Post-ingestion conversion of dietary indoles into anticancer agents

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ABSTRACT
Human health benefits from consuming cruciferous vegetables that release indole-3-carbinol (I3C), but the in vivo transformation of I3C-related indoles remains underinvestigated. Here we present the post-ingestion conversion of I3C into antitumor agents, 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LTr1) and 3,3'-diindolylmethane (DIM), by conceptualizing and materializing the reaction flux derailing (RFD) approach as a means of unraveling these stepwise transformations to be non-enzymatic but pH-dependent and gut microbe-sensitive. In the upper (or acidic) gastrointestinal, LTr1 generates through the Michael addition of 3-methyleneindolium (3MI, derived in situ from I3C) to DIM producing from I3C via the formaldehyde-releasing (major) and CO₂-liberating (minor) pathways. In the large intestine, ‘endogenous’ I3C and DIM can form respectively from couplings of formaldehyde with one and two molecules of indole (a tryptophan catabolite). Acid-producing gut bacteria such as Lactobacillus acidophilus facilitate the H⁺-promotable steps. The work updates the understanding on the merits of I3C consumptions and identifies LTr1 as a drug candidate.

Keywords: indole-3-carbinol, reaction flux derailing (RFD) approach, 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LTr1), anticancer, Lactobacillus acidophilus

INTRODUCTION
Cruciferous vegetable intake is inversely associated with the risk of total mortality and thus conjectured to contribute to longevity (1). Most crucifers biosynthesize indole glucosinolates whose breakdown product, indole-3-carbinol (I3C), plays important roles in plant growth, development and chemical defense (2). As a plant-derived small molecule, I3C regulates some biological processes in mammals, and particularly exerts cancer-preventive action in diverse models (3, 4). However, I3C can be rapidly metabolized in vivo, and becomes undetectable in plasma within an hour after ingested (5). The in vivo I3C metabolites identified include indole-3-carbaldehyde (I3A), indole-3-carboxylic acid (I3CA), indolo[3,2-b]carbazole (ICZ), 3,3'-diindolylmethane (DIM) and 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LTr1) (5) (Table S1). As a major I3C metabolite, DIM has been developed as a key ingredient of commercialized nutraceuticals with anticancer potential (6). LTr1 was also reported to a secondary metabolite of some marine bacteria such as Psychrobacter sp. (7). In this work, LTr1 was shown to have superior antitumor efficacies over DIM in diverse mouse models and cell lines. These facts motivated us to ask how I3C is metabolized in vivo and whether the health-benefit of I3C intakes arises, at least in partly, from the I3C metabolites with antitumor action.

I3C is chemically labile and forms structurally diverse compounds in aqueous acidic media (8) and microbial culture (9, 10). Mammals secrete gastric juice and their gastrointestinal tracts harbor diverse microorganisms, some of which metabolize xenobiotics such as nutra- and pharmaceuticals (11, 12). But little is known concerning the I3C transformation in gastrointestinal and the contribution of such conversion to the cancer prevention resulting from the I3C ingestion. Here we present that I3C, after ingested, is converted successively to the anticancer agents DIM and LTr1, the latter being more effective in diverse cancer cell lines and various tumors in mouse models. The reaction flux derailing (RFD) approach was conceptualized and deployed to reveal the in vivo chemistry underlying the interwined “indole→I3C→DIM→LTr1” conversion chain (Fig. 1). The formaldehyde-releasing, CO₂-liberating and indole-recruiting pathways, through which I3C is formed and transformed, were

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established to be non-enzymatic but pH-dependent, and thus accelerated by gastric acid and acid-producing gut bacteria such as *Lactobacillus acidophilus*. The work symbolizes a movement in understanding the health-beneficial or medical significance of consuming I3C or I3C-releasing diets and updates the knowledge about the *in vivo* chemistry and biofunction of I3C and its metabolites such as LTr1 and DIM.

**RESULTS**

LTr1 is more effective than DIM in diverse cancer cell lines and tumor models in mice

I3C and its metabolites were repeatedly but sporadically tested to be antitumor, thus allowing for some inconsistencies in methodology and activity magnitude (Table S1). To compare their antitumor potency in the same experimental setting, a panel of cancer cell lines was used to evaluate the cytotoxicity of I3C and its *in vivo* metabolites including LTr1, DIM, I3A, I3CA and ICZ (5). DIM was initially expected to be the most effective from its commercialization as nutraceuticals (*e.g.*, DIM Supreme) and its entering into clinical trials as a tumor therapeutic candidate (6). Out of our expectation, LTr1 was demonstrated to be about 10-fold more effective than DIM against the growth of A549 and A375 cell lines with the magnitude comparable to that of doxorubicin, a cancer therapeutic agent coassayed as a reference in the study (Table S2).

The shortage of treatment for lung cancer (11) motivated us to put a top priority on investigating whether LTr1 is efficacious in the A549-grafted nude mice. As illustrated in Fig. S1, the *in vivo* cancer inhibition by LTr1 was evidenced to be superior over DIM from the shrunked size and reduced weight of tumor tissues as well as the lowered expression of Ki67 protein, a tumor proliferation marker (13). With its efficacy ascertained in the tumor cell-grafted mice, we further asked whether LTr1 inhibits the autochthonous lung cancer. The mouse model of Kras*G12D*-driven lung tumorogenesis was developed according to the established protocol (14). To our surprise, the superiority of LTr1 against the cancer over both DIM and pemetrexed disodium (a prescribed therapeutic for non-small cell lung cancer (15)) was corroborated by the lung morphology, microCT, Hematoxylin-Eosin (H&E) staining and Ki67 immunohistochemistry (Fig. S2). Furthermore, LTr1 was revealed to distribute well in autochthonous lung cancer tissues (Fig. S3), indicating that it is stable *in vivo* and thus orally administrable for cancer therapy.

I3C turns into LTr1 via DIM in mouse gastrointestinal tract

The promising anticancer activity of LTr1 intensified our interest in its *in vivo* formation from the ingested I3C molecule. We began the endeavor by determining the pH gradient of the mouse gastrointestinal tract (Fig. 2B). Considering the UV-photolysis capable of generating indole radicals (16), we modeled the *in vivo* conversion of I3C by artificial gastric juice in a lightproof compartment. As shown in Fig. S4A, 10 min after exposed to the gastric juice, around 20% of I3C was converted to LTr1, and 5% to DIM. With the observation in mind, we examined the abundance variation of LTr1, DIM and I3C in the stomach contents sampled 10, 20, 40, and 60 min after the I3C ingestion (Fig. S4B). Comparable to the light-proofed test was the “I3C:DIM:LTr1” ratio change (400:45:150 → 110:10:80) in stomach from 10 to 60 min after the I3C ingestion (Fig. S4B).

If unconverted in stomach, I3C might be transformed in intestine. As expected, 60 min after the I3C gavage, the LTr1 content was increased substantially in small intestine (duodenum, jejunum and ileum) as evidenced from the “I3C:DIM:LTr1” ratio changes in duodenum (20:2:17 → 34:22:135), jejunum (18:6:40 → 28:16:105) and ileum
(7:8:33 → 22:7:53) (Fig. S4B). In particular, LTr1 was (much) more abundant than DIM and I3C in these gastrointestinal compartments. But in large intestine (cecum and colon), I3C remained more detectable than were DIM and LTr1 (Fig. S4B). Such changes rationalized the LTr1 boost in small intestine 60 min after the I3C gavage (Fig. S4B and 4C). This inspired us to investigate whether gut bacteria contribute to the I3C-to-LTr1 conversion. According to the described procedure (17), a panel of gut bacteria was isolated from human feces using the individualized cultivation protocol on diverse media (Table S3). The obtained gut bacteria were evaluated for their I3C metabolizing capacity, indicating that *Lactobacillus acidophilus* was the most efficient converter of I3C into LTr1 and DIM (Fig. S5). *Lactobacillus* strains consist of both true inhabitants and ingested probiotics and are highly populated throughout the human gastrointestinal tract (18–20). Subsequent I3C conversion test in mice was therefore performed with *L. acidophilus*, a representative of lactic acid bacteria residing in human and animal guts (19). Following the described procedure (21), the nude mice were decontaminated by being treated with a mixture of broad-spectrum antibiotics [ampicillin + colistin + streptomycin (ACS)]. Ascertaining the successful ACS eradication of mouse gut microbes through the 16S rRNA analysis, Petri dish culture, dilatation of caecum and H&E staining (Fig. S6), the ACS-decontaminated mice were randomized into two I3C gavage groups, one of which was monocloned with *L. acidophilus* (Fig. 2). As indicated by the 16S rRNA, qPCR and LC-MS measurements, the *L. acidophilus* recolonized mice were disclosed to have an escalated bacterial abundance and an increased LTr1 level in small intestine in comparison to the counterparts of those without the *L. acidophilus* monocolonization (Figs. 2F and 2H). As expected, such *L. acidophilus* monoclonization in the ACS-decontaminated mice acidified its gastrointestinal, as was evident from the lowered pH gradient of its gastrointestinal compartments in comparison to the counterparts of the conventional and ACS-decontaminated animals (Fig. 2B). This set of experiments established the positive correlation of the LTr1 level with the *L. acidophilus* abundance and mouse gastrointestinal acidity, thereby signifying that the lactic acid bacteria can promote the I3C-to-LTr1 transformation.

**The mechanism underlying the *L. acidophilus* promotion of the I3C-to-LTr1 conversion**

Hypothetically, the I3C-to-LTr1 transformation by *L. acidophilus* could be either enzymatic, or non-enzymatic, or spontaneous but accelerated by enzymes as discerned with bacterial pericyclases we found recently (22). To address the ambiguity, the conventional *L. acidophilus* culture was aliquoted into seven fractions for the inactivation test. The first six fractions were respectively boiled for 0 (intact), 1, 5, 10, 30 and 60 min so that the bacterial proteins therein were inactivated in an ascending order. The seventh was autoclaved at 120 °C for 30 min as a definite inactivation of bacterial proteins (Fig. 3). After the inactivation procedure, each of the seven fractions was centrifuged with the precipitate divided into three aliquots which were re-suspended by adding buffers till the volume of the bacterial culture samples. The three suspensions derived from each precipitate were titrated to pHs 4.4, 7.2 and 8.0, respectively. Each of the titrated suspensions was aliquoted into two parts, which were stirred at 37 °C with I3C for 2 and 24 h (Fig. 3A–F), respectively. Subsequent quantification showed that none of these protein inactivation procedures substantially affected the transformation of I3C into DIM and LTr1 which, instead, were formed in a pH- and time-dependent manner (Fig. 3). The observations could be explained by assuming that the conversion of I3C into DIM and LTr1 was non-enzymatic but acidity-dependent. To reinforce the assumption, we tested for the formability of DIM and LTr1 from I3C in the blank MRS (de Man-Rogosa-Sharpe)
medium used for culturing lactic acid bacteria. Surprisingly, LTr1 and DIM produced as well after supplementing I3C into the MRS medium which was measured to have a pH of 5.6 after sterilization (Figs. S5 and S7A). However, the LTr1 generation was fortified in the L. acidophilus culture which was recorded to have a pH range of 4.0 to 4.5 (Fig. S7A). This suggested that the pH reduction by the bacterially secreted lactic acid favored the LTr1 generation. To explore the pH limit for the I3C conversion, a series of transforming experiments were conducted in phosphate buffers at different pHs (Fig. S7B). In a basic pH (> 7) range, DIM and LTr1 formed in reduced and negligible abundance (Fig. 3C-3F), respectively. This is in line with the optimal pH (~ 4) ascertained for the I3C conversion into LTr1 (Fig. S7A and 7B) and confirmed that the I3C-to-LTr1 transformation by L. acidophilus is non-enzymatic, but requires an acidic context (pH < 7). In addition, DIM and LTr1 were detectable in the cells derived from the I3C-exposed culture of L. acidophilus, but none of them could be detected in the conventionally cultured bacterial cells (Fig. S8). The observation underpinned that DIM and LTr1 are not bacterially produced but have their affinity to the bacterial cell membrane.

The above observation aroused our curiosity about the pathways, through which DIM and LTr1 are formed from I3C. Three decades ago (23), DIM was hypothesized to result from the I3C dimerization in concert with the formaldehyde elimination (hereafter referred to as “formaldehyde-releasing” pathway). But to date no experimental evidence was found to support the assumption. To tackle the issue, we conceptualized, designed and deployed the reaction flux derailing (RFD) approach by taking the advantage of the spontaneousness of the I3C-to-LTr1 conversion. The RFD methodology was created and expected to reroute or trap reaction intermediates by using labeled co-substrates with identical or similar reactivities. As a RFD showcase, I3C was stirred in methanol at pH 4 with 5-methoxyindole under the N2-flow that prevented its air-oxidation. The 5-methoxy group of 5-methoxyindole, and other small sized substituents on the benzene moiety of indoles used herein (vide infra), served as the “tag” to indicate the substrate origination. Gratifyingly, the formaldehyde released from the I3C coupling into DIM was incorporated in situ into the predicted molecule (i.e., 5,5'-dimethoxydimedone) with the “signature-like” methylene motif in the middle of the molecule (Fig. 4). Such a RFD experiment afforded “5-methoxydimedone”, too, highlighting the Michael addition of 5-methoxyindole (a nucleophile) to 3-methyleneindolium (3MI), which was formed as an electrophile from the H+ -promoted in situ dehydrogenation of I3C and stabilized by its resonance with (indol-3-yl)methylium (Fig. 4 and Fig. S9). To confirm the 3MI origination, I3C was stirred with thioglycol in an acidic context to give 2-(3-indolymethyl)thioethanol (Fig. S9A). Furthermore, the vulnerability of 3MI to dehydrogenation gained support from its ESI-MS spectrum where the most intense peak at m/z 130.0632 was interpreted to form from the elimination of water from the protonated I3C molecule (Fig. S9B). According to the RFD principle, other substituent-labeled indoles were tested and shown to trap the formaldehyde molecule liberated upon the I3C-to-DIM transformation (Fig. S10), too. Collectively, the RFD approach established herein enabled the capture of reactive intermediates and thus provided the experimental evidence for the DIM formation through the formaldehyde-releasing dimerization of I3C.

LTr1 was hypothesized to generate through the Michael addition of DIM with 3MI followed by a Wagner-Meerwein rearrangement (WMR) with proton abstraction (Fig. 1) (24), although unconfirmable prior to the RFD establishment. Herein, 5-Cl-I3C was selected and stirred with DIM in acidic condition in view of the chlorine isotope abundance that eases the MS analysis of products. As anticipated, we obtained both 5'- and 5''-chlorinated LTr1 analogues to underpin the Michael addition of DIM to 3-methylene-5-chloroindolium (5-Cl-3MI)
which was generated in situ from 5-Cl-I3C as I3C did (Fig. 5 and Fig. S9). Such a condensation was followed immediately by the WMR of one of the two similarly stabilized carbocations, (indol-3-yl)methyl and (5-chloroindol-3-yl)methyl (Fig. 5). The reaction of 5-Cl-I3C with DIM also gave 5,5′-dichlorinated DIM (5,5′-di-Cl-DIM) and 5,5′,5″-trichlorinated LTr1 (5,5′,5″-tri-Cl-LTr1), highlighting that 5-Cl-I3C undergoes the same conversion to form these products as does I3C to yield LTr1 and DIM (Figs. 5). This is why the LTr1 generation from DIM is preconditioned by an acidic pH, at which 3MI and its congeners (e.g., 5-Cl-3MI) could form in situ from I3C and its variants such as 5-Cl-I3C, respectively. In alkaline media, 3MI is deprotonated into 3-methyleneindole whose 3-exomethylene electrophilicity is not competent enough for the efficient addition to DIM (Fig. 4A and Fig. S9A). The rationalization agreed with the observation that, in a basic pH (> 7) range, DIM and LTr1 formed from I3C in highly reduced and negligible yields (Fig. 3), respectively.

In air-exposed media, I3C can be spontaneously but slowly oxidized into I3CA (10, 25). In theory, DIM could be produced alternatively via the decarboxylative Claisen condensation of I3CA with 3MI (formed in situ from I3C in an acidic context), which was termed “CO₂-liberating” pathway (Fig. 1 and Fig. S11A). To confirm the hypothesis, and if so, to examine the competitiveness between the CO₂-liberating and formaldehyde-releasing pathways, 5-methoxy-indole-3-carbinol (5-MeO-I3C) was subjected to the RFD experimentation with exposure to I3CA at different pHs. According to the RFD principle, the expected 5,5′-diMeO- and 5-MeO-DIMs were reasoned to be the “signal products” of the formaldehyde-releasing and CO₂-liberating pathways (Fig. S11), respectively. At pHs 4 and 6, the anticipated products 5,5′-diMeO- and 5-MeO-DIMs formed respectively as major and minor products, signifying that the formaldehyde-releasing pathway was much more dominant than the other. However, at pH 8, none of them could be detected in the reaction mixture (Fig. S11B). Accordingly, the two I3C-to-DIM transforming routes are active in acidic contexts, reaffirming that the H⁺-promoted dehydration of I3C into 3MI is the key step for the formation of DIM in an acidic pH range. On the other hand, the oxygen level is quite low in mammal guts so that numerous anaerobes survive (Table S3). It is therefore reasonable for the CO₂-liberating pathway to play a trivial role in transforming I3C into DIM in the mouse gastrointestinal tract. To probe whether the CO₂-liberating and formaldehyde-releasing pathways are spontaneous, 5-MeO-I3C and I3CA were separately co-supplemented in the L. acidophilus culture and the blank MRS medium. Interestingly, both 5,5′-diMeO- and 5-MeO-DIMs were produced and no difference could be observed between the experiments using the bacterial culture and MRS medium (Fig. S11C and 11D). However, 5,5′-diMeO-DIM was more abundant than 5-MeO-DIM, reaffirming the dominance of formaldehyde-releasing pathway over the CO₂-liberating one in the bacterial culture (Fig. S11C and 11D). A trace amount of 5,5′-diMeO- and 5-MeO-DIMs was detected at pH 7.4, at which 3-methyleneindole existed but underwent the Michael addition very slowly. Collectively, such experiments indicated that the DIM formation from I3C is spontaneous but pH-dependent.

I3C and DIM form alternatively from endogenous indole and formaldehyde

The aforementioned findings were solid enough to explain the production of DIM and LTr1 in the upper gastrointestinal (stomach and small intestine), which was examined to be acidic (Fig. 2B). But, to our surprise, DIM was also detected in large intestine (Fig. 6D), where DIM might be formed via yet-to-be-defined pathways. In healthy individuals, formaldehyde distributes intracellularly at 0.2–0.5 mM as a common metabolite of methanol, creatine and folate (26), and indole exists in human plasma (4.5–9.4 μg/l) (27) and feces (0.25–1.2 mM) (28) as
a tryptophan catabolite (29). Also encouraged by the formation of 5,5′-dimethoxylated DIM from the addition of formaldehyde to 5-methoxyindole (Fig. 4), these reports motivated us to hypothesize that indole and formaldehyde might react to form I3C and/or DIM in their physiological concentration ranges (we thus termed such a route the indole-recruiting pathway). To corroborate the assumption, indole (0.25 mM) and formaldehyde (0.2 mM), both being below the physiological concentration maxima, were stirred at 37 °C at pH 8 in a sealed tube. Subsequent LC-MS analysis indicated that I3C and DIM were generated in a reaction time-dependent manner (Fig. S12B and 12C). This observation could be explained by assuming the base-promoted Friedel–Crafts reaction of indole with formaldehyde to form I3C, and “endogenous” I3C thus formed might undergo a bimolecular nucleophilic substitution (SN2) reaction with indole at its hydroxymethyl carbon to yield DIM (Fig. S13). To ascertain the acidity- and enzyme-dependence of the coupling of indole with formaldehyde, the L. acidophilus cells were collected and aliquoted to two parts, one of which was autoclaved at 120 °C for 30 min. After such pretreatment, each of the two parts was divided into three aliquots which were re-suspended in buffers at pHs 4.4, 7.2 and 8.0, respectively. Each suspension was divided into two aliquots, in which indole and formaldehyde were agitated at 37 °C for 2 and 24 h, respectively (Fig. S12D and 12E). Interestingly, I3C and DIM were both detectable at pH > 7.0 in both living and autoclaved L. acidophilus cultures. However, at acidic pHs, I3C disappeared and DIM was concentrated presumably due to the H⁺-promoted I3C-to-DIM conversion (Fig. S12D and 12E). The observation highlighted that the coupling of indole to formaldehyde was spontaneous but pH- and time-dependent (Fig. S12D and 12E). Moreover, the indole-formaldehyde coupling seems irreversible since we failed to detect any products that might result from the retro-aldol reaction of I3C (Fig. S14).

To ascertain the generalizability of the base-promoted condensation of indole with I3C, indole was individually stirred at pH 8 with 5-chlorinated and 5-methoxylated derivatives of I3C to yield the corresponding 5-chloro- and 5-methoxy-DIMs (Fig. S13B). Analogously, I3C was separately mixed at pH 8 with each of 5-chlorinated, 5-methoxylated, N-benzylated, and N-methylated indoles to afford 5-chloro-, 5-methoxy-, N-benzyl-, and N-methyl-DIMs (Fig. S13B, S35–42), respectively. Such a base-promoted condensation of indole with I3C analogues explained why 5-MeO-I3C reacted with indole to give 5-MeO-DIM (major) and 5,5′-diMeO-DIM (minor) at pH 8.0 (Fig. S13B and S15). However, at basic pHs, LTr1 failed to form from the condensation of I3C with DIM (as a 3-substituted indole), reaffirming that the LTr1 generation could only be realized through the 3Ml-mediated route in an acidic context (Figs. 3 and 5). Similarly, the MRS media with or without L. acidophilus were separately exposed to 5-MeO-I3C and indole. As illustrated in Fig. S15, 5-MeO-DIM was much more abundant than 5,5′-diMeO-DIM, indicating the dominance of the indole-recruiting pathway over the formaldehyde-liberating one.

The above findings motivated us to confirm the DIM formation in large intestine by the in vivo and ex vivo experiments. Mice were gavaged with formaldehyde and 5-chloroindole (chlorine group as tag) at doses within their physiological concentration ranges. As presumed, in mouse feces and urine, 5,5′-diCl-DIM and 5,5′,5″-triCl-LTr1 appeared in an approximate 100:1 ratio (Fig. 6). However, 5,5′-diCl-DIM, but not 5,5′,5″-triCl-LTr1, was detected in mouse large intestine (Fig. 6). In alignment with the observation, both 5,5′-diCl-DIM and 5,5′,5″-triCl-LTr1 were generated by the equally performed ex vivo experiment using mouse stomach and small intestine (Fig. 6). These evidences reinforced that the acidity is essential for the LTr1 generation from DIM, although formaldehyde and indole used herein for experimentation were a bit more concentrated than their ordinary
physiological level. Collectively, I3C and DIM can form alternatively from endogenous indole and formaldehyde, which are more abundant in the lower gastrointestinal tract (26, 28, 30).

DISCUSSION

This work shows that the H⁺-facilitated transformation of I3C into LTr1 via DIM can be realized in the upper gastrointestinal tract of mice through the formaldehyde-releasing (major) and CO₂-liberating (minor) pathways. In the lower mouse gastrointestinal tract, the indole-releasing pathway exists and allows for the 'endogenous I3C' formation via the addition reaction of indole (a tryptophan catabolite (29)) to formaldehyde, a common metabolite of methanol, creatine and folate (26). Endogenous I3C thus formed can be further transformed into DIM, but not LTr1, through its coupling with indole. Concerning the mechanism, the entire "indole→I3C→DIM→LTr1" conversion is non-enzymatic but pH-sensitive and thus depends on the gastrointestinal compartments. In stomach and small intestine, LTr1 produces from the Michael addition of DIM with 3MI generated in situ from the H⁺-promoted dehydration of I3C. In large intestine where pH is around 8 (Fig. 2B), the LTr1 production seems negligible, but DIM forms alternatively from the SN2 reaction of indole with I3C producible from the Friedel–Crafts reaction of formaldehyde to indole (Figs. S12 and S13). This explains why the acid-producing bacteria such as L. acidophilus can escalate the transformation of I3C into LTr1 via DIM in the upper mouse gastrointestinal tract. Because of the dependence on the abiotic factor such as pH, substrate concentration and interaction time, the formaldehyde-releasing, CO₂-liberating and indole-releasing pathways established herein (Fig. 1) may be involved in the conversion of indole-coined compounds by most (if not all) domains of life.

Almost all metazoan taxa harbor a vast ensemble of microbes, but pH seems to be a versatile determinant of the microbiome stratification in a particular niche (31). Our gastrointestinal compartments are pH-differentiated for the utmost of digesting foods and killing ingested pathogens (32), and this is why many gut microbes (e.g., L. acidophilus) produce pH-titrating acids for outcompeting each other in diverse host niches (33, 34). This study assigns the driving force behind the conversion of I3C into DIM and LTr1. In gut, DIM is generated mainly via formaldehyde-releasing pathway although it could be alternatively produced by the indole-I3C coupling in large intestine. However, LTr1 is only formed from I3C in acidic context that allows for the formation of the key protonated intermediate IS-2 (Fig. 1). The fact explains why the I3C-to-LTr1 conversion via DIM is non-enzymatic but depends on the acidity of particular gastrointestinal compartments. Interestingly, such conversion proceeds spontaneously in mouse gastrointestinal tracts. Our protein inactivation experiments showed that no enzyme may accelerate the conversion. Since enzymatic and spontaneous conversions follow identical rules of chemistry (35, 36), we took the advantage of such non-enzymatic feature to create the reaction flux derailing (RFD) strategy to decipher the transformation mechanism. The RFD methodology is generalizable and easy to be practised by adopting substituent-labeled co-substrates with similar reactivity as competent capturing agents (Figs. 4–6 and Fig. S10, S11, S13 and S15). The prowess of the RFD approach was showcased by, but not limited to, our RFD-facilitated confirmation of the acid-triggered/promoted reactions involved in the I3C conversion to antitumor agents LTr1 and DIM (e.g., SN2 reaction, Lewis-acid-promoted dehydration, Michael addition, Friedel–Crafts reaction and Wagner–Meerwein rearrangement). By taking the RFD advantage, we were able to reshape the function of (highly) acidic gastrointestinal compartments in chimerizing some pH-susceptible xenobiotics into functionally and architecturally distinct molecules. Coincidently, the acid production strategy is adopted to
counteract the plant-defensive effect of I3C by some crucifer pathogenic microbes such as *Sclerotinia sclerotiorum* (37), an efficient I3C metabolizer capable of producing oxalic acid (10). It is also noteworthy that the pH-dependence strategy is used in nature by some microbes for constructing skeletally disparate natural products such as merochlorin antibiotics (35). From an evolutionary view point, the pH stratification like that in the mammal gut could be a general strategy for different organisms to survive in nature.

The health-benefit origination of consuming I3C or I3C-containing cruciferous vegetables is a long-standing issue because of the I3C lability (5, 38). As shown by the present investigation, it is the I3C lability that leverages and allows for its *in vivo* transformation into DIM and LTr1. In particular, LTr1 is prominent in the cancer prevention with its *in vivo* efficacy higher than, or at least comparable to, that of DIM which has been approved as a nutraceutical during its clinical trial as a tumor therapeutic candidate (6). Therefore, it is reasonable to conclude that LTr1 and DIM contribute to the anticancer efficacy resulting from the I3C intakes. Concerning the mode of action of anticancer indoles, I3C and DIM function as aryl hydrocarbon receptor ligands (39). DIM was additionally reported to induce endoplasmic reticulum stress and modulate miRNA expressions (40). LTr1 was found to regulate the activity (or expression) of quinone reductase (41), cytochrome P450 and phase II enzymes (42). These reports highlight that, despite the structural comparability, LTr1 might differ from DIM and I3C in its pharmacological mechanism which falls beyond the scope of the work. In correlation with the I3C lability *in vivo* (5) and *in vitro* (8-10), the observation raises the possibility that many bioactivities described for I3C might be actually due to its metabolites such as DIM and LTr1.

This work identifies the endogenous production of I3C from the Friedel–Crafts addition of indole to formaldehyde in the large intestine (Figs. 1, 6 and S12), being distinct from the I3C generation from skatole (3-methylindole) in animals (43, 44). Starting from the finding, the investigation identifies a novel endogenous route to DIM consisting of the base-facilitated steps: (i) the *in situ* synthesis of I3C from the indole-formaldehyde coupling and (ii) the SN2 reaction of thus formed I3C with indole (Fig. 1 and Fig. S13). In view of the origination of indole and formaldehyde from ingested diets (26-28, 30), the aforementioned findings add a new layer of the mechanistic complexity to the metabolism of I3C-related indoles in biosystems, and in particular, help to understand the health-beneficial basis of consuming suitably combined foods. In this sense, the *in vivo* chemistry clarified herein for indoles may serve as an exemplification of a valid model of investigating diverse chemicals we ingest every day.

In conclusion, the work identifies LTr1 as a promising anticancer drug candidate under the inspiration of the health-benefit resulting from consuming cruciferous vegetables that liberate I3C while chopped and processed. The stepwise conversion of I3C into LTr1 via DIM has been addressed by conceptualizing, designing and utilizing the easy-to-follow RDF approach that enabled our establishment of the formaldehyde-releasing, CO2-liberating and indole-recruiting pathways. In view of the involvement of Lewis acid/base chemistry in many biological reactions (36), it can be anticipated that more arrays of health-benefiting compounds can form from diversely structured pH-susceptible chemicals in diets and orally given ethnomedicines, in acidic (micro)environments inside the gastrointestinal compartments including the large intestine (45). While reminding us of rethinking the actual contributor to the reported biological action of I3C from a view of *in vivo* chemistry, the study raises a few interesting topics such as the anticancer spectrum and mechanistic aspect of LTr1 as well as the role of the...
unique pH stratification of our gastrointestinal tracts sense and process countless xenobiotics with diverse structures.

METHODS
Detailed information about cell lines, culture conditions, proliferation assay, treatments and sample collection, X-ray microCT scanning, pharmacokinetics, chromatographic and mass spectrometric analysis, Immunohistochemistry (H&E and Ki67), conversion of I3C into LTr1 in vitro and in vivo, screening for the I3C metabolizer from human fecal bacteria, monoclonization of antibiotic-decontaminated mice with L. acidophilus, 16S rRNA sequencing and data analysis, qPCR, antibiotic treatments for gut bacterial eradication and chemical synthesis are included in the supplementary data online. Variable regions V3–V4 of bacterial 16S rRNA gene were amplified with degenerate PCR primers and the raw data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database under submission number PRJNA657969. Crystallographic data of LTr1 in CIF format have been deposited in the Cambridge Crystallographic Data Centre (no. CCDC-1499138).

AUTHOR CONTRIBUTION
L. P. L., D. L. and R. X. T. designed research and wrote paper; L. P. L., and J. C. Q., evaluated anticancer efficacy and gut microbial transformation; D. L. performed chemical synthesis; L. W. accomplished LC-MS; L. P. L., D. L., Q. Z., and R. X. T. analyzed data.

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Conflict of interest statement. The authors declare no conflict of interest.

SUPPORTING INFORMATION
Supplementary data are available at NSR online.
Figure 1. Three intertwined pathways of converting I3C to DIM and LTr1 in gastrointestinal tract. The CO₂-liberating pathway was characterized by the decarboxylative Claisen condensation of I3C with its oxide I3CA. The formaldehyde-releasing pathway was unique in the formaldehyde liberation from the I3C-coupled intermediate salt (IS-1). The indole-recruiting pathway occurred primarily in large intestine where I3C and DIM formed from couplings of formaldehyde with one and two indole molecules, respectively. I3C, indole-3-carbinol; I3A, indole-3-carbaldehyde; I3CA, indole-3-carboxylic acid; DIM, 3,3'-diindolylmethane; LTr1, 2-(indol-3-ylmethyl)-3,3'-diindolylmethane. 3MI, 3-methyleneindolium; IS-1 and IS-2, intermediate salts 1 and 2, respectively; WMR, Wagner-Meerwein rearrangement.
Figure 2. The *Lactobacillus acidophilus* (*L. acidophilus*)-promoted conversion of I3C into LTr1. (A) Diagram of the treatment regimen and schedule. Four-week-old nude mice were randomized to three groups (n = 5). Group I received I3C ingestion only. Group II was pretreated with a mixture of broad-spectrum antibiotics (ampicillin +
colistin + streptomycin (ACS)), followed by oral administration of I3C. Group III was successively ACS-decontaminated (first week), colonized with *L. acidophilus*, and ingested with I3C (since 2nd week). (B) The deviated pH gradients measured for the gastrointestinal tracts of conventional (group I) and ACS-decontaminated mice without (group II, in red) and with *L. acidophilus* monocolonization (group III, in green). (C) The 16S rRNA-based quantitation of bacteria in mouse feces. Comparison between groups I and II confirmed the ACS-eradication of gut bacteria, and the difference between groups II and III indicated the successful colonization of *L. acidophilus* in the ACS-decontaminated mice. (D) Beta diversity heat map based on the unweighted UniFrac distances using 16S rRNA analysis showed the similarity within, but significant difference among, the three treatments. (E) The inter-group difference in the operational taxonomic unit (OTU) Rank curve demonstrated the robustness of both ACS-decontamination and *L. acidophilus* mono-colonization. (F) The relative abundance (%) of *L. acidophilus* (normalized to 16S rDNA) in different gastrointestinal compartments of group III. The lines within boxes were medians, and the whiskers were the minimal and maximal values of the relative abundance (%) of *L. acidophilus*. Data were means ± SEM of four biological replicates. (G–J) Quantification of DIM and LTr1 from stomach (G), small intestine (H), cecum (I), and colon (J) in mice at indicated time points after the last administration of I3C as in (A). Data were means ± SEM of four biological replicates.
Figure 3. The pH- and time-dependent but non-enzymatic “I3C→DIM→LTr1” transformation. (A–F) The “I3C→DIM→LTr1” conversion by L. acidophilus. With or without boiling (for 0, 1, 5, 10, 30 and 60 min, respectively) or sterilization (120°C, 30 min), the cell filtrates from those L. acidophilus cultures were re-suspended in buffers at pHs 4.4, 7.2 and 8.0. Each obtained suspension was divided into two aliquots which were stirred with I3C (0.15 mg/ml) at 37°C for 2 and 24 h, respectively, followed by LC-MS analysis.
Figure 4. The reaction flux derailing (RFD) approach enabled the capture of 3-methylineindolium (3MI) and formaldehyde liberated from the I3C-to-DIM conversion. (A) The proposed mechanism underlying the incorporation of formaldehyde (liberated from IS-1, see Fig. 1) into 5,5'-Di-MeO-DIM whereas 5-MeO-DIM resulted from the compounding of 5-methoxyindole with 3-MI generated from I3C in methanol at pH 3–4. (B) HPLC detections of products obtained after stirring I3C and 5-MeO-indole in acidic condition. 5,5'-Di-MeO-DIM, 5,5'-dimethoxylated DIM; 5-MeO-DIM, 5-methoxylated DIM.
Figure 5. The RFD-pinpointed stepwise progression of the Michael addition and Wagner-Meerwein rearrangement (WMR) reactions involved in the I3C transformation into LTr1 via DIM. (A) The high 3-methylene electrophilicity of 5-Cl-3-MI (formed via the H⁺-promoted dehydration of 5-Cl-I3C) allowed for its Michael addition with DIM to produce IS-2 (see Fig. 1), which underwent unbiased C₃-to-C₂ WMR migrations of 5-unsubstituted and 5-chlorinated (indol-3-yl)methylums to afford 5'-Cl-LTr1 and 5''-Cl-LTr1, respectively. (B) HPLC monitorings of the reaction of DIM with 5-Cl-I3C to yield 5'-mono-, 5''-mono-, and 5',5'',5'''-trichlorinated analogues of LTr1. 5-Cl-I3C, 5-chlorinated I3C; 5'-Cl-LTr1, 5'-chlorinated LTr1; 5''-Cl-LTr1, 5''-chlorinated LTr1; 5',5'',5'''-Tri-Cl-LTr1, 5',5'',5'''-trichlorinated LTr1; Cl-IS-1 and Cl-IS-2, chlorinated intermediate salts-1 and -2 (see Fig. 1), respectively.
Figure 6. The RFD-facilitated identification of the ex vivo and in vivo coupling of formaldehyde with indole into I3C, DIM and LTr1. (A) The scheme for the ex vivo or in vivo compoundings of 5-Cl-indole with formaldehyde into corresponding chlorinated analogues of I3C, I3CA, DIM and LTr1. (B–E) The LC-MS detection of the chlorinated analogues in stomach and intestine as well as in urine and feces. (B–E) The EICs corresponded to 5-Cl-I3C (m/z 180.03, [M−H]−) (B), 5-Cl-I3CA (m/z 194.01, [M−H]−) (C), 5,5'-diCl-DIM (m/z 313.04, [M−H]−) (D) and 5,5',5''-triCl-LTr1 (m/z 476.06, [M−H]−) (E).
REFERENCES
1. Mori N, Shimazu T and Charvat et al. Cruciferous vegetable intake and mortality in middle-aged adults: A prospective cohort study. Clin Nutr 2019; 38: 631-43.
2. Katz E and Chamovitz DA. Wounding of Arabidopsis leaves induces indole-3-carbinol-dependent autophagy in roots of Arabidopsis thaliana. Plant J 2017; 91: 779-87.
3. Wu Y, Li RW and Huang H et al. Inhibition of tumor growth by dietary indole-3-carbinol in a prostate cancer xenograft model may be associated with disrupted gut microbial interactions. Nutrients 2019; 11:467.
4. Lee YR, Chen M and Lee JD et al. Reactivation of PTEN tumor suppressor for cancer treatment through inhibition of a MYC-WWP1 inhibitory pathway. Science 2019; 364:eaau0159.
5. Anderton MJ, Manson MM and Verschoyle RD et al. Pharmacokinetics and tissue disposition of indole-3-carbinol and its acid condensation products after oral administration to mice. Clin Res 2004; 10: 5233-41.
6. Banerjee S, Kong D and Wang Z et al. Attenuation of multi-targeted proliferation-linked signaling by 3,3′-diindolylmethane (DIM): from bench to clinic. Mutat Res 2011; 728: 47-66.
7. Li H, Li J and Lee CO et al. Indole oligomers from a marine sponge-associated bacterium Psychrobacter sp. Biochem Syst Ecol 2010; 38: 839-41.
8. De Kruijf CA, Marsman JW and Venekamp JC et al. Structure elucidation of acid reaction products of indole-3-carbinol: detection in vivo and enzyme induction in vitro. Chem Biol Interact 1991; 80: 303-15.
9. Lin LP, Yuan P and Jiang N et al. Gene-inspired mycosynthesis of skeletally new indole alkaloids. Org Lett. 2015; 17: 2610-3.
10. Pedras MS and Hossain S. Interaction of cruciferous phytoanticipins with plant fungal pathogens: indole glucosinolates are not metabolized but the corresponding desulfo-derivatives and nitriles are. Phytochemistry. 2011; 72: 2308-16.
11. Zimmermann M, Zimmermann-Kogadeeva M and Wegmann R et al. Mapping human microbiome drug metabolism by gut bacteria and their genes. Nature 2019; 570: 462-7.
12. Wu XM and Tan RX. Interaction between gut microbiota and ethnomedicine constituents. Nat Prod Rep 2019; 36: 788-809.
13. Menon SS, Guruvayoorappan C and Sakthivel KM et al. Ki-67 protein as a tumour proliferation marker. Clin Chim Acta 2019; 491: 39-45.
14. Zhou X, Padanad MS and Evers BM et al. Modulation of mutant KrasG12D-driven lung tumorigenesis in vivo by gain or loss of PCDH7 function. Mol Cancer Res 2019; 17: 594-603.
15. Rossi G, Alama A and Genova C et al. The evolving role of pemetrexed disodium for the treatment of non-small cell lung cancer. Expert Opin Pharmacother 2018; 19: 1969-76.
16. Bloch-Mechkour A, Bally T and Sikora A et al. Radicals and radical ions derived from indole, indole-3-carbinol and diindolylmethane. J Phys Chem A 2010; 114: 6787-94.
17. Hassaninasab A, Hashimoto Y and Tomita-Yokotani K et al. Discovery of the curcumin metabolic pathway involving a unique enzyme in an intestinal microorganism. Proc Natl Acad Sci USA 2011; 108: 6615-20.
18. Dourllard FP and de Vos WM. Biotechnology of health-promoting bacteria. Biotechnol Adv 2019; 37: 107369.
19. Rossi M, Martínez-Martínez D and Amaretti A et al. Mining metagenomic whole genome sequences revealed subdominant but constant Lactobacillus population in the human gut microbiota. Environ Microbiol Rep 2016; 8: 399-406.
20. Heeney DD, Gareau MG and Marco ML. Intestinal Lactobacillus in health and disease, a driver or just along for the ride? Curr Opin Biotechnol 2018; 49: 140-7.
21. Vétizou M, Pitt JM and Daillère R et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 2015; 350: 1079-84.
22. Zhang B, Wang KB and Wang W et al. Enzyme-catalysed [6+4] cycloadditions in the biosynthesis of natural products. Nature 2019; 568: 122-6.
23. Grose KR and Bjeldanes LF. Oligomerization of indole-3-carbinol in aqueous acid. Chem Res Toxicol 1992; 5: 188-93.
24. Lin LP and Tan RX. Bioactive alkaloids from indole-3-carbinol exposed culture of Daldinia eschscholzii. Chin J Chem 2018; 36: 749-53.
25. Sun J, Charron CS and Novotny JA et al. Profiling glucosinolate metabolites in human urine and plasma after broccoli consumption using non-targeted and targeted metabolomic analyses. Food Chem 2020; 309: 125660.
26. Dorokhov YL, Sheshukova EV and Bialik TE et al. Human endogenous formaldehyde as an anticancer metabolite: its oxidation downregulation may be a means of improving therapy. BioEssays 2018; 40: e1800136.
27. Jin M, Zhang X and Zhen Q et al. An electrochemical sensor for indole in plasma based on MWCNTs-chitosan modified screen-printed carbon electrode. Biosens Bioelectron 2017; 98: 392-7.
28. Chimerel C, Emery E and Summers DK et al. Bacterial metabolite indole modulates incretin secretion from intestinal enteroendocrine L cells. Cell Rep 2014; 9: 1202-8.
29. Yang J, Chawla R and Rhee KY et al. Biphasic chemotaxis of Escherichia coli to the microbiota metabolite indole. Proc Natl Acad Sci USA 2020; 117: 6114-20.
30. Swimm A, Giver CR and DeFilipp Z et al. Indoles derived from intestinal microbiota act via type I interferon signaling to limit graft-versus-host disease. Blood 2018; 132: 2506-19.
31. Quinn RA, Comstock W and Zhang T et al. Niche partitioning of a pathogenic microbiome driven by chemical gradients. Sci Adv 2018; 4: eaau1908.
32. Abe K, Irie K and Nakanishi H et al. Crystal structures of the gastric proton pump. Nature 2018; 556: 214-8.
33. Chouaia B, Goda N and Mazza G et al. Developmental stages and gut microenvironments influence gut microbiota dynamics in the invasive beetle Popillia japonica Newman (Coleoptera: Scarabaeidae). Environ Microbiol 2019; 21: 4343-59.
34. Lu Z, Shen H and Shen Z. Effects of dietary SCFA on microbial protein synthesis and urinary urea-N excretion are related to microbiota diversity in rumen. Front Physiol 2019; 10: 1079.
35. Walsh CT and Moore BS. Enzymatic cascade reactions in biosynthesis. Angew Chem Int Ed Engl 2019; 58: 6846-79.
36. Lin CI, McCarty RM and Liu HW. The enzymology of organic transformations: a survey of name reactions in biological systems. Angew Chem Int Ed Engl 2017; 56: 3446-89.
37. Uloth M, You MP and Finnegan PM et al. Seedling resistance to sclerotinia sclerotiorum as expressed across diverse cruciferous species. Plant Dis 2014; 98: 184-90.
38. Nguyen LP and Bradfield CA. The search for endogenous activators of the aryl hydrocarbon receptor. Chem Res Toxicol 2008; 21: 102-16.
39. Schiering C, Wincent E and Metidji A et al. Feedback control of AHR signalling regulates intestinal immunity. Nature 2017; 542: 242-5.
40. Li Y, VandenBoom TG 2nd and Kong D et al. Up-regulation of miR-200 and Let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. Cancer Res 2009; 69: 6704-12.
41. Zhu CY and Loft S. Effect of chemopreventive compounds from Brassica vegetables on NAD(P)H:quinone reductase and induction of DNA strand breaks in murine Hepa1c1c7 cells. Food Chem Toxicol 2003; 41: 455-62.
42. Wortelboer HM, de Kruif CA and van Iersel AA et al. Acid reaction products of indole-3-carbinol and their effects on cytochrome P450 and phase II enzymes in rat and monkey hepatocytes. Biochem Pharmacol 1992; 43: 1439-47.
43. Zhou X, D'Agostino J and Li L et al. Respective roles of CYP2A5 and CYP2F2 in the bioactivation of 3-methylindole in mouse olfactory mucosa and lung: studies using Cyp2a5-null and Cyp2f2-null mouse models. Drug Metab Dispos 2012; 40: 642-7.

44. D'Agostino J, Zhuo X and Shadid M et al. The pneumotoxin 3-methylindole is a substrate and a mechanism-based inactivator of CYP2A13, a human cytochrome P450 enzyme preferentially expressed in the respiratory tract. Drug Metab Dispos 2009; 37: 2018-27.

45. Strandwitz P, Kim KH and Terekhova D et al. GABA-modulating bacteria of the human gut microbiota. Nat Microbiol 2019; 4: 396-403.