Letters

Computer-Aided Rational Drug Design: A Novel Agent (SR13668) Designed to Mimic the Unique Anticancer Mechanisms of Dietary Indole-3-Carbinol to Block Akt Signaling

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Abstract: Indole-3-carbinol (I3C) is a naturally occurring anticancer agent and has entered clinical trials for cancer prevention. However, the clinical development of I3C has been impeded by its poor metabolic profile. The active components of I3C were used to develop a novel class of indole analogs to optimize I3C's anticancer actions, including blocking growth factor-stimulated Akt activation. The most promising of these analogs, SR13668, exhibited potent oral anticancer activity against various cancers and no significant toxicity.

Indole-3-carbinol (I3C, **1**), a dietary component found exclusively in cruciferous vegetables, is known to suppress proliferation and induce apoptosis of various cancer cells, including breast, ovarian, lung, cervical, colon, prostate, and liver, and was the subject of several early-phase clinical trials for cancer prevention.[1] Numerous anticancer mechanisms of I3C have been proposed,[2,3] but the most intriguing is that I3C was able to selectively inhibit activation (phosphorylation) of Akt in the tumor-derived MDA-MB-468 breast and PC-3 prostate cancer cell lines, but not in nontumorigenic human HBL100 breast and CRL2221 prostate epithelial cells.[4,5] I3C was also reported to abrogate epidermal growth factor (EGF)-induced activation of Akt in PC-3 cells and decrease expression of downstream modulators of the Akt survival pathway.[6] The protein kinase Akt is a major therapeutic target for various cancers known to promote cell survival, invasion, and resistance to hormone-, chemo-, and radio-therapy-induced apoptosis.[7] Several kinase inhibitors of the PI3K/Akt pathway, such as wortmannin and LY294002, have suffered from the negative effects of their broad-spectrum kinase inhibition and unacceptable toxicity. Thus, I3C might offer the promise of selectively inhibiting Akt activation in cancer cells.

However, the clinical development of I3C has been impeded by its poor metabolic profile and low potency. I3C (**1**) itself does not possess anticancer activity, but must be acted on by gastric acid in the stomach to form the active metabolites (acid condensation products or oligomers) responsible for its in vivo anticancer effects.[8] Currently, only four of the over 20 oligomers have been shown to have anticancer activity. These four active oligomers, shown in Figure 1, are DIM (**2**, a linear dimer of I3C), LT (**3**, a linear trimer), CT (**4**, a cyclic trimer), and ICZ (**5**, a planar molecule),[9] and DIM, the major I3C oligomer, has been shown to reduce prostate tumor growth in mice.[10] However, these active oligomers represent less than 20% of the total oligomers formed in vivo, and their formation strongly depends on the pH of gastric acid in the stomach, thus, significant interindividual variation was observed in plasma oligomer values in a phase I clinical trial.[11] Because of the unique anticancer mechanism and apparent safety of I3C, we decided to use the four active I3C oligomers as lead compounds to develop a novel class of indole analogs, which are expected to act by I3C-like mechanisms against several cancers and to offer substantially improved potency and activity over I3C that would make them applicable to cancer therapy as well.

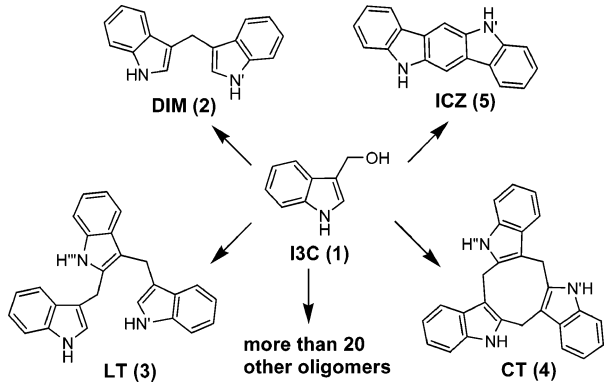

Figure 1. I3C and its four active in vivo oligomers.

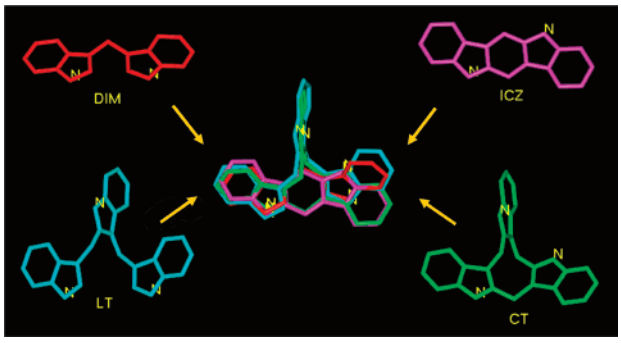

Figure 2. Superimposing the low-energy conformer of each oligomer. To enhance clarity, double bonds and hydrogen atoms are not displayed.

Our lead-based rational drug design was founded on a computer-aided structural analysis of the four chemically distinct active oligomers (DIM, LT, CT, and ICZ) to identify common structural factors responsible for their anticancer activity. Computational analysis of the low-energy conformer of each oligomer, using the SYBYL 7.0 software package (Tripos, St. Louis, MO), revealed that the N−N′ distances were similar in all four active oligomers (5.9 Å for DIM and 5.5 Å for LT, CT, and ICZ). Superimposing their low-energy conformers indeed showed excellent overlap of their indole rings (Figure 2). We speculated that the N−N′ distance might play an important role in their anticancer effects. Our initial structure−activity relationship (SAR) studies thus focused on the effects of the N−N′ distance on the anticancer activity of the oligomers. We chose to use the MCF-7 human breast cancer cell line for our initial biological screen because I3C has entered phase I and II clinical trials for breast cancer prevention.[1] We later expanded our studies to include prostate, ovarian, and lung cancer.

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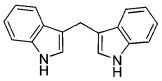
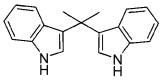
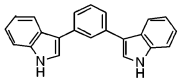
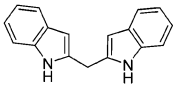
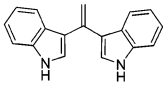
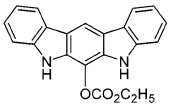
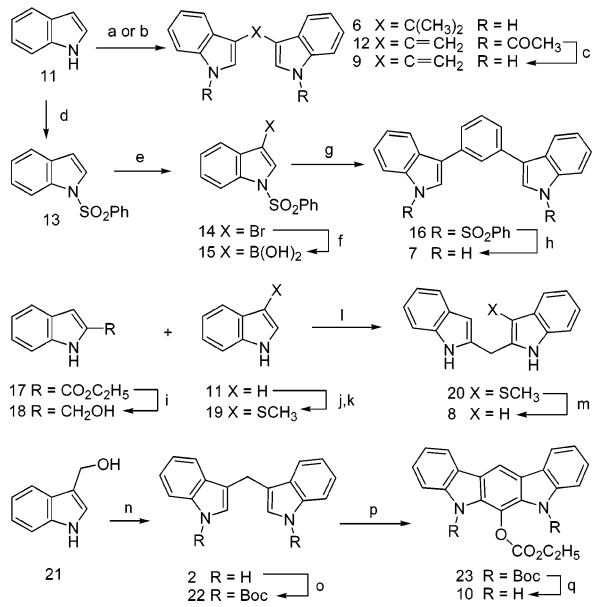
Table 1. Modification of the N−N′ Distance and the Planarity of DIM

Compd	Structure	N-N′ distance (Å)	MCF-7 IC_{50} (μM)[a]
2 (DIM)		5.9	5.5
6		7.0	>10
7		9.0	>10
8		4.7	0.94
9		5.8	3.3
10 (SR13650)		5.1	0.08

[a] IC_{50} is one-half the maximum inhibitory concentration and is the average of quadruple experiments. The highest dose level tested was 10 μM.

Scheme 1[a]



[a] Reagents and conditions: (a) CH_3COCH_3, $Dy(OTf)_3$, EtOH, H_2O, 55%; (b) $(CH_3CO)_2O$, 2-methyl-2-oxazoline, 65%; (c) NaOH, EtOH, H_2O, 80%; (d) $PhSO_2Cl$, NaOH, $(n\text{-}Bu)_4NHSO_4$, CH_2Cl_2, 99%; (e) Br_2, CCl_4, 95%; (f) *t*-BuLi, B(O*i*-Pr)3, THF, H_3O^+, 85%; (g) $Pd(PPh_3)_4$, 1,3-dibromobenzene, aq Na_2CO_3, DME, 75%; (h) K_2CO_3, MeOH, H_2O, 90%; (i) LAH, THF, 98%; (j) NCS, $(CH_3)_2S$, CH_2Cl_2; (k) xylenes, reflux, 63%; (l) $Sc(OTf)_3$, CH_2Cl_2; (m) Raney Ni, EtOH, 65%; (n) 10% aq NaOH, 77%; (o) $(t\text{-}BuOCO)_2O$, DMAP, THF (95%); (p) 2,2,6,6-tetramethylpiperidine (TMP), *n*-BuLi, THF, −78 °C; $ClCO_2C_2H_5$, warmed to 0 °C; (q) CF_3CO_2H, CH_2Cl_2, 72%.

In our lead-based drug design, we tried to minimize structural change in the compounds while optimizing their potency to ensure that our novel indole analogs would retain the safety and unique biological activity of I3C. Analogs **6** to **9** were designed and synthesized to determine the optimal N−N′ distance for anticancer activity (Table 1 and Scheme 1). DIM (**2**) was used as the initial scaffold for chemical modification. Adding a gem-dimethyl group on the one-carbon bridge of DIM afforded **6**, in which steric hindrance caused the N−N′ distance to increase from 5.9 Å to 7.0 Å. Inserting one phenyl ring between the two indole rings of DIM generated **7,** with a minimum -energy N−N′ distance of 9.0 Å. To reduce the N−N′ distance between the two indole rings, we connected the 2 and 2′ positions of indole rings to give **8**, with an N−N′ distance of 4.7 Å. In vitro biological screens showed that both **6** and **7**, with N−N′ distances greater than 6 Å, lacked anticancer activity. Analog **8**, exhibiting improved anticancer activity, was then chosen as the ideal template for further structural modifications.

Unlike the other three active oligomers, ICZ is a planar molecule and was reported to be more potent than DIM in inhibiting the growth of colon cancer cells.[12] We thus designed analog **9** to examine if introducing the planarity into compounds will further enhance their anticancer activity. Changing the hybridization of the carbon bridge atom of DIM from tetrahedral sp^3 to trigonal sp^2 produced **9**, which retained an N−N′ distance similar to that of DIM. In vitro biological results indicate that **9** has retained or slightly improved growth inhibition activity. This interesting result prompted us to design analog **10** (SR13650), in which we incorporated the planarity of **9** into **8**, having an optimal N−N′ distance. Our attempt produced the first potent lead, **10**, with an IC_{50} of 0.08 μM.

To determine if the in vitro anticancer activity of our lead, SR13650, would offer potent tumor growth inhibition in vivo, we conducted a small preliminary tumor xenograft study using MCF-7 cells. SR13650 at 10 mg/kg was administered daily by oral gavage or ip injection for 30 days. We were pleased to find that SR13650 indeed exhibited antitumor effects in animal models at that dose level. We were also disappointed by the low oral antitumor activity of SR13650, as compared to its ip antitumor effect (Table 2). Although the in vivo responses could be different for a given dose depending on the route of administration, a reasonable doubt was that SR13650 might have low oral bioavailability due to its highly lipophilic nature. We thus decided to attempt chemical modifications to improve its oral antitumor effect.

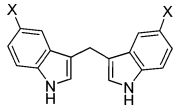
Table 2. In Vivo Antitumor Effect of SR13650

SR13650 (10 mg/kg)	tumor growth inhibition (%)
intraperitoneal	43
oral	26

Increasing the polarity of compounds is known to influence their membrane permeability and thus improve their oral bioavailability. We thus decided to introduce electron-withdrawing groups into SR13650 to increase its dipole moment (polarizability) and possibly its oral bioavailability. To facilitate our SAR study and compound synthesis, we chose to use DIM as a template for selection of a suitable polar substituent that would later be applied on SR13650.

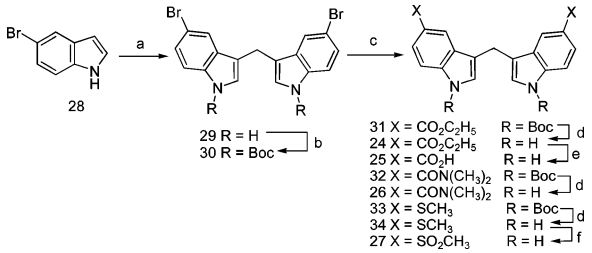
Computational analysis showed that the presence of the polar substituent at the C-5 position of the indole rings would have the most significant effect on their polarizability. The electron-withdrawing groups were thus introduced at the C-5 and C-5′ positions of DIM. Analogs **24** through **27** were designed to evaluate the influence of various functionalities and steric hindrance on the antiproliferative activity of DIM (Table 3). DIM can easily be chemically modified via halogen−metal exchange of 5,5′-dibromo-DIM **29** followed by nucleophilic substitution to give the desired compounds (Scheme 2). Although **24**−**29** all have increased dipole moment, only **24** (ester) exhibited improved antiproliferation activity (Table 3). The lack of activity in **25** (acid), **26** (amide), and **27** (sulfone)

Table 3. Introduction of Polar Substituents into DIM



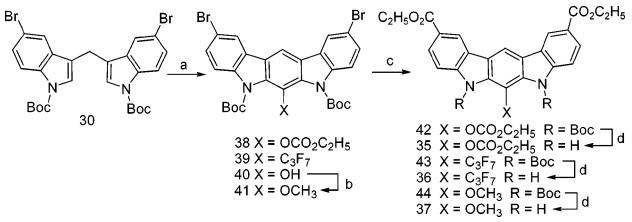
cmpd	R	MCF-7 IC_{50} (μM)	dipole moment (D)
2 (DIM)	H	5.5	0.5
24	$CO_2C_2H_5$	1.75	2.4
25	CO_2H	>10	2.5
26	$CON(CH_3)_2$	>10	7.9
27	SO_2CH_3	>10	5.9

Scheme 2[a]



[a] Reagents and conditions: (a) 37% HCHO, CF_3CO_2H, THF, 60%; (b) $(t\text{-BuOCO})_2O$, DMAP, THF, rt, 94%; (c) *t*-BuLi, THF, −78 °C, $ClCO_2CH_2CH_3$ (**31**; 50%), or $ClCON(CH_3)_2$, THF, −78 °C to 0 °C (**32**; 81%), or CH_3SSCH_3, THF, −78 °C to 0 °C (**33**; 56%); (d) 160 °C, neat, 5 min, 80−90%; (e) aq NaOH, C_2H_5OH, 60 °C, 86%; (f) *m*CPBA, CH_2Cl_2, rt, 82%.

Scheme 3[a]

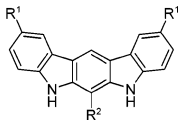


[a] Reagents and conditions: (a) TMP, *n*-BuLi, THF −78 °C, $ClCO_2CH_2CH_3$, warm to −10 °C (**38**; 94%), or $ClCO_2CH_2CH_3$, −78 °C (**40**; 92%), or $(C_3F_7CO)_2O$, warm to 0 °C (**39**; 79%); (b) CH_3I, K_2CO_3, DMF, THF, 91%; (c) *t*-BuLi, $ClCO_2CH_2CH_3$, THF, −78 °C, 80−90%; (d) CF_3CO_2H, CH_2Cl_2, rt, 90−98%.

indicated that, in addition to the dipole moment, the nature of substituents also plays an important role in determining the antiproliferative activity of DIM. The ester functionality was then selected as the best choice for enhancing the dipole moment of indole analogs without diminishing their anticancer activity.

Introducing ester groups into **10** at the C-5 and C-5′ positions on the indole rings created **35** (SR13661), which has an improved dipole moment compared to **10** (Scheme 3 and Table 4). Because fluorine groups are commonly used to increase in vivo drug absorption, we next replaced the carbonate group of **35** with a heptafluoropropyl group to create **36** (SR13667), which had dipole moment comparable with that of **10**. Finally, to maximize the dipole moment of **10**, we introduced both electron-withdrawing ester groups and an electron-donating methoxyl group into the same molecule to create **37** (SR13668), which had greater dipole moment than any other analog. Although all three new indole analogs (**35**−**37**) exhibited in vitro anticancer activity comparable with that of SR13650, in vivo evaluation of their antitumor activity against MCF-7 showed that **35**−**37** indeed exhibited improved tumor growth inhibition (45−60%), as compared to SR13650 (26%), at 10 mg/kg/day after 30 days of treatment. SR13668, with the greatest

Table 4. Modification Dipole Moment of SR13650



cmpd	R^1	R^2	MCF-7 IC_{50} (μM)	dipole moment (D)
10 (SR13650)	H	$OCO_2C_2H_5$	0.08	1.1
35 (SR13661)	$CO_2C_2H_5$	$OCO_2C_2H_5$	0.11	2.4
36 (SR13667)	$CO_2C_2H_5$	C_3F_7	0.09	1.4
37 (SR13668)	$CO_2C_2H_5$	OCH_3	0.19	4.5

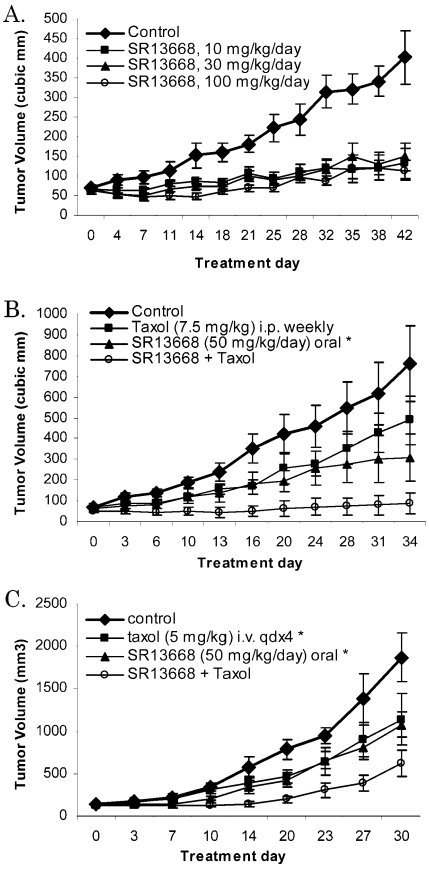


Figure 3. Tumor growth inhibition effects of SR136668 in (A) estrogen-independent MDA-MB-231 breast, (B) androgen-independent PC-3 prostate, and (C) highly drug-resistant SKOV-3 ovarian tumor xenografts. For each study, animals were dosed when tumor sizes reached 70−100 mm^3. The control and test groups consisted of 7−10 mice/group. SR13668 was orally administered daily alone (A) or in combination with taxol (B and C). SR13668 and Taxol alone inhibited tumor growth, but a combination of SR13668 and taxol significantly reduced tumor burden by 70−90% (B and C). qd × 4 means continuous treatment for 4 days (day 1 to day 4); bars, SE; all treatment vs control $P < 0.01$ (ANOVA), except those marked with * ($P < 0.05$).

dipole moment, exhibited the most potent oral antitumor effect in MCF-7 tumor models, so SR13668 was selected for further evaluation in estrogen-independent MDA-MB-231 breast, PC-3 prostate, and SKOV-3 ovarian tumor xenograft models (Figure 3).

In prostate and ovarian tumor models, we also assessed the combination effect of SR13668 and Taxol, a commonly used chemotherapeutic agent. SR13668 exhibited potent oral antitumor activity in all tumor models tested. A pharmacokinetic (PK) study using [^{14}C]-labeled SR13668 in tumor-bearing athymic mice showed that SR13668 was distributed extensively into the

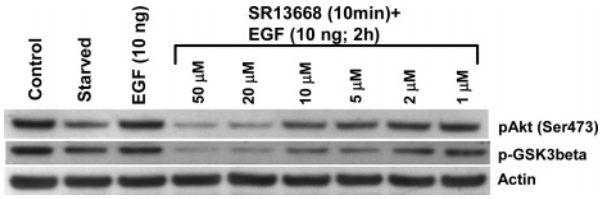


Figure 4. Western blot analysis of pAkt and p-GSK3β in SR13668-treated PC-3 cells. PC-3 cells were starved in serum-free medium. After 24 h, the cells were pretreated with SR13668 at various concentrations for 10 min before stimulation with EGF (10 ng). Cell lysates were prepared 2 h after EGF stimulation and used for Western blot analysis.

tissues and could be detected in tumors 72 h after a single oral dose. However, increasing the oral dose of SR13668 did not proportionally increase its exposure level, which may explained why SR13668 seemed to lack dose-dependent tumor inhibition in the MDA-MB-231 tumor xenograft studies (Figure 3A). We reasoned that this result probably reflected the poor solubility of SR13668 in our oral vehicle (0.5% aqueous hydroxypropylcellulose), where SR13668 formed a suspension that might limit in vivo absorption. As a result of an effort to improve the oral formulation of SR13668, its blood concentration was markedly increased (~30-fold) and its oral antitumor effect could be observed at dose levels as low as 1 mg/kg. To ensure that our novel indole analog also inherited I3C's unique anticancer action to inhibit the Akt signaling pathway, we conducted similar biological studies using SR13668. SR13668 blocked EGF-stimulated Akt activation and inactivated its downstream effector GSK3β in PC-3 prostate cancer cells in a dose-dependent manner (Figure 4). SR13668 was also able to inhibit serum-stimulated Akt phosphorylation in PC-3 and high pAkt expressed MDA-MB-468 breast cancer cells.

Inhibition of the PI3K/Akt signaling pathway by PI3K inhibitor has been shown to induce glucose intolerance and hyperinsulinemia. For example, wortmannin, an irreversible PI3K inhibitor, is known to cause an exaggerated increase in blood glucose concentration. Assessing the effect of SR13668 on glucose metabolism in mice showed that SR13668 has no adverse effects on the fasting glucose levels or body weights after 14 days of oral treatment with SR13668 at 500 mg/kg/day, a dose more than 50 times higher than the dose needed for antitumor activity. Screening a broad selection of 32 kinase targets, including Akt(1,2,3), PI3K, and PDK1, indicated that SR13668 is not a kinase inhibitor. SR13668 appears to inhibit Akt activation by blocking growth factor-stimulated Akt phosphorylation, and it does not target the ATP substrate binding site. Preclinical safety studies showed that SR13668 is not genotoxic in Ames mutagenicity tests or in the mouse micronucleus test. In a 14-day toxicity study in which Sprague−Dawley rats were orally dosed with SR13668 at 25, 75, 200, or 600 mg/kg/day, no drug-related mortality, changes in body or organ weights, or other signs of significant toxicity were seen at any dose level. In summary, SR13668, developed and improved from dietary I3C's active oligomers, appeared to inherit I3C's unique anticancer action to inhibit various cancers while sparing normal, nonneoplastic tissues. These results are exciting because a diet-based compound may be safer for use in long-term cancer treatment to maximize disease control while minimizing undesirable side effects.

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Supporting Information Available: Experimental details, characterization data, and details for in vitro and in vivo assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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