Anticarcinogenic and antioxidant activity of diindolylmethane derivatives[1]

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ABSTRACT

AIM: To investigate the synthesis methods and the bioactivity of diindolylmethane (DIM) derivatives. **METHODS:** 1) A 3D-Quantitative Structure-Active Relationships (QSAR) Comparative Molecular Field Analysis (CoMFA) study of 14 DIM derivatives was investigated to predict their anticarcinogenic activity. 2) Based on CoMFA model, a series of new derivatives of DIM were designed and synthesized. 3) Their free radical scavenging and antioxidant potentials were tested using *in-vitro* DPPH radical scavenging and β-carotene antioxidant models. 4) The anticarcinogenic activities of some compounds were tested by using microculture tetrazolium assay (MTT) and sulforhodamine B (SRB) proteochromosomic assays. **RESULTS:** 1) The CoMFA model derived from DIM analogues proved a good predictive ability with q^2 value of 0.827. 2) New designed compounds 3c and 4c exhibited 3-fold more potent radical scavenging activity than reference substance Vitamin E in DPPH model expressed by IC_{50} values. 3) The primary antitumor screening essay showed that some DIM derivatives designed exhibited the inhibitory activities to some tumor cell growth at relatively high concentration, and DIM was the most effective among them. **CONCLUSION:** DIM's 3D-QSAR model is reliable. According to it, eleven DIM derivatives were synthesized, and two derivatives of them possess potent radical scavenging activities and some showed the inhibitory activities in primary anticancer assay *in vitro*.

INTRODUCTION

The dietary indoles, indole-3-carbinol (I3C), and 3,3'-diindolylmethane (DIM), occur naturally as glucosinolate conjugates in *Brassica* vegetables and are released upon hydrolysis[1]. A number of studies have shown I3C 3a and DIM 4a to be chemopreventive against cancer in multiple target organs such as mammary tissue[2], liver[3], endometrium[4], lung and colon[5] in animal models. They were reported a decade ago to protect against chemical carcinogenesis and chemically induced hepatotoxicity[6]. To define the mechanism by which indoles protect against chemically induced tissue damage, the compounds have been found to inhibit and induce the activities of several enzymes. Although changes in the activities of those enzymes, such as mixed-function oxidases and phase II enzymes, could alter the biological responses of tissues exposed to car-

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cinogens and toxicants, I3C and/or its metabolite DIM have also been shown to be capable of scavenging biologically reactive electrophiles and free radicals[7].

To establish the possible relationship between free radical scavenging and carcinogenic process, a series of DIM derivatives were designed using the predictive model derived from 3D-QSAR CoMFA study[8] then synthesized. Their antioxidant and free radical scavenging potential have been investigated and structure-activity relationship study was conducted. Furthermore, DIM derivatives designed in this report have not been studied to any extent. Their efficiency as radical scavengers was evaluated by their activity toward a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH)[9]. Their potency as antioxidants was evaluated using β-carotene-linoleate model system[10]. Vitamin E and BHA (butylated hydroxyanisole) were used as reference compounds. The anticarcinogenic activity of 5 compounds were tested by using microculture tetrazolium assay (MTT) on HL-60 and P-388 cells and/or sulforhodamine B (SRB) proteochromosomic assays on MCF7 cells.

MATERIALS AND METHODS

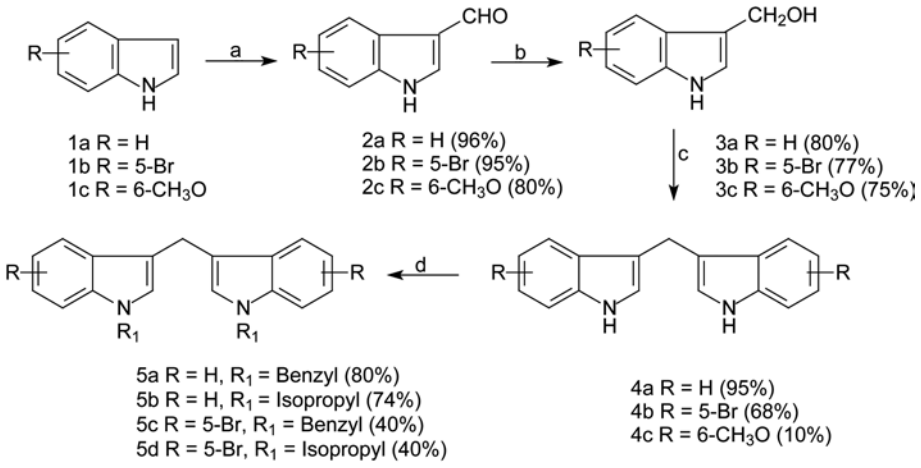
3D-QSAR analysis

CoMFA study From a previous work[11], we have chosen a series of 14 DIM derivatives known for their anticarcinogenic activity by the induction of hepatic cytochrome P450 (CYP) 1A1, 1A2, and their associated catalytic activity ethoxyresorufin-*O*-deethylase (EROD) involved in estrogen metabolism, one of the mechanism responsible of the antitumor activity. Firstly, a DISCO model[12] was derived to guide the superposition of the compounds and identify a common pharmacophore model of the molecules. Secondly, CoMFA analysis was conducted. The CoMFA column value was performed with Sybyl standard parameters[12]. The steric and electrostatic fields energies (AM1 charge) were calculated using an sp^3 carbon probe atom. The statistical analysis was performed using the PLS procedure and regression analysis was performed using cross-validation leave-one-out method.

Chemical synthesis We have chosen a group of the above designed compounds then synthesized them. According to Leete and Marion method[13], the target compounds I3C 3a and DIM 4a were first synthesized, using indole as a starting material and via a four steps sequence: Vilsmeier reaction[14], reduction[15], condensation and alkylation[16] (Schema 1). DIM derivatives were obtained by alkylation on the azote position. Next, other symmetrically substituted DIM (see below), namely, 5,5'-bromo-DIM, 6,6'-methoxy-DIM and derivatives were synthesized from the suitable substituted indole then obtained via the above four steps sequence in good yields. The starting material 5-Br-indole and 6-MeO-indole were prepared using literature methods[17,18].

Biological evaluation Nine compounds were evaluated for their antioxidative potential through 2 *in vitro* antioxidative models: DPPH radical scavenging model and β-carotene-linoleate antioxidative model. Five compounds were evaluated for their anticarcino-genic activity by using microculture tetrazolium assay (MTT)[19] on HL-60 and P-388 cells and/or sulforhodamine B(SRB) proteochromosomic method[20] on MCF7 cells.

Determination of radical scavenging activity



Schema 1. Reagents and conditions: (a) DMF/ $POCl_3$, 35 ºC; (b) KBH_4/MeOH, room temperature; (c) H_2O, reflux 6 h; (d) R_1-X, DMF/NaH, room temperature.

using DPPH method DPPH assay is the simplest method to measure the ability of antioxidants to intercept free radicals. Antioxidants react with DPPH, which is a stable free radical, then scavenge this radical by converting it to α,α-diphenyl-β-picryl hydrazine due to their H-donating ability. The degree of discoloration indicates the scavenging potential of the antioxidant compounds. Experiments were performed according to Blois[9]. Different concentrations of test compounds mixed with methanolic solution of DPPH ranging from 0.1 g/L to 5 g/L were tested. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed in terms of IC_{50} (concentration in mmol/L required for a 50 % decrease in absorbance of DPPH radical). Vitamin E served as reference compounds.

Determination of antioxidative activity using β-carotene-linoleate model system "β-carotene-linoleate test" involves a reaction between a potential antioxidant, β-carotene, and linoleic acid. β-Carotene undergoes rapid discoloration in the absence of an antioxidant. The presence of antioxidant compounds can hinder the extent of β-carotene destruction by neutralizing the linoleate free radical and any other free radicals formed within the system. According to Jayaprakasha[10], different concentrations of the test sample were mixed with the emulsion carotene-linoleate. The tubes were placed at 50 ºC in a water bath, and the absorbance at 470 nm was taken at the beginning (t=0) and continued until discoloration of β-carotene in the control tubes at an interval of 15 min. The antioxidant activity (AA) of the samples was evaluated in terms of IC_{50}. BHA was used as reference compound. Both results of the radical scavenging activity and antioxidative activity are shown in Tab 2.

Determination of antiproliferative activity using SRB assay on MCF7 cells Sulforhodamine B (SRB) is a proteogenic chromosome, it binds to basic amino acid of macrobiomolecules. OD measurement at 515 determines the cell number. The carcinogenic cells were seeded into 96-well plates, each concentration was repeated 3 times. Variance of absorbance produced by the breast cell line was used as an indicator of the efficiency of the sample tested as a cell growth inhibition drug. The OD was measured at a wave-length of 520 nm.

Determination of antiproliferative activity using MTT assay on HL-60 and P-388 cells Measurements of *in vitro* growth in microculture wells by cell mediated reduction of tetrazolium salt to water insoluble formazan crystals showed excellent correlation with measurements of cellular protein in adherent cell line, as well as viable cell count in suspension cell cultures. This assay provides sensitive and reproducible indices of growth as well as drug sensitivity in individual cell lines. Cells were plated into 96-well flat bottomed culture plates in complete culture medium. A period of 24 h after plating, fetal calf serum containing medium was removed and test solution was given to cells in various final concentrations. After incubation with drugs for 24 h, MTT solution was added to the wells and plates and were incubated at 37 ºC for 4 h. Then sodium dodecyl sulphate (10 %, w/v, in 0.01 mol/L HCl) was added and the amount of formazan formed could be determined by photometer at 570 nm. Each concentration was repeated 3 times. Percent of inhibition was calculated from the values of triplicate experiments and the results are expressed as percent of controls.

Statistical methods Correlation and analysis of variance were carried out using the statistical analysis toolPAK in Microsoft Excel program. For both models, all experiments and/or measurements were done in triplicate. All responses were presented as the mean with its standard deviation (n=6) for each case. Two-tailed Student's t-test was used to compare the data. Relationships of parameters were established using a linear regression method. IC_{50} values were obtained from the slope equations ($Y=a+bX$), and all tests were considered statistically significant at $P<0.05$. In SRB assay percent of cell growth inhibition was calculated by the equation: Inhibition=$(A_{540\ control}-A_{540\ sample\ test})/A_{540\ control}\times 100$ %.

RESULTS

CoMFA results The CoMFA model obtained showed a high predictive power with a cross-validation q^2 value of 0.827. The conventionnal correlation coefficient r^2 value was 0.988, the Standard Error Estimate (*SEE*) was 0.044 and the variance ratio F obtained was 103.53.

Predictability of new designed compounds The 3D contour maps were generated to represent the QSAR result produced by CoMFA. The contribution of the steric and the electrostatic field to the activity was 63.2 % and 36.8 %, respectively. Fig 1 and 2 show a stereo colors views of 3D steric and electrostatic map. In Fig 1, regions where increased negative charge is

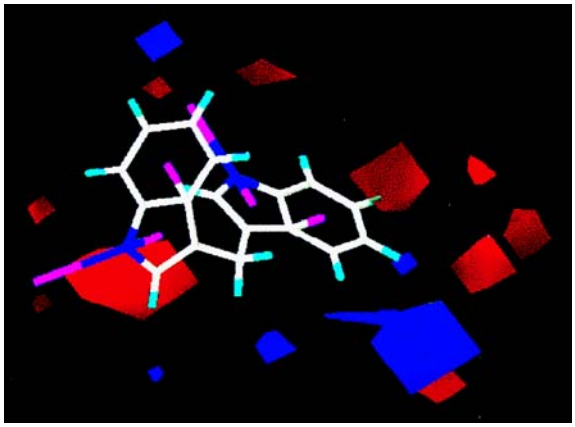


Fig 1. CoMFA electrostatic contour map. Red: negative potential favorable; blue: positive potential favorable.

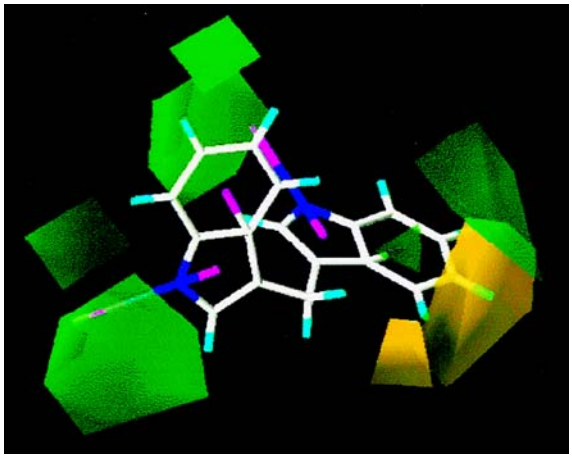
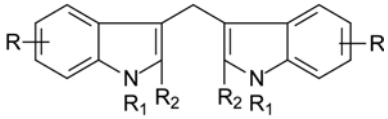


Fig 2. CoMFA steric contour map. Green: sterically favorable regions; yellow: sterically unfavorable regions.

associated with enhanced activity are indicated in red while regions where positive charge is associated with enhanced activity are indicated in blue. In Fig 2, the steric map shows that more bulk is favorable near green area, and unfavorable near yellow area.

DIM was selected as a good candidate for improvement, since it expressed a high activity value, and made some structural changes on the green region, and near the red region where more negative charge is favorable. Substituents were symmetrically added near the green polyhedron where more bulk is desirable, on indole-benzene ring with halogen and alkoxy groups, and on indole-azote position with alkyl groups (Tab 1). They all led to high predictive values compared with the DIM candidate reported activity value (2.835).

Biological evaluation The scavenging effects of I3C, DIM and analogs are shown in Tab 2. Within the group of 9 compounds, two cases could be distinguished.



Tab 1. New designed compounds expressing higher predictive activity.

No	R	R_1	R_2	Pa
1	5-Br	$-CH_2-C_6H_5$	H	3.02
2	5-Br	$-CH(CH_3)_2$	H	3.08
3	5-Br	$-C_2H_5OH$	H	3.08
4	H	$-CH_2-C_6H_5$	H	2.96
5	H	$-CH(CH_3)_2$	H	3.00
6	H	$-C_2H_5OH$	H	3.02
7	H	α-furyl	H	2.93
8	6-OCH_3	H	H	2.90
9	6-OCH_3	$-CH(CH_3)_2$	H	3.06
10	H	H	$-OC_2H_5$	2.96
11	H	H	$-COC_2H_5$	2.88

Tab 2. Antioxidant activities for DPPH· radical and β-carotene model systems. *n*=6. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P* <0.01 *vs* control.

Entry	DPPH model IC_{50}/mmol·L^{-1}	β-carotene model IC_{50}/mmol·L^{-1}
3a	12.69±0.50[c]	94.40±0.06[c]
4a	6.05±0.46[c]	22.03±0.04[c]
5a	>100[a]	33.90±0.03[c]
3b	>100[c]	>100[a]
4b	82.27±0.60[c]	27.11±0.03[c]
5c	>100[a]	29.70±0.10[c]
5d	23.46±0.42[c]	15.06±0.03[c]
3c	10.65±0.20[b]	17.05±0.05[c]
4c	4.91±0.23[c]	9.05±0.04[c]
Vit E	12.56±0.50[c]	–
BHA	–	0.84±0.03[b]

The first case is referring to the presence of *N*-substituents. The antioxidant activity decreased in the following order (4c>4a>3c>3a>5d>5a>5c) with all values significantly different at $P<0.01$ according to the DPPH model assay.

Compounds 3a, 4a, 3c, and 4c with no *N*-substituents, all showed a high radical scavenging activity,

even better than vitamin E. For compounds 4a (DIM) and 4c (6-methoxy-DIM) in DPPH model, their IC_{50} were 50 % and 40 % smaller than that of vit E, due to their hydrogen-donating ability with the presence of two *N-H* group as an *H*-donating group necessary to react with free radical and slightly less potent than the standard phenolic antioxidant BHA in β-carotene model with IC_{50} 4 % and 9 % smaller for 4a and 4c. Compound 5d with a presence of *N-tert*-butyl group substituent showed a moderate activity in both models, 50 % less potent than vitamin E. The tert-butyl group is also known as an electron-donating group, inducing the antioxidant activity, which was somehow decreased by the presence of bromo-atom. Compounds with *N*-benzyl-substituent 5a and 5c showed no activity.

The second case is related to the substitution on the indole-benzene ring. Antioxidant activity decreased in this order: presence of aryl-methoxy substituents (3c and 4c) > no aryl-substituents (3a and 4a) > aryl-bromo-substituents (5a, 3b and 4b). As well known, the methoxy group is a strong electron-donating group, which increase the stability of the benzene ring resulting in increase of radical scavenging activity of compounds 3c (6-methoxy-I3C) and 4c. Without aryl substitution, compounds 3a and 4a showed moderate antioxidant activity due to presence of *N-H* as donating group. Compounds substituted with bromine decreased significantly the activity (5a, 3b, 4b), resulting from the electron-withdrawing property of the halogen. The carbinol 3a which showed a good activity in DPPH model, did not express a significative activity in this model, this is may be due to the sensitivity and instability of carbinol in presence of acids, since the model contain the linoleic acid.

We screened the growth inhibitive or antiproliferative effects of 5 compounds on the cancer cell lines HL 60, P388 and MCF7 in primary. The data show that for MTT assay, 3 compounds have moderate activity at high concentration. Compounds 4a and 4b induced about 12 % growth inhibition of HL60 cells at 1×10^{-4} mol/L. Compound 5d was more effective by inhibiting the growth of 27 % of the same cell line at the same concentration. All three compounds inhibited P-388 cells growth at 1×10^{-4} mol/L with 100 % growth inhibition and compound 4b had some effect at 1×10^{-5} mol/L. Compounds 5a and 5c did not inhibit growth of both cell lines. Substitution on *N*-indole atom seems to lower their activity since none of them showed any antiproliferative effect.

As reported[2-6], the main target compound DIM 4a was effective as an antiproliferative agent. Now, it was confirmed by exposing DIM to SRB assay. In presence of the breast cancer cell line MCF7, DIM induced growth inhibition at different concentrations (for example, DIM showed 35 % inhibition at 1×10^{-8} mol/L).

DISCUSSION

In summary, a first 3D-QSAR study on 14 anticarcinogenic DIMs derivatives was performed by means of CoMFA analysis. The CoMFA model derived from DIM derivatives showed high predictive ability and could describe the steric and electrostatic fields necessary for molecular modeling. According to these fields we designed and synthesized a series of new derivatives of DIM. For the screening of their antioxidant potentiel, one of the mechanisms of chemopreventive effects of I3C and DIM, we have established 2 different antioxidants model system. Nine of our previously synthesized compounds were tested. Results and structure activity relationship (SAR) were discussed and showed that their radical scavenging activity depended on several factors such as presence of electron-donating groups (-NH, -OCH_3, *tert*-butyl), electron-withdrawing groups (-benzyl, -Br) and their chemical stability. We confirmed that I3C 3a and DIM 4a in the DPPH and β-carotene model were pure antioxidant compounds. Another interesting finding of this work is that substitution on indole benzene ring with methoxy group had greatly enhanced the radical scavenging activity of the unsubstituted compounds (3c/4c).

The anti-cancer studies accomplished also confirmed the previous results, where DIM had been shown to inhibit the growth of the breast cancer MCF7 cell line and leukemic HL60, P-388 cell lines at some degree. More works should be done to investigate the activities of other DIM derivatives.

Results of antioxidant activities studied here for a group of compounds were in accordance with the CoMFA analysis. We conclude that antioxidant activity (free radical scavenging) may be associated with anticancer activity and that the new designed and synthesized compounds, 6-methoxy-DIM 4c and 6-methoxy-I3C 3c are suitable candidates to develop further compounds as free radical scavengers.

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REFERENCES

1 De Kruif CA, Marsman JW, Venekamp JC, Falke HE, Noordhoek J, Blaauboer BJ, *et al*. Structure elucidation of acid reaction products of indole-3-carbinol: detection *in vivo* and enzyme induction *in vitro*. Chem Biol Interact 1991; 80: 303-15.

2 Grubbs C, Steele V, Casebolt T, Juliana MM, Eto I, Whitaker LM, *et al*. Chemoprevention of chemically induced mammary carcinogenesis by indole-3-carbinol. Anticancer Res 1995; 15: 709-16.

3 Bailey GS, Dashwood RH, Fong AT, Williams DE, Scanlan RA, Hendricks JD. Modulation of mycotoxin and nitrosamine carcinogenesis by indole-3-carbinol: quantitative analysis of inhibition versus promotion. Anticancer Res 1991; 78: 275-80.

4 Kojima T, Tanaka T, Mori H. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. Cancer Res 1994; 54: 1446-9.

5 Morse MA, LaGreca SA, Amin SG, Chung FL. Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice. Cancer Res 1990; 50: 2613-7.

6 Shertzer HG, Tabor MW, Hogan IT, Brown SJ, Sainsbury M. Molecular modeling parameters predict antioxidant efficacy of 3-indolyl compounds. Arch Toxicol 1996; 70: 830-4.

7 Arnao MB, Sanchez-Bravo J, Acosta M. Indole-3-carbinol as a scavenger of free radicals. Biochem Mol Biol Int 1996; 39: 1125-34.

8 Cramer RD, Patterson DE, Bunce JD. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. J Am Chem Soc 1988; 110: 5959-67.

9 Blois MS. Antioxidant determination by the use of stable free radicals. Nature 1958; 181: 1199-200.

10 Jayaprakasha GK, Singh RP, Sakariah KK. Antioxidant activity of grape seed (*Vitis vinefera*) extracts on peroxidation models *in vitro*. Food Chem 2001; 73: 285-90.

11 Sanderson JT, Slobe L, Lansbergen WA, Safe S, Van Den Berg M. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells. Toxicol Sci 2001; 61: 40-8.

12 Tripos manuel: DISCO, *User Guide*. St Louis (MO). Tripos Associate Inc, 1993.

13 Leete E, Marion L. The hydrogenolysis of I3C and other indole derivative with $LiAlH_4$. Can J Chem 1953; 31: 775-83.

14 James PA, Snyder R. Indole-3-aldehyde. In: Rabjohn N, editor-in-chief. Organic Synthesis; coll. vol IV. New York: John Willey and Sons Inc; 1963. p 539-42.

15 Silverstein M, Ryskiewicz E, Chaikin W. 2-Pyrrolealdehyde and 3-hydroxymethylindole. J Chem Soc 1954; 76: 4485-6.

16 Rubottom GM. The alkylation of indole sodium salt. Synthesis 1972; 1: 566.

17 Russel HF, Harris BJ, Hood DB, Thompson EG, Watkins AD, Williams RD. 5-Substituted indoles via sodium indoline-2-sulfonate, a reexamination. Org Prep Proc 1985; 17: 391-9.

18 Allen MS, Hamaker LK, LaLoggia AJ, Cook JM. Entry into 6-methoxy-*D*(+)-tryptophan. Stereospecific synthesis of 1-benzenesulfonyl-6-methoxy-*D*(+)-tryptophan ethyl ester. Syn Commun 1992; 22: 2077-102.

19 Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 1983; 65: 55-63.

20 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al*. New colorimetric cytotoxicity assay for anticancer drug screening. J Natl Cancer Inst 1990; 82: 1107-12.