Synergy among Phytochemicals within Crucifers: Does It Translate into Chemoprotection?1–3

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ABSTRACT The association between cruciferous vegetables and cancer prevention has been linked to glucosinolate derivatives. These phytochemicals enhance endogenous detoxification, leading to inactivation of potential carcinogens before initiation occurs. Two derivatives, indole-3-carbinol (I3C) and 1-cyano-2-hydroxy-3-butene (crambene) were shown in rats to induce a synergistic enhancement of detoxification enzyme activity. To follow up on these findings, a short-term carcinogenicity study using aflatoxin B1 (AFB1) was performed in which male F344 rats were fed diets supplemented with these 2 compounds alone or in combination. Groups included a negative control group (no AFB1, crambene, or I3C), a crambene group (diet 0.150% crambene), an I3C group (diet 0.165% I3C), a high-dose group (diet 0.150% crambene, 0.165% I3C) a low-dose group (diet 0.030% crambene, 0.033% I3C), and a positive control group (AFB1 treatment only). AFB1 was administered after 2 wk of dietary pretreatment. Liver sections were scored for lesions including karyomegaly, apoptosis, and biliary hyperplasia and evaluated for expression of the preneoplastic marker glutathione S-transferase-π (GSTP). I3C and crambene groups were protected against AFB1 toxicity whereas the low-dose group was not. The high-dose group had scores close to those of the negative controls. For log10 transformed 2- and 3-dimensional GSTP data, the high-dose group demonstrated synergistic reduction in GSTP-positive area and an additive reduction in GSTP-positive volume compared with the crambene and I3C groups. The low-dose group had no effect. In conclusion, high combination dietary doses of I3C and crambene demonstrated enhanced protection from AFB1. Low combination doses, as might be realistically in the diet, were not effective. J. Nutr. 135: 2972S–2977S, 2005.

KEY WORDS: • synergy • phytochemicals • indole-3-carbinol • crambene • aflatoxin B1 • glutathione S-transferase-π • chemoprotection

Diets rich in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage, and kale have been associated with a reduction of the incidence of several types of cancer (1,2). Many of the phytochemicals in these vegetables, most notably those derived from glucosinolates common to all crucifers, induce detoxification enzymes. This induction has been postulated to play a key role in the protection afforded by these vegetables against a variety of cancers (3,4).

Previous research at the University of Illinois identified 2 glucosinolate breakdown products, 1-cyano-2-hydroxy-3-butene (crambene) and indole-3-carbinol (I3C),5 as powerful inducers of detoxification enzymes (5–7). Progoitrin, a glucosinolate within the matrix of certain varieties of Brussels sprouts, broccoli, and related cruciferous vegetables (8), is hydrolyzed by an endogenous enzyme, myrosinase, during mastication or cooking (10) to form crambene, whereas I3C is formed similarly from the glucosinolate glucobrassicin (9).

I3C was found to be protective in rat and mouse models against various chemical carcinogens, including experimentally induced aflatoxin B1 (AFB1) hepatic carcinogenesis (10), and I3C has generated much interest as a potential cancer chemotherapeutic agent (11). I3C induces both phase I and phase II enzymes and hence is termed a bifunctional inducer. Bifunctional inducers such as I3C (and its major metabolite, diindolylmethane) work via the aryl hydrocarbon receptor pathway that ultimately interacts with the xenobiotic response element (XRE). XRE is present not only in the regulatory regions of the genes for many phase II enzymes, such as the glutathione S-transferases (GSTs) and quinone reductase, but also in the regulatory regions of certain cytochrome P450

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5 Abbreviations used: AFB1, aflatoxin B1; ARE, antioxidant response element; CYP, cytochrome P450; GST, glutathione S-transferase; GSTP, glutathione S-transferase-π; I3C, indole-3-carbinol; XRE, xenobiotic response element.
(CYP) genes, such as the gene encoding CYP1A1, a CYP frequently involved in bioactivation of a number of carcino-
gens, including AFB1 (10). Hence, I3C ingestion to prevent
carcinogenesis can be a two-edged sword, actually activating
certain precarcinogens (12,13).

Crambene, on the other hand, is a monofunctional inducer,
having minimal effect on phase I enzymes while remaining a
potent inducer of hepatic phase II enzymes. It is also an
effective long-term inducer of the antioxidant glutathione
(14,15). Crambene affects detoxification enzymes via the
antioxidant response element (ARE) (16), present in the regu-
laratory regions of many phase II enzymes in rats, including α-
and π-GSTs, quinone reductase, and the glutamate cysteine
ligase, the rate-limiting enzyme in glutathione synthesis (17).

Rats treated with I3C and crambene have synergistic rather
than additive induction of phase II enzymes, specifically the
GSTs and quinone reductases (5,6). This synergy occurs at the
gene regulatory level (18), suggesting that this effect may
translate into a synergistic effect in the live animal, allowing
for lower doses of either compound to be used for chemoprotec-
tion. It has been hypothesized that such synergy may explain
why higher doses of each compound individually are
required to achieve protection experimentally than are typi-
cally present in the vegetable plant itself (6). The concept of
synergy among phytochemicals as the mechanism of action
behind chemoprevention and chemoprotection against cancer
afforded by many fruits and vegetables has become increasingly
accepted (19). However, the few in vivo studies specifically
investigating how individual phytochemicals might interact
have been limited to only a few phytochemicals, such as
selenium, α-tocopherol, soy phytoestrogens, and retinoids
(20–23).

The purpose of this study was to examine whether the
synergy between crambene and I3C observed with induction
of phase II detoxification enzymes would translate into actual
chemoprotection, that is, whether the two compounds com-
bined in the diet would interact synergistically to reduce the
number of preneoplastic nodules expressing the biomarker
enzyme, GST-π (GSTP), in the liver after exposure to the
carcinogen, AFB1. The aflatoxin model of hepatocarcinogen-
esis was selected because it is known to be a naturally occur-
ring human carcinogen, is metabolized in the rat by enzymes
having minimal effect on phase I enzymes while remaining a
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Materials and methods

Chemicals. Crambene was isolated and purified from the seeds of
Crambe abyssinica as previously described (27). I3C and other chem-
icals were purchased from Sigma Chemical Company. Animals, study design, treatment, and diet. Sixty weanling, male CDF 344 (crl/BR) rats (Charles River Laboratories), weighing
~75 g, were randomly assigned to 6 groups of 10 rats each. Included
were a negative control group (no AFB1 , crambene, or I3C), a

crambene group (diet 0.15% crambene), an I3C group (diet 0.165%
I3C), a high-dose combination group (diet 0.15% crambene, 0.165%
I3C), a low-dose combination group (diet 0.03% crambene, 0.033%
I3C), and a positive control group fed neither supplement (AFB1,
treatment only) (Table 1). The doses of crambene and I3C were

calculated based on short-term pilot feeding studies (data not shown)
such as to provide each rat ~50 ~56 mg kg body wt
−1 d
−1 doses of crambene and I3C, respectively. Both the high and low
combination doses as well as the individual doses were shown previ-
ously to induce the synergistic response (6). All groups but the first
were treated with AFB1 after 2 wk of dietary pretreatment. Crambene
and I3C were supplied in a semipurified antioxidant-free powdered
diet (AIN 93G; ICN Biomedicals) (28) for 14 d before AFB1 treat-
ment (AFB1, in dimethylformamide via esophageal intubation) on d
14 and 15. After this time, the animals were fed an antioxidant-free
semipurified pelleted diet (AIN 93G) for the duration of the exper-
iment. Dietary intake and body weights were monitored daily through
d 13 and weekly after that time. The animal protocol and all pro-
cedures were approved by the University of Illinois Institutional Ani-
mal Care and Use Committee before the experiment began. For eval-
uation of body weight and feed intake data, 2-way ANOVA was
used to determine differences among groups. For terminal body
weight data and for liver-body weight ratios, 1-way ANOVA followed
by least significant difference for multiple comparisons was performed
using statistical analysis software (SAS; SAS Institute).

Processing and analysis of tissues. At the end of wk 15 (13 wk
after AFB1 treatment), the rats were humanely killed and necrop-
sied. Livers were removed, weighed, fixed in 10% neutral buffered
formalin for 12 h, and then stored in 80% ethanol. After fixation,
tissues were processed in graded alcohols, embedded in paraffin, and
sectioned at 4 µm; sections were stained with hematoxylin and
eosin for evaluation and scoring of karyomegaly, apoptotic index, and
biliary hyperplasia, scores chosen to reflect chronic injury due to
AFB1, and typical of changes induced by the compound. Standard
sections from 3 liver lobes (left lateral, right medial, and caudal) were
examined and scored on a 0–4+ scale for each type of lesion. Nonparametric statistical evaluation (Kruskal-Wallis
analysis (Kruskal-Wallis) was performed for each lesion. Values of P < 0.05 were considered to
indicate significance between groups.

GST immunohistochemistry. For identification of GSTP-posi-
tive foci, sections were baled at 45°C for 30 min before incubation
for 30 min with 1% H2O2. An avidin-biotin system (Vectastain
system, followed by 1° rabbit antiserum with specificity
for GSTP. The area data

TABLE 1
Experimental design

<table>
<thead>
<tr>
<th>Group</th>
<th>Crambene (0.15% diet, 14 d)</th>
<th>I3C (0.165% diet, 14 d)</th>
<th>AFB1 (250 µg/kg BW × 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Negative control</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 Crambene only</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 I3C only</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4 Crambene + I3C, high dose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 Crambene + I3C, low dose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 Positive control</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1 Doses were chosen on the basis of previous studies (data not shown) to achieve doses of 50 mg crambene × kg BW
2 High-dose diet: 0.150% crambene, 0.165% I3C.
3 Low-dose diet: 0.030% crambene, 0.033% I3C.
Results

Although diets were formulated to provide ~50 and ~56 mg·kg body wt$^{-1}$·d$^{-1}$ doses of crambene and I3C, respectively, actual doses of each were ~90 mg·kg body wt$^{-1}$·d$^{-1}$ (data not shown), higher than predicted because of a greater consumption of diet than anticipated by all groups. Accordingly, the high-dose combination group ingested ~90 mg·kg body wt$^{-1}$·d$^{-1}$ of crambene and ~90 mg·kg body wt$^{-1}$·d$^{-1}$ of I3C, whereas the low-dose combination group ingested 20% of that, or ~18 mg·kg body wt$^{-1}$·d$^{-1}$ of both compounds. However, at the time of AFB$_{1}$ treatment and at the conclusion of the study, there were no significant differences in body weight or liver-body-weight ratio among the groups (data not shown).

Histologically, there was evidence that high dietary doses of both crambene and I3C afforded protection from AFB$_{1}$-mediated chronic injury. Livers from the negative control group (group 1) were generally within normal limits (Fig. 1A) although occasional mild biliary hyperplasia and karyomegaly were observed (Fig. 2). By contrast, the positive control group (group 6) had the severest lesions (Fig. 1B), typified by a high level of karyomegaly, biliary hyperplasia, portal hepatitis, and portal fibrosis. The crambene alone, I3C alone, and high-dose combination groups had virtually no lesions and did not differ from the negative control group; these treatments had significant protective effects against both karyomegaly and biliary hyperplasia, in contrast to the low-dose combination, which was nonprotective for all measures assessed. In addition, the degree of protection against biliary hyperplasia afforded by the high-dose combination treatment was also greater than that afforded by either crambene or I3C alone, but the difference was not significant.

Regarding induction of preneoplastic hepatocytes expressing GSTP, only a few scattered, very small foci of GSTP-positive hepatocytes were observed in the negative control group, with GSTP staining confined to cholangiolar epithelium, which normally expresses GSTP (Fig. 3A). By contrast, the positive control group had a significantly greater log$^{10}$ percent area and log$^{10}$ percent volume of GSTP-positive foci and nodules (Fig. 3B). Substantial and significant protection against induction was noted in the crambene and I3C groups, as summarized in Fig. 4 and Table 2, with 2.3- and 3.3-fold reductions in log$^{10}$ mean percent area and volume for crambene and 1.7- and 3.2-fold reductions for I3C, compared with the positive control group. The protection afforded by crambene and I3C alone was less than the protection afforded by the high-dose combination diet, which reduced the log$^{10}$ mean percent area and volume 5.9- and 4.8-fold, respectively. The low-dose combination diet provided no significant protection at all; the small reductions in area and volume did not differ substantially or significantly from the positive control group.

Discussion

These data are the first in vivo evidence that crambene is chemoprotective not only against toxicity but also against initiation of carcinogenesis, supporting previous biochemical and molecular evidence that it is a phytochemical with cancer-protective potential. Although a weak inducer of detoxification enzymes in in vitro systems, crambene is equipotent with sulforaphane in vivo (33). Like sulforaphane, crambene exerts its effect on detoxification enzymes via the Nrf2/keap1 pathway that ultimately interacts with the ARE (16). In addition, the precursor glucosinolate for crambene, progoitrin, is in the same synthetic pathway as sulforaphane (34), being a derivative of methionine, and varieties of broccoli that are low in glucoraphanin, the precursor for sulforaphane, tend to have higher levels of progoitrin (35). Hence, greater dietary exposure to crambene than sulforaphane is possible in some situations.

With regard to crambene and I3C acting synergistically, the present study demonstrated that although dietary crambene and I3C were indeed individually protective against AFB$_{1}$-induced degenerative and/or inflammatory lesions and preneoplastic lesions, high combination doses of I3C and crambene were associated with enhanced protection from AFB$_{1}$ hepatic injury and carcinogenesis. This enhancement approached synergy in the suppression of the area of GSTP induction and achieved additivity in suppression of the volume of GSTP-positive hepatocytes within the liver. However, because of a high degree of variability in response among individual ani-
mals within groups, the apparent additivity and synergy was not significant at the P < 0.05 level. Nevertheless, a clear trend toward enhanced protection by a combination dose of both compounds was present, but the doses producing the effect were supraphysiologic, being far higher than could be realistically encountered even in an exclusively cruciferous diet, where a dose of 10 mg/kg body wt is on the outer realm of possibility (6).

Although the results of this study are somewhat equivocal with regard to synergy, they provide preliminary evidence that crambene and I3C can interact to produce an enhanced protective effect against the initiation stages of carcinogenesis. A longer study (e.g., 24 or 30 wk) may have allowed additional foci and nodules to form in the positive control group and fewer foci and nodules in the treatment groups. This would have enhanced the differences in response and made them stronger statistically. To definitely determine whether synergy can occur between crambene and I3C, a full-blown 2-y carcinogenicity study will have to be undertaken to more clearly define tumor incidence, multiplicity, and growth in animals treated with these compounds alone and in combination.

The low-dose combination group, which nevertheless received doses of crambene and I3C that would be somewhat unrealistic even in a diet very high in crucifers, was not protected, suggesting that synergy does not function at the low doses that might be encountered in the diet. The inability of the low-dose combination treatment to protect is disappointing and does not support the idea that synergistic interactions among phytochemicals that act via XRE and ARE account for the disparity between the high doses of purified phytochemicals needed to provide chemoprotection experimentally and the substantially lower doses of these same phytochemicals present in fruits and vegetables. Rather, the results seem to support a threshold phenomenon that occurs only at relatively high doses of compounds interacting via XRE or ARE. Further dose-response studies are needed to determine whether the threshold occurs and, if it does, at what level it occurs.

Another aspect of synergy not addressed by this study is the blocking versus suppressing properties of crambene and I3C (36). The study investigated the potential for I3C and crambene to block initiation of carcinogenesis rather than to suppress initiated cells from proceeding down the path toward neoplasia. I3C and its major metabolite diindolylmethane, for example, affect the cell cycle and the apoptotic pathways as well (10), pathways that act almost exclusively in the promotional stages of carcinogenesis. Crambene has as yet unspecified effects on the cell cycle (33,37) but is known to induce long-term elevations in pancreas and liver of the antioxidant glutathione as well as induce apoptosis in pancreatic acinar cells (15). At this point, however, it is not known whether the apoptotic effect can be induced in initiated cells or cells of nonpancreatic origin. Important synergistic interactions can and do occur in the promotional stages of carcinogenesis, and these interactions may be of equal or greater importance than interactions that prevent initiation. Additional in vivo investigations using well-defined models of carcinogenesis are needed to determine whether the threshold occurs and, if it does, at what level it occurs.
needed in which potentially synergistic compounds such as crambe and I3C are provided in the diet after initiation.

In any discussion of synergy, however, many complicating factors must be acknowledged, factors affecting any interpretation of results and, more important, their potential applications to humans. As summarized in a recent review on the topic, there are "inadequate chemical identification of compounds, lack of relevant endpoints and inconsistencies in mechanistic hypotheses and experimental methodologies [that] leave many critical gaps in our understanding of the benefits of these compounds" (4). One of these factors is precisely defining the compounds and interactions among these compounds that produce the putative synergistic effect. It is becoming increasingly apparent that it may take >2 bioactive compounds and >1 biochemical pathway to produce chemoprotection (presumably via synergy) by cruciferous vegetables and fruits and vegetables in general (19). I3C and crambe are not the only bioactive phytochemicals in cruciferous vegetables. Sulforaphane, phenylethyl isothiocyanate, goitrin, 1,2-dithiol-2-thiones, and various polyphenols, to name just a few (38–41), are abundant in cruciferous vegetables. In most cases, their interactions with each other as well as with other chemoprotective nutritive compounds, such as vitamins, fiber, and minerals, are unknown or as yet undefined.

A case in point is selenium, which can be readily taken up by cruciferous vegetables (42). Selenium and its metabolites interact synergistically with other chemoprotective compounds, including retinoids and vitamin E, to inhibit carcinogenesis in experimental situations (20,21). However, enrichment of broccoli with selenium recently was shown to substantially decrease sulforaphane and polyphenol content in the enriched broccoli, potentially dampening the chemoprotective contribution of these compounds (43). Interactions of selenium with either I3C or crambe in the process of cancer prevention have yet to be defined, although selenium has been shown to interact with crambe in vitro to synergistically inhibit the growth of and to kill MCF-7 canine mammary cells at doses that leave normal canine mammary epithelium unaffected (44).

The data presented here are preliminary and represent only an early step in the process of truly demonstrating the occurrence of synergy in vivo in carcinogenesis models and then relating this to the underlying biochemical and molecular mechanisms that have resulted from the numerous in vitro studies performed to date. The in vivo studies necessary to identify and prove that the synergistic mechanisms identified in vitro do indeed translate into anticarcinogenesis will be necessary to provide firm and definitive answers to the question of synergy among phytochemicals.

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**LITERATURE CITED**

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