Pharmacological Exploitation of Indole-3-Carbinol to Develop Potent Antitumor Agents

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Abstract: The antitumor activity of indole-3-carbinol is attributable to its ability to interfere with multiple oncogenic signaling pathways governing cell cycle progression, survival, invasion, and other aggressive phenotypes of cancer cells, especially those mediated by EGFR/Src, Akt, NF-κB, endoplasmic reticulum stress, and nuclear receptors. This broad spectrum of antitumor activities in conjunction with its metabolic instability constitutes the rationale for the structural modifications of indole-3-carbinol and its metabolite diindolylmethane to develop novel classes of antitumor agents with improved potency and distinct mechanisms. Thus, this minireview focuses on the chemical biology of the lead optimization of these indole derivatives.

Keywords: Indole-3-carbinol, bis(3'-indolyl)methane, antitumor agents, OSU-A9, C-DIMs, SR13668.

INTRODUCTION

Ever since the reported epidemiological link between high dietary intake of cruciferous vegetables and lower cancer risk [1-4], there has been a growing interest in exploring the chemopreventive potential of indole-3-carbinol, a common phytochemical found in *Brassica* plants. Accumulating evidence indicates that the antitumor activity of indole-3-carbinol is attributable to its ability to interfere with multiple oncogenic signaling pathways governing cell cycle progression, survival, invasion, and other aggressive phenotypes of cancer cells [5-9]. Reported signaling targets of indole-3-carbinol in various cancer cell lines include EGFR/Src [10], Akt/NF-κB [11-16], stress responses [14, 15], elastase [17], and Rho kinase [18]. Moreover, indole-3-carbinol functions as a negative regulator of estrogen action in hormone-sensitive cancer cells through the inhibition of estrogen receptor (ER)-α signaling [19, 20] and/or induction of cytochrome P-450-mediated estrogen metabolism [21], suggesting its clinical use in hormone-sensitive cancers.

From a mechanistic perspective, this diverse spectrum of antitumor actions underlies the *in vivo* efficacy of indole-3-carbinol in blocking spontaneous or chemically induced tumorigenesis in mammary gland, liver, lung, cervix, and gastrointestinal tract in different animal model studies [22-28]. These preclinical findings have led to the human trials of indole-3-carbinol in cervical dysplasia [29], breast cancer [30, 31], vulvar intraepithelial neoplasia [32], and recurrent respiratory papillomatosis [33], some of which have shown positive results.

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DRAWBACKS OF INDOLE-3-CARBINOL AS AN ANTICANCER AGENT

Despite these advances in the preclinical development of indole-3-carbinol, the following factors may hamper its clinical translation.

1. Low Potency and Poor Cellular Uptake

The dose range required for indole-3-carbinol to suppress cancer cell proliferation is 50-100 μM. This supra-pharmacological concentration is difficult to achieve in tumor sites. This low anti-proliferative potency might be attributed to its poor cellular absorption as a recent report indicates that only 0.3% of indole-3-carbinol in the culture medium entered the cell [18]. This poor cellular uptake in conjunction with the metabolic instability of indole-3-carbinol described below severely restricts the intracellular concentrations that can be achieved, rendering its pharmacokinetic behavior unpredictable. For example, a phase I trial in women showed that indole-3-carbinol was not detectable in plasma following escalating oral doses even up to 1,200 mg [31].

2. Metabolic Instability and Pleiotropic Modes of Action of its Metabolites

The intrinsic instability of indole-3-carbinol in acidic milieu arises from the vinyl hemiaminal moiety of the indole ring (Fig. **1A**, enclosed by dashed line), which is readily susceptible to acid-catalyzed dehydration and polymerization to generate a series of oligomeric products [34], including DIM [bis(3'-indolyl)methane, a dimer] and ICZ (indole[3,2*b*]-carbazole, a dimer), LTr_1 (a liner trimer), CTr (a cyclic trimer), and CTet (a cyclic tetramer) (Fig. **1A**).

Moreover, as each of these major metabolites exhibits its own antitumor activities, the observed chemopreventive effect of indole-3-carbinol *in vivo* is, at least in part, attributed to these metabolic products. Among them, DIM induces

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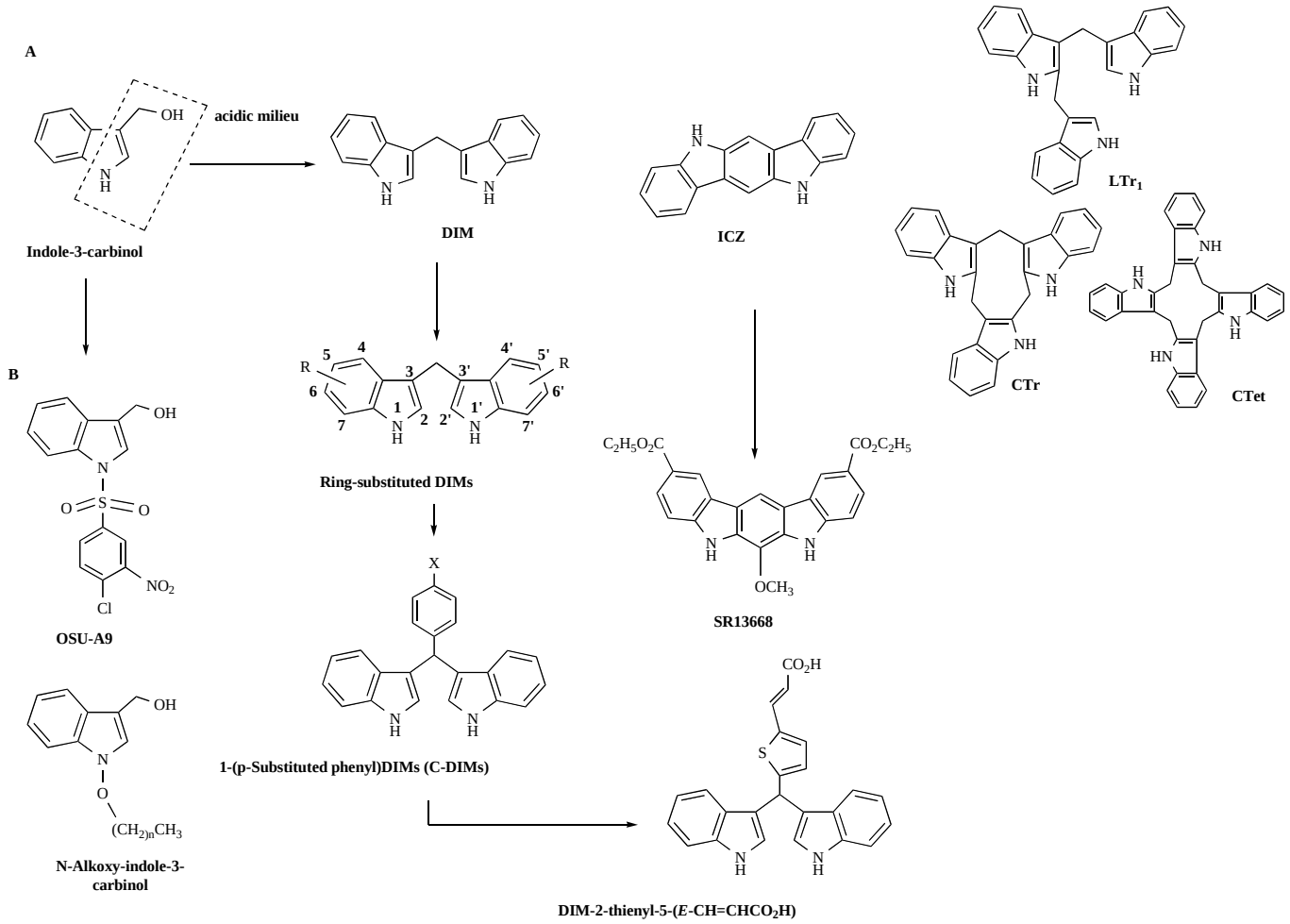


Fig. (1). (**A**) Acid-catalyzed polymerization of indole-3-carbinol to form various oligomeric metabolites. (**B**) Antitumor agents derived from indole-3-carbinol, DIM, and ICZ.

apoptosis and cell cycle arrest through the modulation of signaling targets similar to those affected by indole-3-carbinol, including Akt, NF-κB, endoplasmic reticulum stress, and nuclear receptors, such as ERα and arylhydrocarbon receptor (AhR) [35-47]. In contrast, the functions of the oligomers ICZ, LTr1, and CTr are mainly associated with ERα and AhR [48, 49], while the tetrameric product CTet suppressed breast cancer growth by inhibiting the expression of cyclin-dependent kinase (CDK)6 and other cell cycle regulatory proteins with fivefold higher potency than indole-3-carbinol [50].

3. Hepatotoxicity

Indole-3-carbinol was reported to cause centrilobular hepatocellular hypertrophy in rodents secondary to the induction of the hepatic biotransformation enzymatic system [51, 52]. These pathological changes might underlie the controversy over the role of dietary indole-3-carbinol in increased incidences of uterine adenocarcinoma in animal models [51]. Moreover a recent study using a multi-organ tumorigenesis animal model indicates that, while indole-3-carbinol treatment resulted in an increase in the latency of carcinogen-induced mammary tumor formation, it promoted liver neoplasia [53]. This liver toxicity might limit the long-term use of concentrated indole-3-carbinol supplements for cancer prevention, especially in patients with compromised liver functions.

PHARMACOLOGICAL EXPLOITATION OF INDOLE-3-CARBINOL AND ITS METABOLITES TO DEVELOP NOVEL ANTITUMOR AGENTS

As indole-3-carbinol and its metabolites exhibit low to moderate potencies in suppressing tumor cell proliferation *in vitro*, lead optimization of these compounds to develop novel indole derivatives with improved potency has been the focus of many recent investigations. This drug discovery effort has led to the development of different classes of novel antitumor agents, each of which exhibits a distinct mechanism with enhanced *in vitro* and/or *in vivo* efficacy against different tumor types (Figs. **1B** and **2**).

1. OSU-A9, a Potent, Multi-Targeted Agent with a Pharmacological Profile Identical to that of Indole-3-Carbinol

OSU-A9 was developed in the authors' laboratory from the scaffold of indole-3-carbinol *via N*-substitution of the vinyl hemiaminal function of the indole ring with a benzenesulfonyl moiety, which blocks acid-catalyzed dehydration

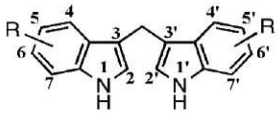
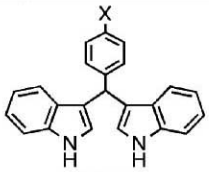
General structure	Derivatives (R =)	Ref.
 Ring substituted DIMs	***Dihalo-substituted DIMs*** 4,4'-dichloro-; 5,5'-dichloro-2,2'-dimethyl-; 6,6'-dichloro-; 5,5'-dibromo-; 5,5'difluoro-	*62*
	Methyl-substituted DIMs 1,1'-dimethyl. 2,2'-dimethyl, 1,1',2,2'-tetramethyl; 5,5'-dimethyl; 6,6'-dimethyl-; 7,7'-dimethyl	*63*
 C-DIMs	***PPARγ-active C-DIMs*** *p*-trifluoromethyl (C-pPhCF$_3$)-; *p*-t-butyl (C-pPhtBu)-; p-phenyl (pPhC$_6$H$_5$)-	*65, 67*
	Nur77-active-DIMs *p*-methoxy (C-PhOCH$_3$)-; H (C-Ph)-	*66, 68*

Fig. (2). Ring-substituted DIMs and C-DIMs.

and polymerization [14]. This modification not only improves the acid stability, but also results in a 100-fold increase in apoptosis-inducing potency as compared to its parent compound. It is noteworthy that OSU-A9 retains all of indole-3-carbinol's characteristic effects on signaling pathways to mediate cell cycle arrest and apoptosis induction. In breast tumor cells, these signaling mechanisms could be categorized into two functional linkages: the Akt-NF-κB axis and stress response signaling (Fig. **3**) [15].

In light of the molecular heterogeneity of human tumors, the ability of OSU-A9 to target multiple signaling pathways by interfering with the interplay between these two signaling networks underscores its therapeutic potential in cancer treatment.

The Akt-NF-κB Axis

Similar to indole-3-carbinol, OSU-A9 blocked signaling pathways mediated by Akt and NF-κB in prostate cancer, breast cancer, and hepatocellular carcinoma cells [14-16]. It is noteworthy that these two indole derivatives inhibited NF-κB signaling through two distinct mechanisms. First, both agents caused accumulation of the NF-κB inhibitor IκB as a result of drug-induced inactivation of IκB kinase (IKK)α, a downstream effect of Akt inhibition, and consequent decrease in IκB degradation. Second, these agents exhibited a unique suppressive effect on the expression of RelA/p65 subunit of NF-κB. The consequent inhibition of NF-κB led to changes in the expression level of a series of NF-κB-regulated gene products, including the downregulation of the antiapoptotic proteins survivin, Bcl-2, Bcl-xL, and Mcl-1, and the upregulation of the proapoptotic protein Bax. Moreover, the repression of the cytokine receptor CXCR4 and the oncoprotein Her2 is noteworthy, as both effects are therapeutically relevant to the treatment of HER2-mediated breast cancer metastasis.

Stress Response Signaling

OSU-A9 and indole-3-carbinol activated p38 and JNK and induced the expression of GADD153, a well-recognized endoplasmic reticulum stress-inducible transcription factor, suggesting the involvement of cellular stress responses in the antitumor effects of these agents [14, 15]. Endoplasmic reticulum stress might also underlie the enhanced expression of breast cancer susceptibility genes BRCA1 and BRCA2 observed in OSU-A9- and indole-3-carbinol-treated breast cancer cells [15], both of which have been identified as tumor suppressors for hormone-sensitive cancers [54]. Changes characteristic of cellular responses to oxidative

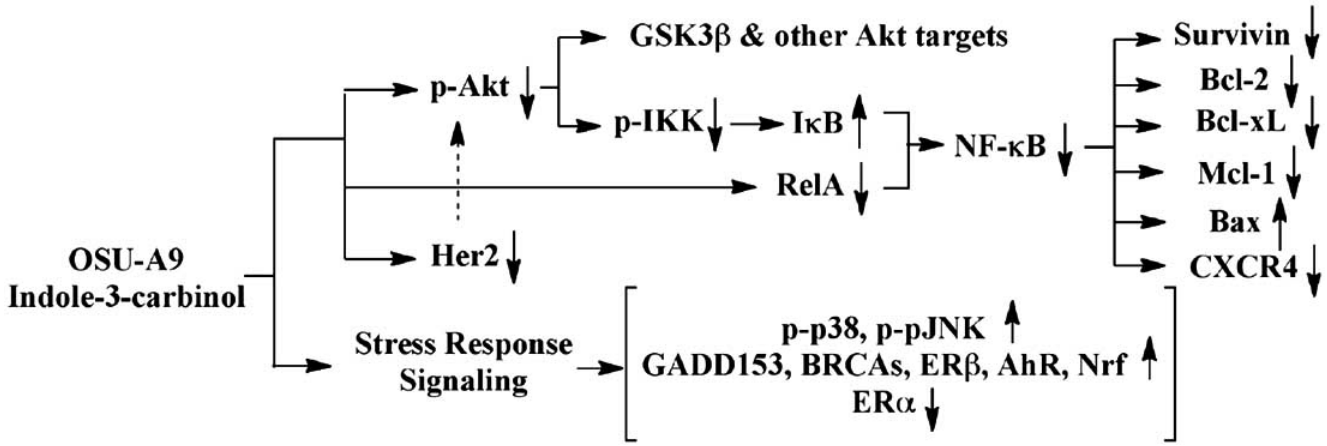


Fig. (3). Schematic representation of the inhibitory and activating effects of OSU-A9 and its parent compound indole-3-carbinol on the Akt-NF-κB signaling axis and stress response signaling, respectively.

stress were also detected in drug-treated breast cancer cells, including the upregulated expression of AhR and its downstream target NF-E2 p45-regulated factor (Nrf2) and the increased expression ratio of ERβ to ERα [15]. These findings are therapeutically relevant as it is well recognized that AhR and Nrf2 control the transcription of genes encoding antioxidants and xenobiotic detoxification enzymes, such as glutathione-S-transferases and NAD(P)H:quinone oxidase 1 [55, 56], and that suppressed ERβ expression characterizes the malignant progression of breast cancer [57].

Despite this broad spectrum of pharmacological activities, nonmalignant cells were less sensitive to the antiproliferative effect of OSU-A9 relative to cancer cell lines. Moreover, oral OSU-A9 has been shown to suppress xenograft tumor growth in various animal models, including that of prostate, breast, and liver, without causing overt toxicity or the hepatic changes associated with indole-3-carbinol [14-16].

2. *N*-Alkoxy Derivatives of Indole-3-Carbinol, Potent Inhibitors of CDK6 Gene Expression

Based on the report that the naturally occurring *N*-methoxy-indole-3-carbinol was a more potent inducer of cytochrome P450 activity in cultured cells than the parent compound [58], Firestone and coworkers developed a series of *N*-alkoxy-indole-3-carbinol derivatives with alkyl chains of one to four carbons in length [59]. Relative to indole-3-carbinol, these *N*-alkoxy derivatives showed a significant increase in the ability to induce G_1 cell cycle arrest in breast cancer cells, which directly correlated with the alkyl chain length, i.e., C_1, 23-fold; C_2, 50-fold; C_3, 217-fold; C_4, 470-fold. The ability of these *N*-alkoxy derivatives to block cell cycle progression at the G1 phase was attributable to the transcriptional suppression of CDK6 expression and the inhibition of CDK2 kinase activity. This growth arrest, however, was not noted when the electron-withdrawing *N*-alkoxy group was replaced by an electron-donating *N*-methyl function, suggesting an essential role of the *N*-alkoxy group in interacting with target proteins(s). The investigators proposed that these *N*-alkoxy derivatives inhibited CDK6 transcription by targeting Sp1 binding to a composite element within the CDK6 promoter, reminiscent of the mechanism of indole-3-carbinol [60].

3. Synthetic DIM Analogues, Modulators of Nuclear Receptors

Safe and coworkers have used DIM to develop a series of analogues with unique pharmacological activities in targeting various nuclear receptors (review: [61]). Structurally, these DIM analogues can be classified into two subclasses (Fig. **2**).

a. Ring-Substituted DIMs

Symmetrical disubstitutions at various positions (1,1', 2,2', 4,4', 5,5', 6,6', and 7,7') of DIM with methyl or halo (Br or Cl) groups gave rise to a series of structural variants, among which 5,5'-diMeDIM and 5,5'-diBrDIM are noteworthy (Fig. **2**) [62, 63]. Both ring-substituted DIMs showed *in vivo* efficacy in suppressing carcinogen-induced rat mammary tumor growth in female Sprague-Dawley rats. Despite structural similarity with DIM, 5,5'-diMeDIM and 5,5'-diBrDIM exhibited mechanisms distinct from that of the parental compound in suppressing cancer cell growth, indicating a subtle structure-activity relationship [64]. For example, 5,5'-diBrDIM is a mitochondrial poison that induced cell death by decreasing mitochondrial membrane potential and inducing endoplasmic reticulum stress, whereas minimal effects on mitochondrial integrity were noted for DIM.

b. 1-(p-Substituted Phenyl)DIMs (C-DIMs)

Substitutions of a proton with bulky aromatic substituents on the methylene group of DIM alter the activity of resulting compounds, i.e., C-DIMs, in interacting with various types of nuclear receptors [65-68]. It is noteworthy that these C-DIMs are no longer AhR agonists, but could activate peroxisomal proliferator-activated receptor (PPAR)γ and/or Nur77 [also known as nerve growth factor (NGF)I-Bα] (PPARγ C-DIMs: [65, 67]; Nur77 C-DIMs: [66, 68]). For example, of the representative C-DIM derivatives depicted in Fig. **2**, DIM-C-pPhtBu and DIM-C-pPhOCH$_3$ are PPARγ-specific and Nur77-specific agonists, respectively, while DIM-C-pPhCF$_3$ transactivates both PPARγ and Nur77. Transactivation of these nuclear receptors activates downstream responses, including the induction of p21, KLF-4, and caveolin 1 [69, 70], leading to cell cycle arrest and induction of cell death pathways as indicated by caspase activation and poly(ADP-ribose)polymerase (PARP) cleavage. In addition, a number of PPARγ- or Nur77-independent signaling mechanisms have also been reported for the proapoptotic activities of these agents. These include inhibition of androgen receptor-mediated signaling [71], induction of proapoptotic genes [68], decreased mitochondrial membrane potential [72, 73], activation of JNK [72, 74], and endoplasmic reticulum stress [75]. Through these complicated modes of action, these agents exhibited *in vivo* efficacy in suppressing xenograft tumor growth in nude mice bearing various types of cancer, including that of pancreas, bladder, and colon. Beyond cancer therapy, DIM-C-pPhtBu also showed neuroprotective effects against apoptosis induced by the Parkinsonian neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in primary striatal neurons by suppressing NF-κB-dependent expression of inflammatory genes in astrocytes [76].

More recently, in light of the intimate relationship between PPARγ/Nur77 and retinoid X receptor (RXR), DIM-C-pPhOCF$_3$ was further used as a scaffold for developing RXR ligands, which led to the development of DIM-2-thienyl-5-(*E*-CH=CHCO$_2$H) a specific RXRα agonist (Fig. **1B**) [77]. This finding underlines the versatility of DIM as a template to develop novel ligands for various nuclear receptors.

4. SR13668, an Akt Inhibitor

Structural modifications of ICZ generated a novel class of antitumor agents, of which SR13668 represents an optimal agent [78] (Fig. **1B**). The antitumor effect of SR13668 was mediated the inhibition of growth factor-stimulated Akt phosphorylation. However, the mode of action of SR13668 in blocking Akt activation is distinct from that of many other Akt inhibitors, i.e., it does not target the ATP binding site.

SR13668 is currently being developed as a chemopreventive agent. A recently published pharmacokinetic analysis indicates that this Akt-targeted agent exhibits poor oral bioavailability in both rats and dogs (< 1%) presumably due to its low absorption [79].

CONCLUSION

Recent studies in different laboratories have clearly demonstrated the versatility of using indole-3-carbinol and its dimeric metabolites DIM and ICZ as scaffolds to develop novel antitumor agents with distinct mechanisms of action. As many of these agents show impressive *in vivo* efficacy in suppressing xenograft tumor growth without causing acute toxicity in various cancer models, there is urgency in the clinical translation of these agents into therapeutic and/or chemopreventive agents.

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ABBREVIATIONS

AhR	=	arylhydrocarbon receptor
BRCA	=	breast cancer susceptibility gene
CDK	=	cyclin-dependent kinase
DIM	=	bis(3'-indolyl)methane
C-DIM	=	1-(p-substituted phenyl)DIM
ER	=	estrogen receptor
ICZ	=	indole[3,2*b*]-carbazole
IKKα	=	IκB kinase α
Nrf2	=	NF-E2 p45-regulated factor
PARP	=	poly(ADP-ribose)polymerase
PPARγ	=	peroxisomal proliferator-activated receptor γ
RXR	=	retinoid X receptor

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