THE MICRONUTRIENT INDOLE-3-CARBINOL: IMPLICATIONS FOR DISEASE AND CHEMOPREVENTION

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CONTENTS

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SUMMARY

This review provides a historical perspective for the development of indole-3-carbinol (I-3-C) as a chemopreventive or therapeutic agent. Early experiments in animal models clearly showed that feeding cruciferous vegetables reduced the incidence of chemical carcinogenesis. Excitement was generated by the finding that these vegetables contained a high content of indole-containing compounds, and I-3-C could by itself inhibit neoplasia. The mechanism of action was linked primarily to the ability of I-3-C and derived substances to induce mixed-function oxidases and phase II antioxidant enzymes by binding and activating the aryl hydrocarbon receptor. Most of the literature on chemoprotection by dietary indole compounds relates to this mechanism of action. Other mechanisms, however, are notable for this class of compounds, including their ability to act as radical and electrophile scavengers; the various ascorbate conjugates of I-3-C (ascorbigens) may be important in this regard. Exciting recent findings have demonstrated that I-3-C and its reaction products can affect cellular signaling pathways, regulate the cell cycle, and decrease tumor cell properties related to metastasis. It does not appear that I-3-C *per se* is the primary active compound in chemoprotection or chemoprevention. Rather, I-3-C and ascorbate provide the parent compounds for the formation of a myriad of nonenzymatic reaction products that have strong biological potency. We conclude with our thoughts regarding the current status and future directions for the use of I-3-C and related compounds.

KEY WORDS

aryl hydrocarbon receptor, ascorbigen, biotransformation, cancer, carcinogenesis, chemoprevention, chemoprotection, cytochromes P450, estrogen, glucobrassicin, indole-3-carbinol, metabolism, micronutrient, review

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Abbreviations: AHR = aromatic hydrocarbon receptor; CTr = 5,6,11,12,17,18-hexahydrocyclononа[1,2-*b*:4,5-*b*′:7,8-*b*′′]triindole, cyclic triindole; DIM = 3,3′-diindolylmethane, di(indol-3-yl)methane; I-3-C = indole-3-carbinol, 3-hydroxymethylindole, indole-3-methanol; ICZ = 5,11-dihydroindolo[3,2-*b*]carbazole; LTr-1 = 2-(indol-3-ylmethyl)-3,3′-diindolylmethane, linear triindole-1.

1. INTRODUCTION

This review provides a historical perspective, as well as insights into the current status and future directions, of the development and utilization of indole-3-carbinol (I-3-C) as a chemopreventive or therapeutic agent in humans. It is not intended to be a comprehensive review of the literature. In 1988, McDannell and co-workers published a succinct, yet comprehensive, review of the chemical and physiological properties of glucobrassicins (indole-containing glucosinolates) and their breakdown and condensation products /1/. A number of exhaustive reviews have recently appeared that cover several aspects of research in the area. The chemistry of glucobrassicins was discussed in detail in two reviews by Preobrazhenskaya and co-workers /2,3/. Verhoeven *et al.* /4/ carefully tabulated and evaluated the literature up to 1997 on the anticarcinogenic components of *Brassica* vegetables. In 1998, Broadbent and Broadbent /5/ wrote a chronologically-ordered review that was highlighted by the excellent summary descriptions of key papers in all research disciplines related to I-3-C chemistry and toxicology. In that same year, Zhu and Conney published a comprehensive review on the physiological and toxicological implications of estrogen metabolism in target cells /6/. Although the review by Zhu and Conney did not focus on I-3-C or *Brassica* vegetables, it did provide the framework for a brief review published in the following year by Bradlow *et al.* /7/, which focused primarily on I-3-C modulation of estrogen metabolism and its relationship to cancer prevention.

The more recent literature, summarized in this review, is presented as Table 1, which focuses on 1990 to the present.

2. METABOLIC ACTIVATION OF CHEMICALS

Early research in the field of micronutrient research occurred in an age of optimism, that all complex systems could be broken down into their simplest components. The essential components could then be reformulated into a system that would function as the original complex system. This approach, termed *resolution and reconstitution*, was extremely successful for determining the structure and function of such complex systems as the mitochondrial respiratory chain and oxidative phosphorylation, as well as mixed-function oxidase

TABLE 1

I-3-C - biotransformation, enzyme induction and biological responses

A. I-3-C and Chemical Carcinogenesis

Model	*Tissue*	*Reaction or Response*	*Ref.*
Trout	Liver	Dose response study. I-3-C suppression of AFB_1 tumorigenesis was dominant over promotion.	/76/
Mouse	Lung, Liver	I-3-C increased liver metabolism of NNK, decreased lung DNA methylation and lung tumors.	/77/
Rat	Liver	I-3-C inhibited NDEA induced tumors.	/78/
Trout	Liver	Dietary I-3-C, at concentrations that did not alter various biotransformation enzyme activities, inhibited the metabolic activation of AFB_1, as did I-3-C ARXM	/79/
In vitro	*Salmonella*	I-3-C generated mutagens at pH 3 in the presence of nitrite and without metabolic activation.	/80/
Rat	Hepatocytes	I-3-C inhibited toxicity from the mutagens MNNG and MMS. Inhibition correlated with antioxidation potency and membrane stabilization efficacy.	/41/
Rat	Liver	Oral I-3-C induced various oxidative and phase II enzyme activities. High dosages reduced GSH levels and produced some neurological toxicity.	/46/
Mouse	Liver	I-3-C protected isolated rat hepatocytes from CCl_4, MNNG and MMS toxicity. Oral I-3-C induced various oxidative and phase II enzymes, decreased glutathione peroxidase and SOD, and enhanced CCl_4 hepatotoxicity.	/47/
Trout	Liver	I-3-C inhibited AFB_1 DNA adducts and liver tumors when administered before or concurrent with carcinogen. If given after the carcinogen, I-3-C acted as a tumor promoter.	/81/
Rat	Liver	Dietary I-3-C decreased GSTP positive foci 40 weeks after treatment with MNU, NDEA or NDBA	/82/
Trout	Liver	I-3-C promoted AFB_1 induced tumors.	/83/
Rat	Tongue	I-3-C completely inhibited 4-NQO carcinogenesis.	/84/
Rat	Liver, Lung, Intestine	Oral I-3-C or ARXM of I-3-C induced P450-associated enzyme activities in all three organs, and reduced BaP-DNA binding in liver and lung. IP injection of I-3-C did not alter enzyme activities or BaP binding to lung DNA.	/85/

In vitro	CHO cells	I-3-C protected against cytotoxicity produced by nitropyrenes.	/86/
Rat	Liver	Dietary I-3-C induced protein levels of CYP1A1, 1A2, 2B1/2 and 3A1/2. I-3-C also promoted the de oxification of AFB_1 to AFM_1.	/87/
Rat	Liver	Dietary I-3-C inhibited *in vivo* AFB_1-DNA adduction. I-3-C substantially elevated protein levels of GST-Yc2.	/88/
Rat	Liver	Dietary I-3-C given before initiation with DEN decreased GSTP positive foci but when given postinitiation, increased number and area of GSTP-positive foci	/89/
Trout	Liver	I-3-C ARXM injected into embryos protected against AFB_1-DNA binding and liver tumors after one year.	/90/
Rat	Mammary	Oral I-3-C decreased DMBA and MNU induced tumors.	/91/
Rat	Colon, Liver, Mammary	Dietary I-3-C decreased PhIP-DNA adducts in the three tissues examined.	/92/
Trout	Liver	Dietary I-3-C decreased AFB_1-DNA binding which was not associated with EROD activity.	/93/
Trout, *in vitro*	Liver, *Salmonella*	I-3-C ARXM, DIM and CTr inhibited AFB_1 mutagenesis with trout liver S9 activation. AFB_1-8,9-epoxide mediated DNA binding and mutagenicity were also inhibited.	/94/
Rat	Colon	Dietary I-3-C was a potent inhibitor of PhIP mediated aberrant crypt foci formation.	/95/
Mouse	Melanoma	Dietary I-3-C or I-3-C AXRM reversed activation of *MDR-1* and thereby sensitized MDR tumors to chemotherapeutic drugs.	/96/
Rat	Colon, Liver	Dietary I-3-C inhibited hepatic IQ-DNA adducts and colonic aberrant crypt foci formation from IQ. Western blot and enzyme assay showed that I-3-C induced CYP1A1 over 1A2 and as a result altered the metabolic profile of IQ.	/97/
Mouse	Lung	Dietary I-3-C decreased the incidence and multiplicity of lung tumors produced by NNK.	/98/
Rat	Liver, Thyroid	Dietary I-3-C promoted hepatic GSTP positive foci and thyroid gland tumors produced by combination treatment with the carcinogens NDEA, MNU and DHPN.	/99/

Human	Urine	Oral I-3-C decreased levels of urinary NNK metabolites, which was associated with a steady-state increase in the metabolism of this lung carcinogen.	/100/
Mouse	Liver	Chronic dietary I-3-C decreased NDEA initiated liver tumors at eight months.	/101/
Rat	Colon	Dietary I-3-C decreased the formation of aberrant crypt foci by IQ, associated with ring hydroxylation and inactivation by CYP1A1, and associated with *N*-hydroxylation and activation by CYP1A2.	/102/
Rat	GI tract; Liver	Dietary I-3-C altered expression and activity of GST isoforms, which partly explained its anticarcinogenic effects in the GI tract.	/103/
Mouse	Skin	Topical I-3-C, after applying DMBA and prior to TPA application, decreased the number and multiplicity of skin tumors and delayed tumor onset.	/104/
Rat	Liver	Dietary I-3-C inhibited AFB_1 liver tumors by blocking initiation and inhibiting ODC and tyrosine kinase activity. Tumorigenesis was predicted by expression of the late biomarker cytokeratin 18.	/52/
In vitro	Mammalian cell lines	I-3-C inhibited proliferation in several cell lines and this may be related to the inhibition of ODC activity.	/53/
Mouse	Bone marrow	Oral I-3-C protected against cyclophosphamide-mediated bone marrow cell micronuclei formation.	/105/
Rat	Liver	In tissue slices DIM induced CYP1A, 2B and 3A and decreased AFB_1 cytotoxicity. I-3-C had no effect.	/106/
Trout	Liver	Dietary I-3-C promoted AFB_1 liver tumors, an effect better correlated with estrogenicity (vitellogenin induction) than with CYP1A induction	/107/
Rat	Many	Dietary I-3-C inhibited DNA adducts formed from PhIP and IQ in several non-target organs and the target organ, mammary gland. This appeared to be mediated by increasing PhIP metabolism through CYP1A1 and 1A2.	/108, 109/
Mouse	Bone marrow	Oral I-3-C protected against cyclophosphamide-induced chromosomal aberrations in bone marrow cells.	/110/
Rat	Many	Oral I-3-C diminished the formation of major and minor DNA adducts, determined by ^{32}P-postlabeling, in lung, trachea and bladder of rats exposed to second-hand cigarette smoke.	/111/

B. I-3-C and Hormone-mediated Carcinogenesis

Model	*Tissue*	*Reaction or Response*	*Ref.*
Human	Urine	Dietary I-3-C increased estradiol 2-hydroxylation, as indicated by urinary metabolites.	/55/
Human	Urine	Dietary I-3-C increased the amount of urinary 2-hydroxyestrone relative to estriol in both men and women.	/58/
Rat	Liver	Oral I-3-C increased mixed function oxidase mediated production of 2,3- and 3,4-catechol estrogens from estradiol.	/57/
Mouse	Liver, Mammary	Hepatic P450 mediated 2-hydroxylation of estradiol is correlated with a decrease in mammary tumor incidence and multiplicity, and prolonged tumor latency in chronic I-3-C feeding studies.	/56/
Mouse	Liver	Dietary I-3-C induced increased EROD, 2-hydroxylation of estradiol and 6α-hydroxylation of testosterone. I-3-C ARXM directly inhibited microsomal 6β-hydroxylation of testosterone	/112/
Mouse	Larynx	Oral I-3-C increased the ratio of 2-hydroxyestrone to 16α-hydroxyestrone, which correlated with a decrease in laryngeal papilloma cysts produced by HPV-11.	/59/
In vitro	Human breast cancer cell lines	I-3-C enhanced 2-hydroxylation of estradiol and induction of CYP1A1 in estrogen-sensitive MCF-7 cells, but not in estrogen-insensitive MDA-MB-231 cells. These observations correlated with I-3-C anti-growth effects in MCF-7 cells, but not in MDA-MB-231 cells.	/63/
Rat	Mammary, Endometrium, Uterus	Dietary I-3-C inhibited endometrial and uterine adenocarcinoma. I-3-C also inhibited pre-neoplastic endometrial lesions and the incidence of mammary fibroadenoma. These effects correlated with the induction of estradiol 2-hydroxylation.	/113/
Rat	Liver	Oral I-3-C or ascorbigen increased the production of 2-hydroxyestradiol and 2-hydroxyestrone from estradiol in microsomes.	/62/
In vitro	MCF-7 cells	I-3-C induced 2-hydroxylation, but not 16α-hydroxylation of estradiol.	/61/
Human	Urine	Dietary I-3-C produced a sustained (3 month) increase in the ratio of urinary metabolites, 2-hydroxyestrone to estriol.	/114/

Human	Urine	Oral I3-C in men and women increased excretion of 2-hydroxylation products of estradiol, with concomitant decreases in urinary estradiol, estrone, estriol and 16α-hydroxyestrone.	/65/
In vitro	Human mammary carcinoma cells	I-3-C inhibited aberrant proliferation and the percentage of cells undergoing proliferation, and increased cellular apoptosis. These effects correlated with an increase in 2-hydroxylation of estradiol.	/67/
Mouse	Liver	I-3-C induced 2-hydroxylation of estradiol and EROD activity.	/115/
Human	Respiratory tract	Preliminary results from a phase I trial showed that oral I-3-C slowed or abated respiratory tract papilloma growth. Results correlated with an increase in the ratio of urinary 2-hydroxylation to 16α-hydroxylation products of estradiol.	/70/
Human	Urine	Dietary I-3-C increased the ratio of estradiol metabolites 2-hydroxyestrone to estriol.	/116/
In vitro	Breast cancer cell lines	DIM and I-3-C inhibited growth in MCF-7 cells. Growth supression with DIM correlated with increased DNA fragmentation and apoptosis.	/117/
In vitro	Human breast cancer cells	I-3-C inhibited [^{3}H]-thymidine incorporation and cell cycle progression, correlating with the dose and time dependent decrease in CDK6. This occurred in both estrogen sensitive MCF-7 and estrogen receptor deficient MDA-MB-231 cells.	/118/
In vitro	Cervical cancer cells	In CaSki cells infected with HPV, I-3-C or 2-hydroxyestrone inhibited estradiol binding to the estrogen receptor, an effect that correlated with induction of CYP1A1.	/68/
Mouse	Cervix, Vagina	Dietary I-3-C inhibited development of cervical-vaginal cancer, and bladder fluid retention, in estradiol treated mice that express transgenes for HPV under a keratin promoter.	/119/
In vitro	Breast cancer cells	LTr-1 inhibited estrogen dependent growth of MCF-7 cells and estrogen independent growth of MDA-MB-231 cells. LTr-1 was a weak antagonist of the estrogen receptor and the aryl hydrocarbon receptor, and competitively inhibited EROD activity.	/120/
In vitro	MCF-7 cells	I-3-C, in combination with tamoxifen, reduced CDK2 enzyme activity but not protein levels, and ablated the phosphorylation of Rb. Neither compound was effective individually. I-3-C did not compete with estradiol for the estrogen receptor.	/73/

In vitro	Breast cancer cells	CTr stimulated proliferation of estrogen-responsive MCF-7 cells, but not estrogen-independent MDA-MB-231 cells. CTr displaced estradiol from the estrogen receptor suggesting that it acts analogous to tamoxifen.	/71/
In vitro	Breast cancer cells	I-3-C inhibited fibronectin-stimulated cell adhesion, migration and invasion in estrogen responsive MCF-7 and estrogen receptor-negative MDA-MB-468, human breast cancer cell lines. I-3-C also suppressed estradiol-stimulated migration and invasion of MCF-7 cells, and increased E-cadherin, major catenins and BRCA1 expression.	/74/

C. I-3-C and Non-cancer Endpoints

Model	*Tissue*	*Reaction or Response*	*Ref*
Rat	Colon, Liver	Oral I-3-C increased CYP1A1 and 1A2 mRNA in colon and liver.	/121/
Rat, *In vitro*	Feces, Hepa-1 cells	In the presence of fecal bacteria, either I-3-C or tryptophan form AHR ligands. Tryptophan metabolites increased EROD activity in Hepa-1 cells but not in ARNT defective *c4* cells	/122/
Mouse, *In vitro*	Liver, Hepa-1 cells	I-3-C ARXM induced various drug-metabolizing enzymes through the AHR. ICZ was the most effective inducing agent, with a K_d = 190 pM for AHR binding and EC_{50} = 269 nM for induction of CYP1A1.	/35/
Mouse	Liver	The I-3-C acid condensation product ICZ is produced *in vitro*, and also *in vivo* following oral intubation with I-3-C. ICZ and related compounds are responsible for AHR-mediated enzyme induction.	/123/
In vitro	Cell-free	In acidic aqueous solution, I-3-C forms DIM, CTr, LTr-1 and ICZ. Linear and cyclic tetramers were also tentatively identified.	/34/
Monkey, Rat, *In vitro*	Primary hepatocytes	DIM, CTr, and BII induced EROD, 7α-hydroxylation of testosterone, and NQO1 in rat hepatocytes, but decreased activities of 16α- and 2α-hydroxylation of testosterone. Similar, but not identical patterns of change were observed in monkey hepatocytes.	/124/

Rat, *In vitro*	Liver, Small intestine, Primary hepatocytes	Dietary I-3-C induced hepatic CYP1A1, CYP1A2 and CYP2B1 apoprotein levels, and levels of CYP2B1 and CYP1A1 in the small intestine. Dietary I-3-C induced GST, UGT and NQO1 in both the liver and small intestine. DIM and I-3-acetonitrile but not I-3-C enhanced EROD and decreased 16α- and 2α-hydroxylation of testosterone.	/125/
Mouse	Liver	GST isoforms are induced by I-3-C, while I-3-C ARXM inhibit methyltrienolone (steroid) binding to GST	/126/
Mouse	Blood	Oral I-3-C reduced serum cholesterol levels.	/54/
Rat	Liver	Oral I-3-C increased the 6β-hydroxylation of andosterone, but decreased the amount of CYP3A1/2, the major P450 isoform considered responsible for 6β-hydroxylation.	/127/
Trout	Liver	I-3-C, DIM, CTr, and I-3-C AXRM were injected into trout embryos. Hepatic CYP1A1 and EROD were induced through the AHR, and irreversibly inactivated by I-3-C AXRM.	/128/
Rat	Liver	Hepatic levels of I-3-C metabolites, following dietary exposure, were individually insufficient to induce CYP1A1 or inhibit activation of AFB_1. However, the combination of I-3-C derivatives may be sufficient to produce these biological responses.	/36/
In vitro	Human breast cancer cells	I-3-C or DIM did not induce CYP1A1, but inhibited TCDD-induced EROD and CYP1A1 mRNA in T47D human breast cancer cells.	/129/
In vitro	Cell-free	I-3-C inhibited lipid peroxidation, a parameter that was predicted by the heat of formation of the indole cation radical and partition coefficient values.	/44/
In vitro	Cell-free	I-3-C scavenged a metastable synthetic free radical, and generate indole-3-aldehyde	/130/
Rat	Intestine, Liver	Dietary I-3-C inhibited FMO1 activity and expression in both the intestine and liver.	/131/
Rat	Testes	Oral I-3-C administered to pregnant rats produced a decrease in sperm production in male offspring.	/132/
Yeast		I-3-C was an exceptionally potent AHR agonist in yeast (EC_{50} values approximately 10 μM).	/133/

Human	Urine	Men fed Brussels sprouts had a decreased ratio of trimethylamine to trimethylamine *N*-oxide in the urine, indicative of a decrease in hepatic FMO activity. I-3-C ARXM were potent competitive inhibitors of human FMO3, the major human isoform	/134/
Rat, *In vitro*	Liver, Hepa-1 cells	*N*-methoxy-I-3-C induced EROD activity and CYP1A1 mRNA in Hepa-1 cells with 10 fold greater efficacy than I-3-C. However, oral *N*-methoxy-I-3-C was not as effective as I-3-C in inducing hepatic CYP1A1 and 1A2 in rats. Ascorbigen was a weak AHR inducing agent *in vivo*.	/39/
Rat	Immune	Dietary I-3-C inhibited natural killer cell activity and increased T-cell mediated delayed-type hypersensitivity.	/135/

The table includes literature regarding I-3-C effects on biotransformation, chemoprotection and chemoprevention from 1990 to the present.

Abbreviations: AFB_1, aflatoxin B_1; AFM_1, a DNA binding reactive metabolite of aflatoxin B_1; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; ARXM, acidified reaction mixture; BII, 2,3-bis[3-indolylmethyl]indole; CDK, cyclin dependent kinase; CHO, Chinese hamster ovary cells; CTr, 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b'*:7,8-*b''*]triindole, cyclic triindole; DHPN, dihydroxy-di-*N*-propyl-nitrosamine; DIM, 3,3′-diindolylmethane; di(indol-3-yl)methane; DMBA, dimethylbenzanthracene; EROD, 7-ethoxyresorufin *O*-deethylase; FMO, flavin-containing monoxygenase; GSH, glutathione; GST, glutathione S-transferase; GSTP, glutathione S-transferase π form, GSTP1-1, GST3, GST-Yf; HPV, human papilloma virus; IQ, 2-amino-3-methylimidazo[4,5-*f*] quinoline; MDR, multi-drug resistance gene; MNS, methylmethanesulfonic acid; MNU, methylnitrosourea; NDBA, *N*-nitroso-dibutylamine; NDEA, *N*-nitrosodimethylamine; NNK, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone; NQO1, NAD(P)H:quinone oxidoreductase; 4-NQO, 4-nitroquinoline 1-oxide; ODC, ornithine decarboxylase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine; Rb, retinoblastoma protein; SOD, superoxide dismutase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TPA, 12-*O*-tetra-decanoylphorbol 13-acetate; UGT, UDP-glucuronosyltransferase.

activities related to cytochromes P450. The concept of bioactivation (the process of metabolic activation of compounds to proximate toxicants and carcinogens) became firmly established in the 1960s. Many such activation events were linked to carcinogen activation by cytochromes P450-mediated mixed-function oxidase activities.

In the early 1970s Wattenberg and co-workers performed their pioneering work in establishing that *Brassica* vegetables of the family Cruciferae (cruciferous vegetables) were capable of reducing the risk of tumor development in experimental animals /8-15/. Work by other investigators, notably Stoewsand, Babich and co-workers, supported and extended these findings /16-20/. These studies also established that dietary cruciferous vegetables could induce the activities of aryl hydrocarbon hydroxylase and other mixed-function oxidase activities that were associated with metabolic activation of carcinogens of the breast liver, lung and intestinal tract organs. Phase II conjugation enzymes were also induced by feeding cruciferous vegetables to experimental animals /21-23/.

The component of cruciferous vegetables responsible for enzyme induction was soon identified as glucobrassicin (indolylmethylglucosinolate), the structure of which had been identified by the Virtanen laboratory in 1961 /24/. This family of compounds is metabolized both enzymatically and nonenzymatically to produce a large number of diverse reaction products. Among these products, Loub *et al.* /15/ identified three mixed-function oxidase-inducing indoles, namely indole-3-carbinol (I-3-C), indole-3-acetonitrile and diindolylmethane (DIM). I-3-C appeared to be the major reaction product and became the primary focus indolic compound in chemoprevention research.

During the 1970s and early 1980s, evidence accumulated on the ability of I-3-C to induce mixed-function oxidase enzyme activities associated with metabolism, and especially activation, of carcinogens and toxicants /15,16,25-27/. Many of these activities became associated with cytochromes P450 1A1 (CYP1A1) and P450 1A2 (CYP1A2), enzymes that are encoded by genes under the control of the aryl hydrocarbon receptor (AHR) /28,29/. This cytosolic ligand-activated AHR sheds its chaperone proteins, translocates to the nucleus and binds aryl hydrocarbon receptor nuclear translocase (ARNT), to form a heterodimeric transcription factor that binds the aryl hydrocarbon response element. A number of genes are controlled

by the AHR, including genes of the *Ah* battery (CYP1A1, CYP1A2, CYP1B1, and four phase II genes) /30/.

An intriguing finding in some early studies related to AHR-mediated induction by I-3-C was that the efficacy of induction was greatest when the compound was administered *p.o.* (gavage) /25,31/. Such studies suggested that modification of I-3-C in the digestive tract generated products that were the true inducing compounds *in vivo*. Such enzyme-inducing acid condensation products (Fig. 1) were described about a decade ago /32-35/, and have since been shown to be responsible for many of the biological responses that had previously been ascribed to I-3-C. In a carefully-performed pharmacokinetics study, Stresser *et al.* /36/ showed that following oral administration of high dosages of I-3-C to male rats, the concentrations of the three major putative AHR-inducing acid condensation products (DIM, LTr-1 and ICZ [see Fig. 1]) did not individually reach sufficient hepatic concentrations to account for the observed induction of CYP1A1. If this finding were extrapolated to other animal models, it could suggest that activation of the AHR occurs by the synergistic action of many I-3-C-derived products. Alternatively, it might suggest that other acid condensation products are formed from I-3-C that were not considered in the study. The latter possibility was strengthened by the identification of additional indole-containing acid reaction products derived from glucobrassicin (or I-3-C) and ascorbic acid. The structure of the parent compound ascorbigen (3-skatyl ascorbate) was elucidated in 1966 /37/, and the group of compounds includes a number of acid and alkaline reaction products, as well as *N*-alkoxy (especially *N*-methoxy) derivatives (Fig. 1) /2,3/.

3. BIOTRANSFORMATION OF GLUCOBRASSICIN

Recent reviews have described the complex behavior of the myrosinase reaction products of glucobrassicin metabolism /2,3/. The electronic charge-directing properties of the indolic nitrogen, and the aromaticity of the fused ring system, make the 3-position carbon particularly reactive, with lesser reactivity at the 5-carbon and 2-carbon of the indole ring. These reactive centers, especially under acidic conditions and in the presence of ascorbic acid, produce a myriad of nonenzymatic derivatives, with the major acid condensation reaction products shown in Figure 1. The reaction products that have

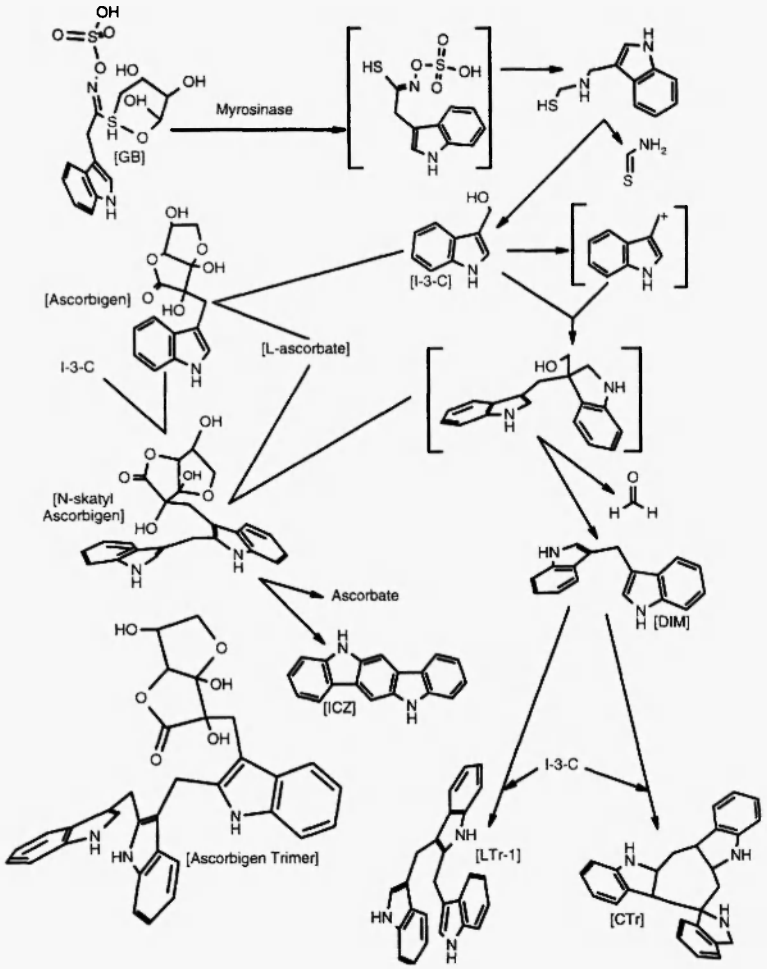

Fig. 1: The major indole reaction pathways from glucobrassicins [GB].
The pathways depicted in the figure were adapted from references /2,3,10,33/. With the exception of the initial myrosinase reaction, all of the reactions occur nonenzymatically. In order to depict the representative structures shown in the figure, we performed molecular modeling for each compound. The lowest energy molecular conformations were calculated with HyperChem Molecular Modeling software (Hypercube, Inc.), using the Austin Model 1 (AM-1) with Polak-Ribiere conjugate gradient algorithm and UHF spin pairing with a 0.01 convergence limit in vacuo. The figure, using the energy-optimized configurations, was created using ISIS Draw (MDL Information Systems, Inc.). Unstable intermediates are enclosed in large brackets, and the compound identities are enclosed in small brackets. **Abbreviations:** CTr = 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*′′]triindole, cyclic triindole; DIM = 3,3′-diindolylmethane, di(indol-3-yl)methane; I-3-C = indole-3-carbinol, 3-hydroxymethylindole, indole-3-methanol; ICZ = 5,11-dihydroindolo[3,2-*b*]carbazole; LTr-1 = 2-(indol-3-ylmethyl)-3,3′-diindolylmethane, linear triindole-1, skatole, 3-methylindole.

received the most attention, due to their cytochrome P450 inducing capabilities, are 3,3′-diindolylmethane (DIM), 5,6,11,12,17,18-hexahydrocyclononа[1,2-*b*:4,5-*b*′:7,8-*b*′′]triindole (CTr), and 5,11-dihydroindolo[3,2-*b*]carbazole (ICZ). ICZ is a particularly noteworthy I-3-C metabolite, since it has an exceptionally high binding affinity for the AHR, comparable to 2,3,7,8-tetrahydrodibenzo-*p*-dioxin (TCDD, dioxin) /38/.

Although they are similar, the pattern of indole-containing acid condensation products derived from cruciferous vegetable and those derived from I-3-C are not identical. Significant differences may exist between the indole-containing acid condensation products derived from cruciferous vegetables, and those derived from I-3-C. Such differences may be attributed, in part, to differing availabilities of L-ascorbic acid to condense with glucobrassicin and 1-3-C reaction products. Additionally, other indolic species are present in plants. For example, the *N*-alkoxy indole (tertiary amine) metabolites are present at about 10% of the levels of the secondary amine forms, and have received little attention in toxicological research. *N*-Methoxyindole-3-carbinol is a relatively more potent AHR agonist and inducer of CYP1A1 than I-3-C in cultured mouse hepatoma cells, but less effective than 1-3-C *in vivo* /39/. Other *N*-alkoxy ascorbigens and indole acid condensation products should be examined for biological responses.

Important differences may also exist in the pharmacokinetics of the products formed from plants, versus those formed from pure I-3-C. For example, 1-3-C as a bolus may have untoward biological activity associated with its rate of metabolism and absorption, that is not associated with vegetable consumption. Thus, I-3-C-mediated tumor promotion, that is observed in animal models under very restrictive conditions, may not be associated with eating vegetables under any condition. The biological responses relating to the formulation of indole-containing compounds in the diet, or as dietary supplements, clearly requires additional research.

4. OTHER BIOCHEMICAL PROPERTIES OF I-3-C RELATED TO CHEMOPROTECTION

In addition to enzyme induction, I-3-C was found to have a number of interesting biochemical properties that were associated with its

chemoprotective efficacy. One such property is an increase in nucleophilicity of liver tissue extracts following treatment of mice with I-3-C /40/. The nucleophilicity was not due to the parent compound, but presumably due to metabolite(s) derived from I-3-C. Nevertheless, increased tissue nucleophilicity was strongly associated with protection of mice from *N*-nitrosodimethylamine-mediated cytotoxicity and genotoxicity. Another interesting property of I-3-C is its ability to reduce membrane fragility and stabilize biological membranes from osmotic damage /41/. This property is closely correlated with the ability of I-3-C to inhibit cytotoxicity produced by methylmethanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. A mechanistic relationship between chemoprotection and membrane stability may be related to the destabilizing effects of many cytotoxic compounds, leading to loss of cellular compartmentation.

Another important biochemical property, shared by all compounds containing the indole nucleus, is that of radical scavenging /41-45/. The chemical center for antioxidant activity resides on the anilinic nitrogen atom containing a lone pair of electrons. Antioxidant potential for indolic compounds depends on electron delocalization over the aromatic system, as well as the ease of oxidation (electron abstraction to form the indolic cation radical). While I-3-C has an efficacy for inhibition of lipid peroxidation about 16-fold less than that of α-tocopherol, other indole-containing compounds, especially indenoindoles, have efficacies 10-fold to 100-fold greater than α-tocopherol /41,45-48/. The property of radical scavenging appears to contribute to the chemoprotective properties of indole-containing compounds toward chemical toxicity /26,27,41,42,49,50/, as well as benzo[*a*]pyrene-induced skin tumors in mice /51/.

A potentially important target for chemoprevention by I-3-C is its ability to inhibit ornithine decarboxylase (ODC). This enzyme is the rate-limiting step in polyamine synthesis and is required for DNA replication and cell proliferation. It was shown that long-term feeding of 0.5% I-3-C to rats inhibited the activity of ODC, and this was related to the observed protection against aflatoxin B_1 liver cancer /52/. Similarly, another indole chemoprotective compound, 4*b*,5,9*b*,10-tetrahydroindeno[1,2-*b*]indole, protected against benzo[*a*]pyrene-mediated skin cancer, and this correlated with an inhibition of ODC /51/. While I-3-C itself inhibited ODC activity in cultured tumor cell lines only at very high concentrations /53/, there is the likelihood that

in vivo the effectors of ODC activity are acid condensation products derived from I-3-C, rather than the parent compound. Additional work is therefore needed in this area of research.

5. EMERGING AREAS OF RESEARCH

The research performed to date regarding chemoprevention and chemoprotection by dietary indoles has focused primarily on the ability of these compounds to modify the metabolism of environmental chemical carcinogens. In recent years, attention has turned in part to effects not directly related to environmental exposures, but rather modification of endogenous metabolic and cell signaling pathways. In this regard, LeBlanc *et al.* /54/ demonstrated that I-3-C could alter the hepatic metabolism of cholesterol and modify serum cholesterol levels. Perhaps the greatest attention in recent years has concerned the modification of endogenous estrogen metabolism and the risk of developing tumors that are dependent on estrogen signaling pathways.

The work of Fishman, Michnovicz, and others, and especially Bradlow and co-workers /55-67/ has clearly shown that the metabolism of β-estradiol is altered by I-3-C, or by I-3-C reaction products, to increase 2-position hydroxylation, and concomitantly decrease the formation of the genotoxic metabolites 16α-hydroxyestrone and 4-hydroxyestrone (leading to 3,4-catechol estrogen). This alteration in endogenous estrogen metabolism has been confirmed in human studies, and has important implications in diagnosing and decreasing the risk of developing estrogen-associated cancer of the breast /58,65/, endometrium /64/, cervix /68,69/, and larynx /59,60,70/. These studies have moved the use of I-3-C to clinical chemoprevention trials. The question of whether I-3-C, or a reaction product generated from I-3-C, would be a better chemoprotector remains an issue. In this regard, Riby *et al.* /71/ has recently demonstrated that CTr, the major cyclic acid condensation product of I-3-C, is a strong estrogen receptor ligand that may act as an estrogen receptor antagonist in a manner analogous to tamoxifen.

An exciting recent report by Cover *et al.* /72/ showed that growth of cultured MCF-7 human breast cancer epithelial cells could be arrested in G_1 phase by I-3-C. This effect appeared to be related to an inhibition of the phosphorylation of cyclin-dependent kinase-6, a G_1-

S-promoting cyclin in its phosphorylated form. I-3-C also inhibited phosphorylation of the tumor suppressive retinoblastoma protein, an E2F-binding protein in the hypophosphorylated state (associated with G_1-S cell cycle arrest). In a sequel, Cover *et al.* /73/ demonstrated that I-3-C and tamoxifen, in combination, ablated the expression of phosphorylated retinoblastoma protein, down-regulated the expression of cyclin-dependent kinase-6, and inhibited the enzyme activity (but not the gene expression) of cyclin-dependent kinase-2.

Meng *et al.* /74/ has very recently demonstrated that I-3-C had the potential to act as an anti-carcinogen at the metastatic stages of cancer development. At non-toxic concentrations, I-3-C inhibited fibronectin-stimulated cell adhesion, migration and invasion in human breast cancer epithelial cells. Inhibition occurred in both estrogen-responsive MCF-7 cells, as well as in estrogen receptor-negative MDA-MB-468 cells. I-3-C also suppressed β-estradiol-stimulated migration and invasion of MCF-7 cells, and increased E-cadherin, three major catenins and BRCA1 expression. Thus, I-3-C has the potential to suppress the invasion and the migration of human breast cancer cells, and thereby diminish the metastatic potential of the primary invasive tumor.

6. SYNOPSIS AND PERSPECTIVE

The basic tenet in the science of toxicology is that all things are toxic in some concentration. All forms of nourishment contain potentially deleterious components, yet we do not normally worry about toxicity inherent in a normal diet. Similarly, the finding that a specific component of the diet may prove beneficial under certain conditions does not imply that it is generally beneficial under all conditions. We may think of a micronutrient as a drug, with a dose-response relationship for benefit and for toxicological risk. We need to evaluate benefit-risk relationships for micronutrients in order to determine the therapeutic window of dose that maximizes the benefit-risk ratio. Moreover, we can view micronutrient chemoprevention as a polygenetic trait, whereby a complex phenotype is under the control of combinations of many different genes, with each individual gene being subdominant. In an analogous fashion, a single micronutrient may have a small effect on mitigating a disease trait, but combinations of micronutrients may show substantially greater effects.

For more than a decade, dialog has existed regarding the most promising reaction products derived from consumption of cruciferous vegetables, that could be used in clinical trials in populations at risk for developing cancer. At the center of this discussion was the observation that I-3-C *per se* is not produced upon ingestion of chemoprotective cruciferous vegetables. This is because indole-containing myrosinase-generated breakdown products of glucobrassicin condense rapidly following damage to the vegetable material by mastication or crushing /2,3/. Such products are formed with and without ascorbic acid (found in the ingested plant as well as in the host), especially in the acid environment of the stomach. On the other hand, I-3-C ingestion is sufficient to generate the reaction products derived from cruciferous vegetables, providing ascorbic acid is present in the stomach. A coherent discussion of these issues can be found in the paper by Preobrazhenskaya and Korolev, with a response by Michnovicz and Bradlow /75/.

In order to evaluate I-3-C, and I-3-C precursors and products, as potentially beneficial micronutrients, additional information is required. For example, we were unable to find any studies that systematically compared the content of available I-3-C, or its reaction products, in cruciferous vegetables grown in different regions or countries, or under standardized growth conditions. Similarly, there are no studies that evaluate human individual or racial variability in digestive processing or the pharmacokinetics of glucosinolates in the diet. Since enzymatic and nonenzymatic processing of glucobrassicin generates indolic products that far exceed I-3-C in biological potency, what are the dietary and host-specific modulators of glucobrassicin biotransformation? How does ascorbate status (dietary intake, blood and tissue levels) affect the spectrum of indolic products formed from glucobrassicins?

Considering the complexity of indolic metabolism of glucobrassicins, how should proper dosage be established? The answer is clearly related to the mechanism of action of the compound(s) in question. Thus, the use of a glucobrassicin-rich diet, or even I-3-C itself, for management of risk associated with diseases related to estrogen-stimulated cell proliferation (i.e., breast cancer in high-risk individuals or populations) should be based on an established biomarker. In this case, for example, dosing could be based on preliminary noninvasive testing, such as changes in urinary estrogen

metabolites. If a governmental regulatory agency were to recommend the use of I-3-C-containing diets for risk management, then it would seem important to standardize the content of foods for biologically-available indole content. One may argue that a good candidate for standardization is total ascorbigen content of acidified, ascorbate-fortified food homogenates. If I-3-C-supplemented diets were to be recommended, then perhaps ascorbic acid should be part of the formulation.

The emphasis of studies regarding the chemotherapeutic and chemoprotective properties of I-3-C and other micronutrients has shifted in recent years. Prior to 1990, most studies focused on the manner by which I-3-C altered the biotransformation profiles of putative environmental toxicants and carcinogens. Current studies tend to emphasize alteration in the metabolism of endogenous compounds (e.g., estrogen) and cellular signaling pathways (e.g., cell cycle regulation). Such mechanism-based approaches to defining the etiology of I-3-C activity will likely be combined with new genetic approaches (e.g., quantitative trait loci analyses, DNA microarrays, and transgenic animal models) that will provide the experimental basis for translational research and the development of new clinical approaches to risk management through the use of micronutrients such as I-3-C and other forms of indole-containing diets.

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