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Effect of 4-Methoxyindole-3-carbinol on the Proliferation of Colon Cancer Cells in Vitro, When Treated Alone or in Combination with Indole-3-carbinol

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Consumption of cruciferous vegetables and cancer prevention seem to be positively associated. We present an easy two-step synthesis for 4-methoxyindole-3-carbinol (4MeOI3C), the expected breakdown product of 4-methoxyglucobrassicin during ingestion. 4MeOI3C inhibited the proliferation of human colon cancer cells DLD-1 and HCT 116 with IC_{50} values of 116 μM and 96 μM, respectively, after 48 h in vitro, and is therefore a more potent inhibitor than indole-3-carbinol (I3C). 4MeOI3C and I3C combined in different molar ratios inhibited proliferation in a nearly additive to slightly synergistic manner. Proliferation was inhibited by 100 μM 4MeOI3C after 48 h without affecting cell cycle phase distribution, indicating an overall-slowdown effect on the cell cycle. However, 200 μM 4MeOI3C caused a very high level of cell death and an accumulation of living cells in the G_0/G_1 phase, indicating a concentration-dependent mode of action. We conclude that 4MeOI3C might play a role in the cancer preventive effect of cruciferous vegetables.

KEYWORDS: 4-Methoxyindole-3-carbinol; indole-3-carbinol; glucosinolates; cruciferous vegetables; colorectal cancer; combination index analysis

INTRODUCTION

For years, a positive relationship between the high intake of cruciferous vegetables and prevention of cancer has been observed (*1*−*3*). The content of glucosinolates in such vegetables is believed to cause this positive relationship. When disrupting the plant cells, e.g., during food preparation or chewing, the enzyme myrosinase is introduced to the glucosinolates and cleaves off glucose, leaving a compound that rapidly converts to a thiocyanate, an isothiocyanate or a nitrile. The isothiocyanate products formed from indolic glucosinolates (**Figure 1**) are unstable in aqueous solutions, and further conversion rapidly takes place, leaving hydroxymethyl (carbinol) compounds as the main initial products of indolic glucosinolate breakdown (*4*). These products might further react in the acidic milieu of the stomach, forming a range of polyaromatic indolic metabolites including several dimers and oligomers (*2*, *5*).

So far, the focus in cancer prevention research of the indoles has almost exclusively been on 3-hydroxymethylindole (indole-3-carbinol, I3C), the breakdown product of the glucosinolate glucobrassicin (GB) (**Figure 1**). The compound has been shown to inhibit proliferating cancer cells from a range of human tissues in vitro, including breast (*6*−*8*), prostate (*9*−*11*), skin (*12*), and pancreas (*13*) among others. As reviewed elsewhere (*14*−*16*), the inhibitory effect of I3C can be ascribed to the involvement of the compound in multiple critical pathways of, e.g., the cell cycle and/or apoptosis. I3C was previously shown to have a relatively high IC_{50} value around 250 μM in human colon cancer cells after 48 h of treatment (*17*). However, 3-hydroxymethyl-1-methoxyindole (NI3C), the breakdown product of 1-methoxy-GB (NeoGB) (**Figure 1**), was about 10-fold more potent, when comparing the IC_{50} value to that of I3C in these cells (*17*).

Using a rat model studying the modulation of xenobiotic metabolism, it was reported that a mixture of indolic glucosinolates, i.e., 48% GB, 36% NeoGB, and 16% 4-methoxy-GB (4MeOGB) (**Figure 1**) myrosinase-treated or untreated, was significantly a more powerful inducer of microsomal hepatic CYP1A1 protein than solutions of GB or NeoGB alone (*18*), and the presence of 4MeOGB was ascribed as being possibly responsible for this effect. This observation has stimulated our interest to test the biological activity of the 4MeOGB breakdown product, 3-hydroxymethyl-4-methoxyindole (4-methoxyindole-3-carbinol, 4MeOI3C) (**Figure 1**). We present here an easy two-step synthesis of 4MeOI3C from commercially available 4-methoxyindole. Additionally, we demonstrate the effect of the compound, when exposed to proliferating DLD-1 and HCT 116 human colon cancer cells in vitro, either alone or combined with I3C. Finally, the effect of 4MeOI3C on cell cycle phase distribution and the ability of the compound to cause cell death are elucidated.

MATERIALS AND METHODS

Synthesis. For the synthesis and structure elucidation, highest grade solvents and chemicals were used exclusively. 4MeOI3C was synthesized in a two-step synthesis. First, a formyl group was introduced to 4-methoxyindole (99%, Aldrich, USA) through a Vilsmeier−Haack reaction inspired by others (*19*, *20*). Phosphorus oxychloride (0.8 mL (8.7 mmol)) was mixed

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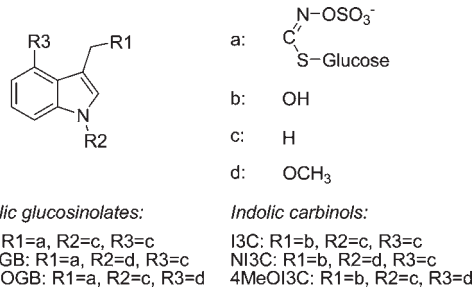

Figure 1. Structures of Indolic Glucosinolates and Carbinols of Relevance for This Study.

with 3.5 mL of ice-cold dimethylformamide. To this mixture, 1.0 g (6.8 mmol) of 4-methoxyindole dissolved in 23 mL of ice-cold dimethylformamide was added under magnetic stirring. The compounds reacted for 1 h at 40 °C. To the pale yellow reaction mixture, 35 mL of ice-cold water was added, and the color changed to burgundy. After basification with 5% aqueous sodium hydroxide, the color of the solution again changed to yellow, and an additional 30 mL of water was added. The aqueous solution was extracted three times with a 5% (v) solution of methanol in dichloromethane. Then the combined organic extracts were washed with 150 mL of brine and dried over sodium sulfate. After evaporating the organic layer under reduced pressure into a small volume of about 10 mL, pure 3-formyl-4-methoxyindole was subsequently obtained by preparative TLC (Silica gel 40 F254, layer thickness 0.25 mm) using as eluent a solution of 5% (v) methanol in diethylether/dichloromethane (1:5, v/v). The yield of product was 745 mg (63%). Mp = 153−155 °C.

A subsequent reduction reaction led to the formation of 4MeOI3C. Seven hundred milligrams of 3-formyl-4-methoxyindole (4.0 mmol) reacted with 150 mg of sodium borohydride in 12.5 mL of a solvent mixture of methanol/ethanol/chloroform (1:8:5, v/v/v) for 6 h under magnetic stirring. Afterward, the solvent was evaporated under reduced pressure. The solid residue was dissolved in 7 mL of 0.1 M aqueous sodium hydroxide, and the solution was extracted three times with 35 mL of diethylether. The combined organic extracts were dried over sodium sulfate, and the solvent was evaporated under reduced pressure. The yield of the crude product was 662 mg (93%). Recrystallization from methanol gave 432 mg of beige crystals. The purity was acceptable for biological testing (>98%) with the main impurity being water shown by NMR. Mp = 91−92 °C.

Structure Determination. Different methods of NMR were used for structure determination. 3-Formyl-4-methoxyindole was dissolved in acetone-d_6 for ^{1}H NMR performed at 300 MHz or in 10% (v) CD_3OD in $CDCl_3$ for ^{13}C NMR at 75 MHz (Varian Mercury 300 NMR spectrometer operated by VNMR 6.1B software). 4MeOI3C was dissolved in $CDCl_3$ and similarly examined at 300 and 75 MHz. In the ^{1}H NMR spectrum obtained, the compound purity was found from integrals. In addition, a NOE-dif experiment was performed in order to find the chemical shift of 4MeOI3C H-5 (the proton closest to the methoxy group). In order to determine the chemical shift of 4MeOI3C C-5, a HMQC 2D NMR experiment was performed (Varian Inova 600 NMR spectrometer operated by VNMR 6.1B software) in $CDCl_3$. 2D NMR data were analyzed using Mestrelab Research MestRe-C software.

Cell Growing. DLD-1 and HCT 116 human colon cancer cells (obtained from American Type Culture Collection, ATCC) were grown under similar conditions. Except when handled during experimental performance or when stated otherwise, cells were kept in an incubator (37 °C, 5% carbon dioxide atmosphere with maximal humidity) and grown in McCoy's 5A medium w GlutaMAX (Gibco, USA) (containing 10% FCS (lot 1046 FF, Biochrom AG, Germany) and 45 μg/mL gentamicin (Gibco, USA)). Subcultivation was done every second or third day before 80% confluence was reached. During subcultivation, cells were treated with Versene (containing 0.05% trypsin) (from (Gibco, USA)) for 10 min. At passage 30, cells were discarded.

Growth Inhibition. To investigate the time- and concentration-dependent effect of 4MeOI3C on proliferating cells, 24 well plates were used (Nunclon Δ 24-well plates (Nunc, Denmark)); 3.5×10^4 cells in 1 mL medium were seeded in each well. After an overnight incubation, the cells of one plate were trypsinized for 20 min and counted on Coulter Particle Counter and Size Analyzer Z_2 (Beckmann-Coulter Inc.) operated by Multisizer AccuComp 3.01 software. On the remaining plates, 4MeOI3C (0−200 μM in dimethyl sulphoxide (DMSO, Sigma-Aldrich Chemical Co. (USA)), 4 replicates per concentration) was introduced in new medium. DMSO exposure never exceeded 0.1%. After 24, 48, 72, or 96 h of incubation, cells were counted using a Coulter counter. Cells with a diameter smaller than 10.6 μm were considered dead cells and were subtracted from the cell number.

Combinatorial Treatment. For the combinatorial treatment analysis, another source of suppliers was used. McCoy's 5A medium with L-glutamine, PBS without Ca^{2+} and Mg^{2+}, and gentamicin 50 mg/mL were purchased from Lonza (Belgium) and FCS (lot 0739 L) from Biochrom AG, Germany. EDTA solution was made as 0.02% EDTA (Sigma-Aldrich Chemical Co.) in PBS. The affected cell number was estimated by Neutral Red (NR) analysis. Similarly distributed on four 96-well plates (Black Nunc F96 MicroWell 96-well plates (Nunc, Denmark)), five series of test compound concentrations (8 replicates per concentration, 2 on each plate) were prepared. In one series, 4MeOI3C (80−200 μM) was tested alone and in another I3C (140−450 μM) alone (I3C from Sigma-Aldrich Chemical Co. (USA) was recrystallized before use). In addition, three molar 4MeOI3C/I3C ratios, i.e., 1:1 (60−135 μM 4MeOI3C), 1:2 (40−100 μM 4MeOI3C) and 2:1 (60−165 μM 4MeOI3C), were examined. Each plate contained 10 control wells and 12 background wells. The experiment was performed as follows: 8000 DLD-1 or HCT 116 cells in 100 μL of medium were seeded in all wells except background wells, which received 100 μL of medium without cells. After an overnight incubation, 50 μL of medium containing DMSO and test compounds in concentrations 3-fold the desired final concentrations was added to each well. Control and background wells received 50 μL of medium containing 0.3% DMSO only. After 48 h of incubation, all wells were washed with 100 μL of PBS, and there was added 100 μL of medium containing 50 μg/mL NR (Sigma-Aldrich Chemical Co. (USA)) (freshly made from a stock solution of 1 mg/mL NR in Milli-Q water), followed by incubation for three hours. All wells were washed twice in 100 μL of PBS. Then, 100 μL of 96% ethanol was added, and the plates were covered with parafilm and placed on a rocking table for 20 min before scanning in a fluorescence-reader (excitation 530 nm and emission 590 nm) (BioTek Synergy HT, Biotek Instruments Inc., operated by BioTek KC4 3.4 software). On each plate, the background mean value was subtracted from all other values. The NR uptake of cells treated with 4MeOI3C and/or I3C was estimated relative to that of control cells (nontreated) as being the surviving fraction (SF) of cells. The affected fraction (AF) was the fraction of living cells missing, when compared to the control; thus, AF + SF = 1. From the concentration−effect relationship within the AF interval 0.2 to 0.8, the Combination Index (CI) introduced by Chou and Talalay (*21*, *22*) was found. The following equation was used under the assumption of a mutually exclusive compound interaction: $CI = C_{4MeOI3C}/IC_{x\ 4MeOI3C} + C_{I3C}/IC_{x\ I3C}$, where $IC_{x\ 4MeOI3C}$ and $IC_{x\ I3C}$ are the inhibitory concentrations of 4MeOI3C and I3C, respectively, affecting a certain fraction (x %) of the cells, when treated separately, and $C_{4MeOI3C}$ and C_{I3C} are the concentrations of the two compounds also affecting x % of cells, when used in combination. CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively. In a review by Chou (*23*), the terms nearly additive (CI: 1.10−0.90), slight synergism (CI: 0.90−0.85), and moderate synergism (CI: 0.85−0.70) are used before plain synergism (CI: < 0.70). For each of the series of 1:1, 1:2, and 2:1 ratios of 4MeOI3C and I3C of each cell line, an AF−CI relationship was described using these terms.

Influence on Cell Cycle and Cell Death. HCT 116 cells were seeded in 56.7 cm^2 dishes (6.5×10^5 cells in 10 mL of medium each) (Nunclon Δ 56.7 cm^2 dishes (Nunc, Denmark)) and incubated overnight. The medium was removed and replaced with medium containing 0.1% DMSO and no compound (controls), 100 μM or 200 μM 4MeOI3C, or 250 μM I3C. Three replicates per concentration were used. After 48 h of incubation, the medium and 5 mL of washing PBS were combined in centrifuge tubes in order to collect floating cells. The remaining cells were trypsinized in 1 mL of Versene and resuspended in 5 mL of medium before adding to the tubes, which were spun at 300*g* and 4 °C for 5 min (standard) afterward. The pellet was dissolved in PBS, and the cell number was estimated. At least 5.0×10^5 cells were transferred to a new tube, washed in PBS, and

resuspended in 200 μL of PBS. Under whirl mixing, 2 mL of ice cold 70% ethanol was slowly added, and the tubes were kept at −20 °C until FACS analysis was performed. For the analysis, cells in ethanol were spun, washed in PBS, and resuspended in 400 μL of PBS. This was followed by whirl mixing with 50 μL of 1 mg/mL RNase in PBS, and subsequently, 50 μL of 0.4 mg/mL propidium iodide (PI) in PBS was added, and the cells were incubated in the dark for 30 min at room temperature. All samples kept in the dark were analyzed on a flow cytometer within 2 h (FACSCalibur, Becton Dickinson used with BD Bioscience CellQuest Pro 4.0.2 operating software and Verity Software House Inc. ModFit *LT* data processing software). Living cells were categorized in the G_0/G_1 phase, S phase, and G_2/M phase from their content of DNA. Registrations in the sub-G_0/G_1 area in the histograms were considered as the sum of dead/dying cells and debris.

Data Processing and Statistics. Unless otherwise stated, all experiments were performed three times, and the results are given as the mean with standard deviation (SD) . Microsoft Excel 2007 in combination with SYSTAT 11 for Windows was used for data processing and statistics. All data were tested for being normally distributed according to the Shapiro-Wilk test. When appropriate, variance analysis was performed using one-way ANOVA with Bonferroni post hoc test at statistical significance levels $p < 0.05$ and $p < 0.01$.

RESULTS

Structure Elucidation. NMR spectral data of 3-formyl-4-methoxyindole were in agreement with assignments given elsewhere (*19*) (data not shown). The final product, 4MeOI3C, was dissolved in $CDCl_3$, and spectral data were as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.27 ppm (t, 1, J = 6.8 Hz, OH), 3.98 (s, 3, OCH_3), 4.80 (dd, 2, J = 6.8 Hz, 0.6 Hz, CH_2), 6.55 (dd, 1, J = 7.8 Hz, 0.4 Hz, H-5), 6.93 (dd, 1, J = 2.4 Hz, 0.3 Hz, H-2), 6.97 (dd, 1, J = 8.2 Hz, 0.7 Hz, H-7), 7.11 (dd, 1, J = 8.2 Hz, 7.8 Hz, H-6), 8.30 (s, br, 1, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 55.5 ppm (OCH_3), 58.2 (CH_2OH), 99.7 (C-5), 105.2 (C-7), 116.4 (C-3), 116.8 (C-3α), 121.0 (C-2), 123.2 (C-6), 138.4 (C-7α), 153.2 (C-4). Calculations could not predict which of the 4MeOI3C protons H-5 and H-7 should have chemical shifts at 6.55 ppm and 6.97 ppm, respectively. For this reason, we performed a NOE-dif experiment in order to point out the H-5 shift. The doublet at 6.55 ppm was heavily affected when the methoxy group was irradiated. Neither could our calculations predict the C-5 and C-7 chemical shifts. HMQC 2D NMR showed couplings between H-5 and the 99.7 ppm carbon. Also, a coupling between H-7 and the 105.2 ppm carbon was revealed, thus confirming the position of these critical carbons. Furthermore, couplings between H-2 and C-2 as well as H-6 and C-6 were confirmed. Finally, H−C couplings within the methoxy group and the hydroxymethyl group were observed.

4MeOI3C Inhibits Cell Growth in a Time- and Concentration-Dependent Manner. Using cell counting, we estimated the effect of 4MeOI3C on proliferating DLD-1 and HCT 116 cells. Cells were grown for 24, 48, 72, or 96 h under exposure to increasing concentrations of 4MeOI3C (25−200 μM). Surviving cells at different concentrations were compared to control cells percentage-wise (**Table 1**). The cell number was inhibited in a concentration- and time-dependent manner by 4MeOI3C. Already after 24 h of treatment, a statistically significant effect of 50 μM 4MeOI3C on DLD-1 and of 100 μM on HCT 116 cells was observed. Furthermore, these data revealed that 200 μM kept the cell number almost unchanged throughout the experimental period. From concentration−effect curves, we calculated the median inhibitory effect concentrations (IC_{50}) for each cell line at all time points (**Table 2**). In order to reach the median effect after only 24 h, a relatively high dose of 189 ± 23 μM 4MeOI3C was necessary in DLD-1 cells. Extending the time of exposure reduced the concentrations needed for the median inhibitory effect concentration. In HCT 116 cells, a relatively large standard deviation at 24 h blurs this time-dependent pattern. However, the compound seems to be an equally potent inhibitor of both cell lines.

Table 1. Time- and Concentration-Dependent Effect of 4MeOI3C on Proliferating Human Cancer Cells in Vitro (Mean of 3 Identical Experiments ± SD)

		living cells in percent of control			
cell line	concentration (μM)[b]	24 h	48 h	72 h	96 h
DLD-1	25	89.4 ± 1.7	88.6 ± 0.3	92.7 ± 1.4	89.6 ± 3.6[a]
	50	83.1 ± 4.9[a]	75.4 ± 6.1[a]	80.6 ± 3.0[a]	79.6 ± 3.7[a]
	100	73.8 ± 3.4[a]	56.5 ± 4.3[a]	56.5 ± 3.9[a]	58.5 ± 1.3[a]
	200	45.0 ± 6.7[a]	23.5 ± 7.6[a]	12.1 ± 3.9[a]	6.08 ± 1.7[a]
HCT 116	25	89.0 ± 4.8	89.3 ± 1.7	93.3 ± 2.4	92.7 ± 4.1
	50	79.1 ± 11.7	71.5 ± 5.9[a]	76.0 ± 8.5[a]	82.4 ± 3.6[a]
	100	63.5 ± 12.0[a]	46.3 ± 5.9[a]	44.2 ± 5.9[a]	59.6 ± 6.8[a]
	200	43.7 ± 9.4[a]	16.9 ± 2.9[a]	7.33 ± 1.9[a]	4.90 ± 2.0[a]

[a] $p < 0.01$ significant from the control. [b] All cells, including control cells, were exposed to 0.1% DMSO.

Table 2. Median Effect Concentrations (IC_{50}) of 4MeOI3C in Micromolar over Time (Mean of 3 Identical Experiments ± SD)

cell line	24 h	48 h	72 h	96 h
DLD-1	189 ± 23	116 ± 14	119 ± 8	116 ± 5
HCT 116	162 ± 62	96 ± 13	98 ± 15	120 ± 10

Additionally, we calculated the effect of 4MeOI3C on cell doubling time (T_d). Forty-eight hours of treatment with 100 μM 4MeOI3C increased T_d to 27.2 h (±1.1 h) for DLD-1 cells compared to T_d = 18.5 h (±0.4 h) in untreated cells. Treating HCT 116 cells similarly, we found that T_d was increased to 26.2 h (±1.7 h), compared with T_d = 16.3 h (±0.9 h) in untreated cells. In both cases, the T_d at 100 μM treatment was statistically significantly different from that of controls at the $p < 0.01$ level. Increasing the concentration to 200 μM 4MeOI3C, we found that the cell number was more or less unchanged.

4MeOI3C and I3C Exhibit Nearly Additive to Slightly Synergistic Effects. In three molar 4MeOI3C/I3C ratios, i.e., 1:1, 1:2, and 2:1, we investigated whether additive effects were achieved during the combinatorial treatment of the compounds. The CI was plotted for each series with respect to AF (**Figure 2C**,**D**), which is positively related to the effect concentrations of the compounds (**Figure 2A**,**B**). In **Figure 2C**,**D**, the line CI = 1 represents additive effects, whereas values below and above represent synergism and antagonism, respectively. In DLD-1 cells, near-additive to slightly synergistic effects were achieved, when using equal or half amounts of 4MeOI3C relative to I3C, whereas the responses achieved were closer to an additive effect when 4MeOI3C was in double amounts relative to I3C. All series in HCT 116 cells exhibited near-additive effects.

4MeOI3C Influences Cell Cycle Phase Distribution in a Concentration-Dependent Manner. The distribution of HCT 116 cells in the phases of the cell cycle was investigated by FACS analysis. Cells were untreated (control) or treated with a medium (100 μM) or a high (200 μM) concentration of 4MeOI3C or with a medium concentration of I3C (250 μM) for 48 h, and the distribution is shown in **Figure 3**. Although our cell counting indicated that 100 μM 4MeOI3C is close to IC_{50}, no differences in phase distribution were observed, when compared to that of the control cells after 48 h. Increasing the 4MeOI3C to 200 μM, a large build up in the G_0/G_1 phase was observed (increased from 44% to 74%), and cells in S phase were reduced dramatically (from 37% to 8%). These observations indicate that new cellular targets of 4MeOI3C are introduced along with increasing concentrations.

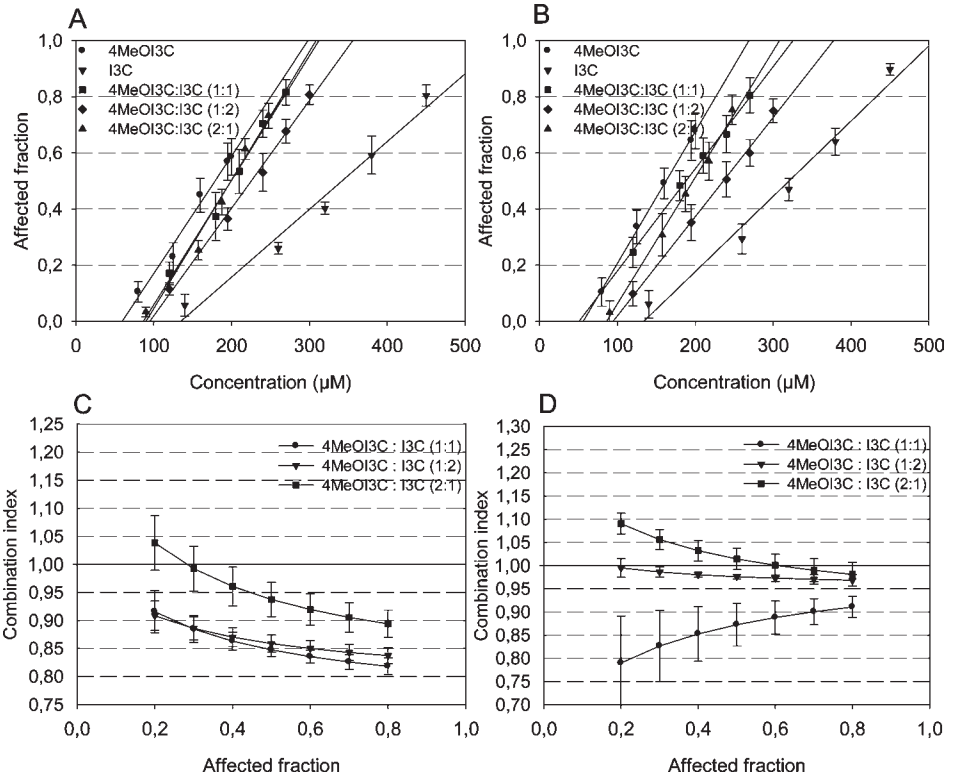

Figure 2. Combinatory effect of treatment of 4MeOI3C and I3C. The affected fraction (AF) of cells compared to controls after 48 h of compound treatment is plotted with respect to total compound concentration in micromolar for DLD-1 (**A**) and HCT 116 (**B**) cells, respectively. The concentration is in this way either pure 4MeOI3C or I3C, or it is the sum of concentrations in a series of molar 4MeOI3C/I3C ratios 1:1, 1:2, or 2:1. Means of four identical experiments ± STD are given, and the lines are tendency lines. The Combination Index (CI) is plotted with respect to AF, as well for DLD-1 (**C**) and HCT 116 (**D**) cells. CI relationships of three molar 4MeOI3C/I3C ratios are given for each cell line as the means of four identical experiments ± STD. CI < 1, CI = 1, and CI > 1 represent synergism, additive effect, and antagonism, respectively. See text for further explanations of the effect.

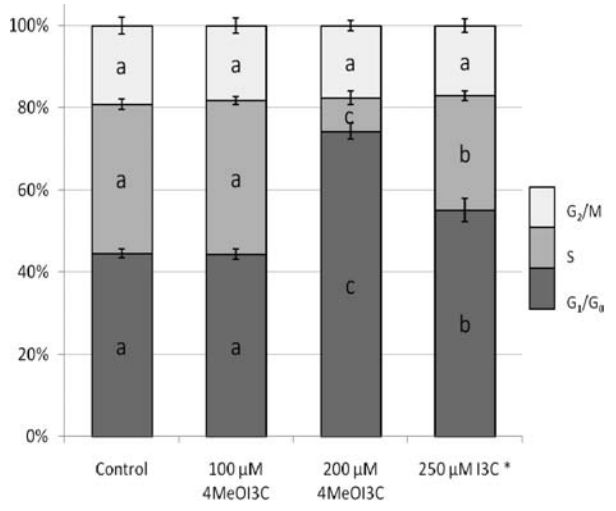

Figure 3. Effect of 4MeOI3C and I3C on the cell cycle of HCT 116 cells. Living cells (100%) distributed in phases of the cell cycle after 48 h of treatment with 100 or 200 μM 4MeOI3C or 250 μM I3C. No test compound was introduced to control cells, and all cells were exposed to 0.1% DMSO. Means of three identical experiments ± STD are given. * Means of only two identical experiments ± half of the range are given. Within each phase (i.e., horizontally compared), only groups not sharing identical letters (a−c) are statistically significantly different at the $p < 0.01$ level.

After 48 h of culture, 9% (±2%) of all registrations were in the sub-G_0/G_1 area for control cells. Exposure to 100 μM of 4MeOI3C increased this number to 21% (±2%), which was not statistically significant, indicating milder cytotoxic effects, whereas 200 μM exhibited strong cytotoxicity by increasing sub-G_0/G_1 registrations dramatically to 88% (±5%), statistically significantly different from controls at $p < 0.01$ level (data not shown). However, the sub-G_0/G_1 area of histograms is very poorly defined, and further investigations are necessary in order to evaluate the ability of 4MeOI3C to induce apoptosis. Thus, 100 μM 4MeOI3C can be considered as a cytostatic concentration with an overall-slowdown effect not related to a specific cell cycle phase.

Following 48 h of treatment with 250 μM I3C, which corresponds to IC_{50}, an increased accumulation in the G_0/G_1 phase was observed. The level of counts in the sub-G_0/G_1 area was 17% (±1%).

DISCUSSION

In cruciferous vegetables, various indoles have been identified, and they may in part explain the proposed cancer preventive activity of these vegetables. The cancer preventive effects of indoles, mainly I3C, have been related to the modulation of metabolism of procarcinogens, but in the last 10 years, the inhibitory effect on cell proliferation has provided a relevant additional explanation.

In general terms, the parental compounds of I3C and NI3C are present at the highest concentrations in, e.g., broccoli (*24*), but in some cases, the level of 4MeOGB in broccoli and Brussels sprouts is shown to exceed the GB and NeoGB levels (*25*). Previous multivariate analysis of the effect of broccoli showed significant contribution from all of the glucosinolates found in broccoli (*26*), which is why a closer analysis of the cellular effects of 4MeOI3C is relevant compared with I3C. Previously, such a comparison was done for NI3C and I3C (*17*).

Pure 4MeOI3C was produced in two steps from 4-methoxyindole in an overall yield of 38% after the final recrystallization. Likewise, pure NI3C has been produced in an overall yield of 20% in a similar two-step synthesis (*27*). However, the synthesis of NI3C differed from that of 4MeOI3C in that it involved two distillations, one after each step, as both NI3C and the intermediate compound 3-formyl-1-methoxyindole are oils. 3-Formyl-4-methoxyindole was purified on p-TLC plates and therefore not further recrystallized before use in the second reaction step. That the synthesis of 4MeOI3C is about doubly as effective as that of NI3C might in part be a consequence of the different purification methods and in part of the different reactivities of the reactant indoles.

This is the first analysis of the cellular effect of 4MeOI3C, and the potency is about 2-fold higher than I3C on the basis of IC_{50} values in both colon cell lines. NI3C was previously found to be about 10-fold more potent compared with that of I3C in the same cell lines as those used here (*17*). These differences in potency indicate that the actual combinations of various indoles in the cruciferous vegetables may be crucial for the biological effects of the vegetable.

I3C is highly sensitive to the acidic milieu of the stomach but also the near-neutral pH (*28*). As reviewed elsewhere (*5*), the indolic carbinols likely react to form a range of products throughout the digestive system, e.g., di- and oligomers and products from reactions with other plant substances present such as ascorbic acid. Therefore, our in vitro model does not perfectly reflect the in vivo situation following the ingestion of cruciferous vegetables but is useful for evaluating the combinatory effect of the indoles.

Even though several studies have been performed on the in vitro effect of I3C, it has been proposed that little or no effect can be ascribed to the compound itself. Bradlow (*29*) states that I3C in cell culture medium under normal conditions might be so unstable that the effect can only be ascribed to spontaneously formed condensation products of I3C, mainly, 3,3′-diindolylmethane (DIM). We observed well-defined, concentration-dependent inhibitory effects of 4MeOI3C and I3C, separately treated to cells or combined, and the CI analysis was performed on the basis of these observations. The calculations of CI were based on initial indole concentrations, as the compounds dissolved in medium were only added once at the beginning of the testing period. The experiments do not give information on the conversion of the indoles included in the analyses, but we suggest that future experiments should focus on this aspect.

As indicated above, the combined effect will strongly depend on the actual combination of the indoles present. Therefore, we wanted to show the combined effect of 4MeOI3C and I3C. Near-additive to slightly synergistic effects were achieved in DLD-1 cells, whereas the combination of the two indoles only exhibited a near-additive effect in HCT 116 cells. The results indicate that the strongest effect is obtained, when the level of I3C matches or, in DLD-1 only, exceeds that of 4MeOI3C; however, the effect is only slightly synergistic. In extracts from some cruciferous plants such as watercress (*30*), nozawana (*31*), and broccoli sprouts (*32*), 4MeOGB levels higher than GB levels have been observed, but in most plant extracts, the level of GB exceeds that of 4MeOGB (*33*−*35*). No strong synergism is achieved during combinatorial treatment, which may indicate that the two compounds to a high degree have a similar mode of action. Neave et al. (*17*) proposed a higher degree of combinatory effect of NI3C and I3C, on the basis of the fact that they act differently on the cell cycle. Both the cell cycle analysis and the combinatory experiments indicate that the mode of action depends on the concentration of 4MeOI3C.

In these experiments, two different methods of estimating the cell number were used. Comparing these two assays, we observed 55−70% higher IC_{50} values for 4MeOI3C at 48 h, when performing the NR assay instead of the cell counting experiments (compare **Figure 2A**,**B** and **Table 2**). When counting the cells, the limit between living and dead cells is set manually and may therefore differ from the NR assay. In the NR assay, only living cells take up NR, and the measure of light emission defines the surviving fraction of cells relative to untreated cells. These two assays are in fact two different ways of viewing the same problem, but different results may well appear from the differences in experimental conditions. Different plates are used (24-well versus 96-well dishes), trypsination for Coulter counting may damage some of the living cells, and in the Coulter counting experiment, the indole-containing medium is replacing the medium used for seeding the cells the day before. However, both our Coulter counter experiments and the CI analysis were quite consistent when reproduced, indicating reliable results.

The combinatory effects of several compounds have been described by different models: Chou and Talalay introduced the Combination Index method and included the median effect plot (MEP) as a mathematical tool to improve the linearity of the concentration−effect curves (*22*). The concentration−effect curves after 48 h of treatment with the indoles exhibited nearly perfect linearity in all series. When these data were transformed to MEP, the lines were slightly disturbed, and the use of MEP transformation did not alter the final AF−CI relationship. For these reasons, the MEP was not included in our final data processing.

The CI analysis was chosen over other methods for describing combinatorial treatments, e.g., fractional product method and isobologram analysis, in part because the analysis seems kinetically independent of the two compounds, in part because further investigations can include more compounds which are still comparable to results achieved so far, and in part because the degree of synergism, additive effect, and antagonism is easily described in a curved relationship to an interval of cellular inhibition (expressed as AF).

Comparing the effects of the three indoles on the cell cycle distribution in HCT 116 cells indicates that they act by different mechanisms: I3C causes an accumulation in G_0/G_1 phase, NI3C accumulates the cells in G_2/M phases (*17*), and the present study showed no change of cell phase distribution, when cells were exposed to 100 μM 4MeOI3C, whereas a higher concentration caused G_0/G_1 phase accumulation. The major difference in the response to I3C and NI3C was the fact that NI3C besides the p21 protein also induces the p27 protein (*17*). However, we performed no analysis of the modulation of cell cycle related proteins by 4MeOI3C. The fact that 100 μM 4MeOI3C increased the doubling time by about 50%, combined with the observation that the cell cycle phase distribution was not affected, indicates that 4MeOI3C at 100 μM has an overall-slowdown effect on the cell cycle. Continued studies should reveal, whether cell cycle related proteins are targets of 4MeOI3C during this slowdown.

4MeOI3C was continuously found to be about 2-fold as potent as I3C on the cells investigated. However, only when tested at a high concentration (200 μM) does 4MeOI3C seem to have the same effect as I3C, in that cells are arrested in the G_0/G_1 phase or killed. Even if 4MeOI3C and I3C have different molecular targets, the end point effects of both compounds are crucial to the cell, as proliferation is inhibited, and the compounds are, therefore, in agreement with the statements of Chou (*23*), most likely at least partly dependent on the presense of each other. This supports our use of the Combination Index equation for mutually exclusive compound interaction, i.e., when the compounds are not totally independent of each other.

In conclusion, 4MeOI3C was successfully synthesized and purified. Treatment of the compound on human colon cancer cell lines DLD-1 and HCT 116 in vitro resulted in inhibition of their proliferation in a time- and concentration-dependent manner. The compound was about 2-fold as potent as the structurally familiar I3C in performing this inhibition. Combinatorial treatment of the two compounds exhibited nearly additive to slightly synergistic effects after 48 h. 4MeOI3C levels close to IC_{50} induced an overall-slowdown mechanism in HCT 116 cells. However, when treated at the double concentration, 4MeOI3C caused the cells to accumulate in the G_0/G_1 phase, and the cytotoxicity was increased dramatically, possibly through the induction of apoptosis. Thus, the mode of action seems to be concentration-dependent.

On the basis of our observations, we find 4MeOI3C to be an interesting substance, which does not antagonize the anticancer effect of I3C but contributes by being both cytostatic and cytotoxic to cancer cells in vitro; however, we suggest future investigations to elusidate the molecular fate in vivo and the role of possible condensation products of the compound following cruciferous vegetable consumption.

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