copper-mediated DNA DSBs by colibactin-645 (13) (Supplementary Fig. 27). After being secreted from a producing bacterium that localizes close to or in contact with the intestinal brush border\(^1^0\), 13 binds to exchangeable copper in the intestinal lumen, likely coming from diet\(^5^0\), to form a colibactin-Cu(II) complex. The macrocyclic colibactin was determined to have a higher binding affinity for Cu(II) than some of the known copper-binding natural products such as bleomycin and tambjamine\(^5^1\), supporting the physiological relevance of the suggested complex formation with Cu(II). This complex is quickly transported into the epithelial cell while reduced to a colibactin-Cu(I) complex, and the coordination of O\(_2\) to this cuprous complex in cells generates ‘activated colibactin’ that attacks DNA and initiates DNA cleavage. Cu(II)—O\(^-\) (or Cu(III)==O) is proposed to be the active species in the ‘activated colibactin’ complex susceptible of DNA carbon–hydrogen bond activation\(^3^9\), which is consistent with the observed inhibitory effects of H\(_2\)O\(_2\) scavengers on the DNA cleavage reaction \textit{in vitro} as colibactin-Cu(II)—OOH is a key intermediate to colibactin-Cu(II)—O\(^-\) (Supplementary Fig. 27). Additionally, we do not exclude the possibility that 13 quickly enters the epithelial cell and then binds the intracellular copper to exert its activity. This mechanism is analogous to the proposed one for the generation of ‘activated bleomycin’ \textit{in vivo}, differing mainly in the metal usage and the intrinsic metal reduction activity of compounds\(^3^7,3^8\).

The unusual heterocycle-fused macrocycle in 13 is important for copper binding and reduction, as only macrocyclic colibactins, such as 10 and 11, demonstrated a strong and comparable Cu(II) reduction activity. In addition, the comparison between the DSBs activity of 10 and 11, as well as 15 and 13, highlights the significance of the terminal 5-hydroxy oxazole moiety for DNA DSBs activity. We speculate that the thiazole/5-hydroxy oxazole tail found in 11 and 13 may serve as the DNA intercalating element, similar to the function of the bithiazole moiety found in bleomycin\(^3^7,3^8\). Based on the comparative DSBs activity of 11 and 13 \textit{in vitro} but a drastically different solubility as well as a significantly lower activity of 11 in cellular assays, we further propose that the loss of the N-terminal fatty acyl-asparagine residue as the prodrug motif facilitates the access of mature colibactin-645 to target eukaryotic cells\(^1^5\). Although many secondary metabolites have been reported to induce DNA DSBs, a majority of them function via indirect mechanisms (such as by inhibiting topoisomerase complexes\(^5^2\)), and few of them cleave DNA double-strand directly\(^5^3\). 13 thus represents a novel molecular scaffold exerting a direct DNA DSBs
activity, providing a model for designing and synthesizing potent DNA cleaving agents, from synthetic restriction ‘enzymes’ to chemotherapeutic agents.

Considering that DNA cross-linking was also observed in human cell lines incubated with colibactin-producing *E. coli*, we speculate that the overall genotoxic effect of the *clb* island may arise from a mixture of metabolites with different modes of action, including but not limited to DNA cross-linking and DSBs activities. This is consistent with the predicted comprehensive role of colibactins in mediating diverse bacteria-host interactions.

In summary, we have identified and characterized a novel genotoxic colibactin metabolite, provided the conclusive evidence for macrocyclic colibactin directly mediating DNA damage, and shed light on the long-standing mystery of the molecular mechanism underlying colibactin-induced DNA DSBs. Our discoveries thus lay out a framework for future investigations that could enhance our understanding of the *clb* pathogenicity island from human gut microbes, and enable further mechanistic interrogation of colibactin-induced DNA DSBs and colibactin-related CRC incidence and prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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**Fig. 1** Structures and proposed biosynthesis of precolibactins.

**a.** Structures of precolibactin-546 (5), precolibactin-712 (7), precolibactin-795a (8), precolibactin-795b (9), precolibactin-886 (10) and precolibactin-969 (11).

**b.** Proposed biosynthetic pathway of precolibactins. Extending from ClbJ, the dimodule PKS/NRPS ClbK shows diverse functions in the production of clb metabolites. The clb pathway utilizes ClbK\(_{\text{PKS}}\) and ClbL to produce 8 (Route A); or skips ClbK\(_{\text{PKS}}\) but utilizes ClbK\(_{\text{NRPS}}\) to produce 9 (Route B); or utilizes both of ClbK\(_{\text{PKS}}\) and ClbK\(_{\text{NRPS}}\) modules to produce 10 which is the precursor for the assembly of 11 (Route C). A, adenylation; ACP, acyl carrier.
protein; Am, amidase; AT, acyltransferase; Cy, cyclization; KS, ketosynthase; Ox, oxidase; PCP, peptidyl carrier protein. AT* domains are predicted based on structural topology as ancestral inactive relics.
Fig. 2 | Genes and proposed mechanisms of aminomalonate-utilizing PKSs in the biosynthesis of precolibactins.

a, A comparison of LC–MS extracted ion chromatogram traces of the metabolic extracts from ΔclbPΔclbQ, ΔclbPΔclbQΔclbS and its ten mutants, and ΔclbPΔclbQΔclbS::clbR, showing the impact of gene knockout or knockin on the yield of 11, and the requirement of clb pathway genes for the biosynthesis of 11. EIC+ = 970.38 ± 0.01, which corresponds to 11. b, Proposed mechanism of ClbKPKS underlying the production of 10. The chain elongation is achieved through C–C bond formation by decarboxylative Claisen condensation. c, A comparison of LC–MS extracted ion chromatogram traces of the metabolic extracts from clbPΔclbQΔclbS and its ten mutants. EIC+ = 796.37 ± 0.01 and 796.35 ± 0.01, which correspond to 8 and 9, respectively. d, Proposed mechanism of ClbKPKS and ClbL underlying the production of 8. The chain elongation is achieved through C–N bond formation by nucleophilic attack of the amine in the aminomalonate extender unit, followed by synchronous cyclization and release of 8. e, Proposed mechanism of ClbO and ClbL, underlying the production of 11 with a similar biosynthetic logic to that of 8. b, d and e, A, adenylation; ACP, acyl carrier protein; Am, amidase; AT, acyltransferase; Cy, cyclization; KS, ketosynthase; Ox, oxidase; PCP, peptidyl carrier protein. AT* domains are predicted based on structural topology as ancestral inactive relics.
Fig. 3 | Maturation of colibactin.

a. Proposed pathway for colibactin maturation. A prodrug mechanism is involved in colibactin biosynthesis. Precilbactin-969 (11) is biosynthesized in the cytoplasm of *E. coli* strains by the *clb* biosynthetic pathway and transported via ClbM into the periplasm, whereby the membrane-bound peptidase, ClbP, cleaves 11 to generate mature colibactin-645 (13) and a prodrug motif N-myristoyl-D-asparagine (14), followed by outer membrane translocation. b. A comparison of LC–MS extracted ion chromatogram traces shows the production of 13 resulting from its precursor 11 cleavage by *E. coli* strains expressing the
peptidase gene clbP in the presence or absence of metal chelators; and the detection of metabolite identical to 13 from either cell-free culture extracts or cellular extracts of cultured wild-type clb+ E. coli CFT073 and its clb− mutant. EIC+ = 646.14 ± 0.01 and 343.26 ± 0.01, which correspond to 13 and 14, respectively.
Fig. 4 | Analysis of DNA damage by colibactin in vitro.

a. The effect of colibactin-645 (13) on the plasmid pBR322 DNA cleavage. Reactions were performed at 15 μM 13 in the absence or presence of Cu(II) (3 μM or 30 μM) for 12 hours at 37 °C. DNA cleavage by 13 is observed only in the co-incubation of Cu(II) and 13, in which nicked (Form II) and linearized (Form III) DNA forms from the supercoiled plasmid DNA (Form I).

b. The time-dependent DNA damage induced by precolibactin-969 (11) (15 μM) is observed in the presence of Cu(II) (30 μM). Reactions were performed at 37 °C with different incubation times.

c. The effect of a specific Cu(I) chelator neocuproine (1 mM), a reductant β-mercaptoethanol (β-ME) (5 mM), or a reductant dithiothreitol (DTT) (5 mM) on the DNA cleavage by 11 (15 μM) in the presence of Cu(II) (30 μM). Reactions were performed at 37 °C for 4 h.

d. The effect of various reactive oxygen species (ROS) scavengers, including potassium iodide (KI) (1 mM), catalase (0.1 mg/mL), superoxide dismutase (SOD) (10 units), mannitol (50 mM), and dimethyl sulfoxide (DMSO) (10%), on the 11-induced DNA cleavage in the presence of Cu(II) (30 μM). Reactions were performed at 15 μM 11, 37 °C for 12 h. All of the controls (reactions without 11) of each reagent or scavenger show no DNA cleavage similar to the negative control presented in the figure (the lane with DNA only).

a–d, Top band, nicked DNA (Form II); middle band, linearized DNA (Form III); bottom band, supercoiled DNA (Form I). 

EcoRI-linearized pBR322 DNA is shown as the linearized DNA standard.
Fig. 5. Colibactin-induced DNA damage in cell cultures.

a. Immunofluorescence imaging of γH2AX and 53BP1 foci in HeLa cells that are treated with precolibactin-969 (11, 50 nM), colibactin-645 (13, 50 nM) or 15 (50 nM). Columns from left to right, nucleus (blue), γH2AX (green), 53BP1 (red), and merge. In control, only DMSO solvent was added. b. Accrued DNA lesions are induced by increased concentrations of 13, as measured by the neutral comet unwinding assay. c. The effect of either ethylenediaminetetraacetic acid (EDTA) (2.5 mM) or bathocuproinedisulfonic acid (BCS) (2 mM) on the DNA damage in HeLa cells after incubation with 13 (50 nM), as measured by
the neutral comet assay. **d**, The effect of EDTA (2.5 mM) or BCS (2 mM) on the DNA damage in HeLa cells after incubation with the wild-type *clb*+ *E. coli* CFT073, as measured by the neutral comet assay. **e, d**, Tail moment was obtained in the neutral comet unwinding assay, which represents the extent of DNA cleavage and is defined as the product of the tail length and the fraction of DNA in the tail. Bars represent mean tail moment (50 cells were randomly selected), error bars represent s.e.m. ***P < 0.001*** (one-way ANOVA).