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Research paper

Selective cytostatic and cytotoxic effects of glucosinolates hydrolysis products on human colon cancer cells *in vitro*

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Glucosinolates hydrolysis products are attracting increasing attention since many studies have suggested that they may be involved in the anticarcinogenic property of cruciferous vegetables. In this study, we show that diindolylmethane (DIM) and sulforaphane, produced during the hydrolysis of glucobrassicin and glucoraphanin, respectively, exert a dose-dependent cytotoxicity on human colon adenocarcinoma HT29 cells. Moreover, these products are able to inhibit quiescent cells to re-enter the cell cycle. Interestingly, our results clearly show that low doses of DIM and sulforaphane, although very effective on undifferentiated intestinal HT29 cells, do not affect the viability of the differentiated CaCo2 cells. The reversibility of their effects has also been tested and is discussed. [© 1998 Rapid Science Ltd.]

Key words: Colon carcinoma cells, cruciferous vegetable, diindolylmethane, glucosinolate, HT29 cells, sulforaphane.

Introduction

The case for implicating diet in the etiology of colorectal cancer is growing stronger. A number of observational epidemiologic studies indicate that the risk of developing this malignancy is influenced by dietary factors. They are either initiatory or promotor (essentially found in fat and meat), or protector (generally found in vegetables, fruits and seeds).[1,2] Glucosinolates are sulfur-containing compounds found in high amounts in cruciferous vegetables.[3,4] Intact glucosinolates themselves exhibit low bioactivities, but some of their hydrolytic breakdown products obtained under myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) action are attracting increasing attention as potent chemical and dietary protectors against cancer.[5–7] In numerous laboratory animal feeding studies some of these compounds have been proven to be effective chemopreventive agents in a variety of tumor models.[8] The biological effects of glucosinolate breakdown products have been also studied *in vitro*, and some of them have been shown to inhibit the growth of some mouse and human cancer cells. This is the case for indole-3-carbinol (I3C), the major indole derivative occurring after glucobrassicin hydrolysis, or its acid condensated product 3,3′-diindolylmethane (DIM) as well as some isothiocyanates.[9–12]

Altogether these data suggest protective and/or preventive properties of some glucosinolate breakdown products, especially in the gastrointestinal tract where they are supposed to be present in high amounts. It is therefore of interest to study their anticancer activity on intestinal-derived cell lines. In this study, we have tested the effect of an isothiocyanate produced by the hydrolysis of glucoraphanin (i.e. sulforaphane), the effect of glucobrassicin and of its four main hydrolysis products, I3C, indole-3-acetic acid (IAA), indole-3-carboxylic acid (ICA) and DIM, on the growth of two human colon cancer cell lines in culture, HT29 and CaCo2 cells. These cells provide interesting intestinal models as they differ by their capacity to differentiate upon confluency. Indeed, HT29 cells are well undifferentiated whatever the stage of culture, whereas post-confluent CaCo2 cells undergo intestinal differentiation.[13]

Materials and methods

Drugs and chemicals

Dulbecco's modified Eagle's medium (DMEM) and

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fetal calf serum (FCS) were obtained from Gibco Life Technologies (France). All other chemicals were purchased from Sigma (St Louis, MO) or from Merck (France) and were of analytical grade. Glucobrassicin was synthesized according to the method of Viaud and Rollin.[14] Sulforaphane was synthesized according to the method described by Schmid and Karrer[15] after some improvements. The product was purified just before use by HPLC (Hewlett Packard System Series 1050) on a reverse-phase column [Ultrabase C_{18} (250 × 4.6 mm)] using elution starting with 100% of solution A (20%, acetonitrile, 80% H_2O v/v) for 10 min, followed by a 10 min linear gradient to reach 100% of solution B (90%, acetonitrile, 10% H_2O, v/v). Sulforaphane elution was detected at 245 nm; the product was collected, dried under nitrogen, and then resuspended in ethanol at a concentration of 30 mM and stored at −20 C. DIM was synthesized from I3C according to the technique of Leete and Marion[16] modified by Latxague *et al.*[17] Moreover, at the end of the preparation, in order to improve the purity of DIM, the final solution was then recrystallized in methanol, filtered, lyophilized and stored at −20 C. For the experiments DIM was diluted in ethanol at the various indicated concentrations.

Cell culture

HT29 and CaCo2 cell lines have been established in permanent culture from a human colon carcinoma by Dr Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY).[18] CaCo2 cells were obtained from Unité INSERM 317 (Toulouse, France) and HT29 cells were purchased from the European Collection of Cell Culture (Salisbury, UK). Routinely, stock cells were cultured in DMEM containing 25 mM glucose, 43 mM bicarbonate, 60 μM/ml penicillin and 100 μg/ml streptomycin at 37 C, under an air/CO_2 (9/1) atmosphere. The HT29 cells medium was supplemented with 5% heat-inactivated FCS. CaCo2 cell medium was supplemented with 10% heat-inactivated FCS and 1% non-essential amino acids. For both cell lines, the medium was changed every 48 h. For the experiments, HT29 cells were seeded at low density (5×10^4 cells/ml) in 35 or 120 mm diameter Petri dishes in standard medium containing 5% FCS. One day after seeding, HT29 cells were placed in serum-free medium for 20 h in order to arrest cell growth. The maximum proliferative effect of FCS is obtained in HT29 cells in the presence of 3% FCS, which induces a doubling of the cell population within a 30 h period of culture (data not shown). So the next day, to check the possible effect of glucosinolate-derived products on exponentially growing HT29 cells, quiescent cells were stimulated with sub-optimal doses of FCS (1%) and treated simultaneously with the indicated concentrations of the glucosinolate breakdown products diluted in ethanol. An equivalent amount (0.2%) of the solvent (ethanol) was added to the control cells. In assays requiring confluent conditions, CaCo2 cells were plated at 1×10^5 cells/ml and allowed to grow to confluency in 10% FCS-containing medium. Then, 3 weeks after they have reached confluency, CaCo2 cells were fully differentiated[13] and treated with 1% FCS in the presence of the various compounds or 0.2% ethanol alone (control).

Cell viability assay

Drug effects on cellular viability were evaluated using an assay based on the cleavage of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to purple formazan crystals by deshydrogenase activity in mitochondria, a conversion which occurs only in living cells.[19] After different periods of treatment, cells were first rinsed with phenol red-free RPMI medium, then they received MTT diluted in RPMI over 4 h. The cells were then solubilized in SDS-HCl, and the optical density of the cellular homogenate was measured at 570 and 690 nm.

Cell proliferation assay

Drug effects on proliferation were evaluated after measuring cell cycle distribution by flow cytometry. Cell cycle distribution was determined based on DNA content which was measured after labeling with propidium iodide according to the method of Vindelov and Christensen,[20] slightly modified. Cell suspensions from either preconfluent or confluent cultures were prepared by trypsinization and washed twice with 0.9% NaCl by centrifugation at 300 *g*. Cells (1×10^6) were resuspended in 220 μl solution A (3.4 mM trisodium citrate, 0.1% Nonidet P-40, 552 mg/ml spermine tetrahydrochloride and 0.5 mM Tris) containing trypsin (30 mg/l) at pH 7.6 over 10 min at room temperature. Then 180 μl of solution B containing a trypsin inhibitor was added. After a 10 min incubation period at room temperature, cells were labeled by addition of 180 μl of solution C containing propidium iodide (416 mg/l), RNase A (100 mg/l) and spermine tetrahydrochloride (1160 mg/l) at pH 7.6. The suspension was incubated overnight at 4 C to allow for maximum labeling of DNA. Cell cycle was

analyzed on a Coulter Elite flow cytometer. Propidium iodide fluorescence was measured through a 630 LP filter. Debris and doublets were eliminated by gating on peak versus integrated signals; 1.5×10^4 cells were collected by each sample. DNA content analysis was performed with Multicycle AV Software (Phoenix Flow System, San Diego, CA).

Results

Effect of glucosinolates hydrolysis products on HT29 cellular viability

Figure 1 shows the effect of 100 μM of either glucobrassicin (GB), I3C, IAA, ICA, DIM or sulforaphane on the viability of preconfluent HT29 culture after a 24 or 48 h period of treatment. Results show clearly that for such a high dose and whatever the duration of the treatment, glucobrassicin, ICA or IAA did not affect HT29 cell viability in a significant manner. In contrast, 100 μM of I3C decreased the percentage of viable cells by nearly 70% after 48 h of incubation, whereas it did not affect cellular viability after a 24 h period of treatment. DIM and sulforaphane were the most potent products, decreasing the cell viability by nearly 80% compared to untreated cells, as soon as after 24 h of incubation.

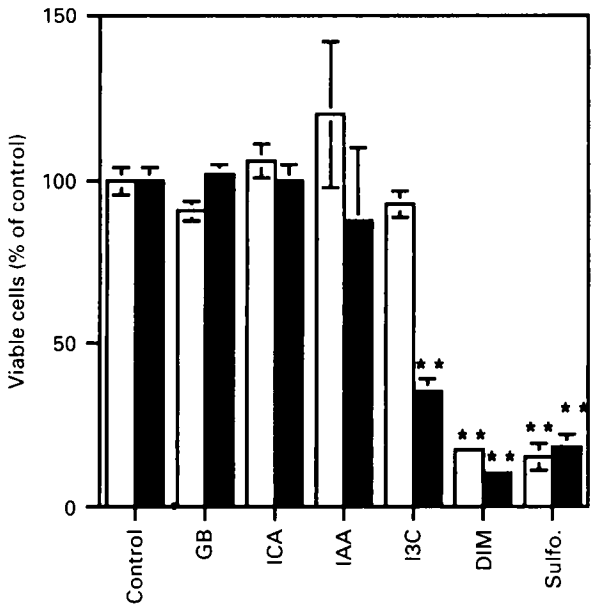

Figure 1. Effects of sulforaphane and of the main hydrolysis products of glucobrassicin on HT 29 cells viability. At time 0, quiescent cells were stimulated by 1% FCS and treated simultaneously with 100 μM GB, ICA, IAA, I3C, DIM, sulforaphane (sulfo) or with 0.2% ethanol (control). After a 24 (□) and 48 (■) h period of treatment, cellular viability was determined using the MTT assay as described in Materials and methods. Results are expressed as percent of control and are the mean ± SEM of four separate experiments (**$p < 0.01$).

Dose–response experiments and reversibility of the effect of DIM and sulforaphane

Dose-response experiments (Figure 2) demonstrate that the cytotoxicity of I3C was not observed when it was added to HT29 culture at concentrations below 100 μM (Figure 2a). In contrast, Figure 2(b and c) clearly shows that, after 48 h of incubation, DIM and sulforaphane reduced viable HT29 cells numbers in a dose-dependent manner, with an IC_{50} value of approximatively 10 and 15 μM, respectively.

As shown in Figure 3, we then tested the reversibility of the cytotoxic effect of 10 μM DIM (Figure 3a) or 15 μM sulforaphane (Figure 3b). DIM and sulforaphane were added to HT29 cell culture medium over 6, 12, 18, 24 or 48 h. At the end of each period of treatment, half of the HT29 cells were used for viability assays, whereas remaining cells were cultivated for another 30 h in DIM- or sulforaphane-deprived media. Cell viability was measured after the 30 h period of culture. Figure 3(a and b) clearly show a time-dependent decrease of viable HT29 cells in sulforaphane- and DIM-treated cultures, which was evident after 18 and 24 h of exposure, respectively. Moreover, our results demonstrated that the cytotoxic effect of 10 μM DIM could be reversed by removing it from the culture medium only if the duration of the treatment was less than 12 h. On the other hand, HT29 cells were allowed to grow in a sulforaphane-free medium even after a 18 h period of treatment with 15 μM of this isothiocyanate. However, incubation of HT29 cells with 15 μM sulforaphane for 24 h led to an irreversible decrease of viable cell numbers.

Effect of DIM and sulforaphane on HT29 cell cycle

The percentage of proliferating preconfluent cells was then measured after early drug treatment (10 μM DIM and 15 μM sulforaphane over 15 h) by flow cytometric analysis of DNA content (Figure 4a) using similar culture conditions as for cell viability experiments. At the beginning of the experiment, preconfluent HT29 cultures contained a high percentage of resting cells in G_0/G_1, which was maintained even after a 15 h period of culture in FCS-free medium, as seen in Figure 4(a).

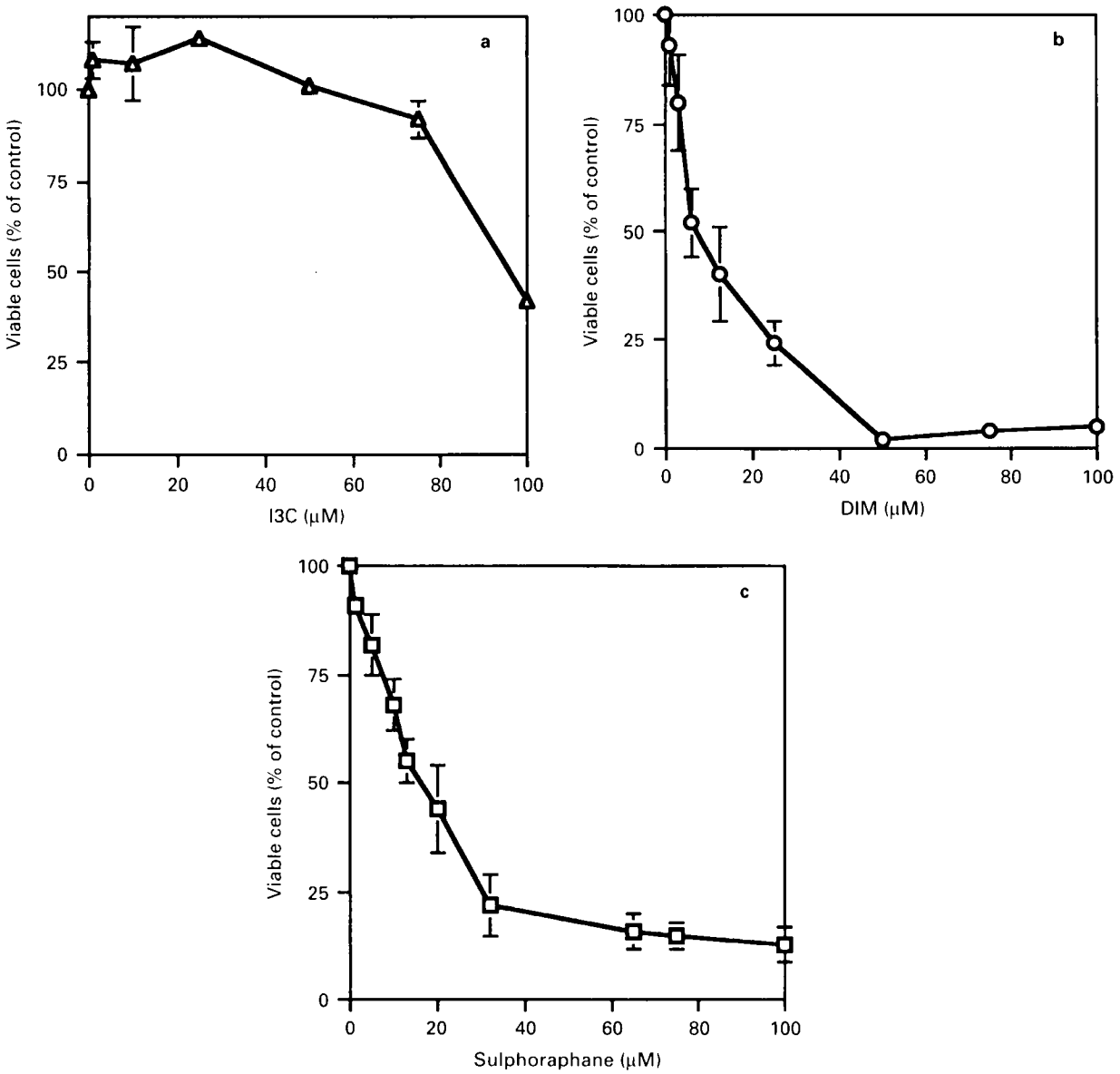

Figure 2. Effects of increasing doses of I3C (a), DIM (b) and sulforaphane (c) on HT29 cell viability. At the beginning of the experiment quiescent cells were stimulated to grow in the presence of 1% FCS and treated with 0.2% ethanol alone (control) or with increasing concentrations of DIM and sulforaphane diluted in ethanol. Cell viability was estimated as described in Materials and methods after 48 h of treatment. Results are expressed as the mean percentage of control cells ± SEM of three separate experiments. When they do not appear, error bars are smaller than the symbol size.

Following replenishment with 1% FCS-containing medium, a sizable fraction of cells was synchroneously stimulated to undergo proliferation. As shown in Figure 4(a), the percentage of cells in G_0/G_1, within 15 h following replenishment with serum-containing medium, decreased as the percentage of cells in S phase increased (from approximatively 25 to 60% of the population). In contrast, our results clearly show that addition of DIM or sulforaphane inhibited the reinitiation of FCS-induced DNA synthesis. Figure 4(b) indicates that their effects were not the result of an early delay in cell cycle progression since the arrest in G_0/G_1 for DIM- or sulforaphane-treated cells was maintained after 48 h of treatment. We have also studied the reversibility of the DIM- and sulforaphane-induced G_0/G_1 arrest (data not shown). The G_0/G_1 growth arrest observed in DIM-treated HT29 cells was not reversible whatever the time of incubation. Moreover, the percentage of HT29 cells arrested in G_0/G_1 30 h after replenishment with DIM-free medium

was increased when the period of treatment with this compound exceeded 15 h (data not shown). However, removal of sulforaphane from the culture medium after a 15, 24 or 30 h period of treatment with this isothiocyanate even led to reinitiation of the cell cycle (data not shown), although its effect on cell

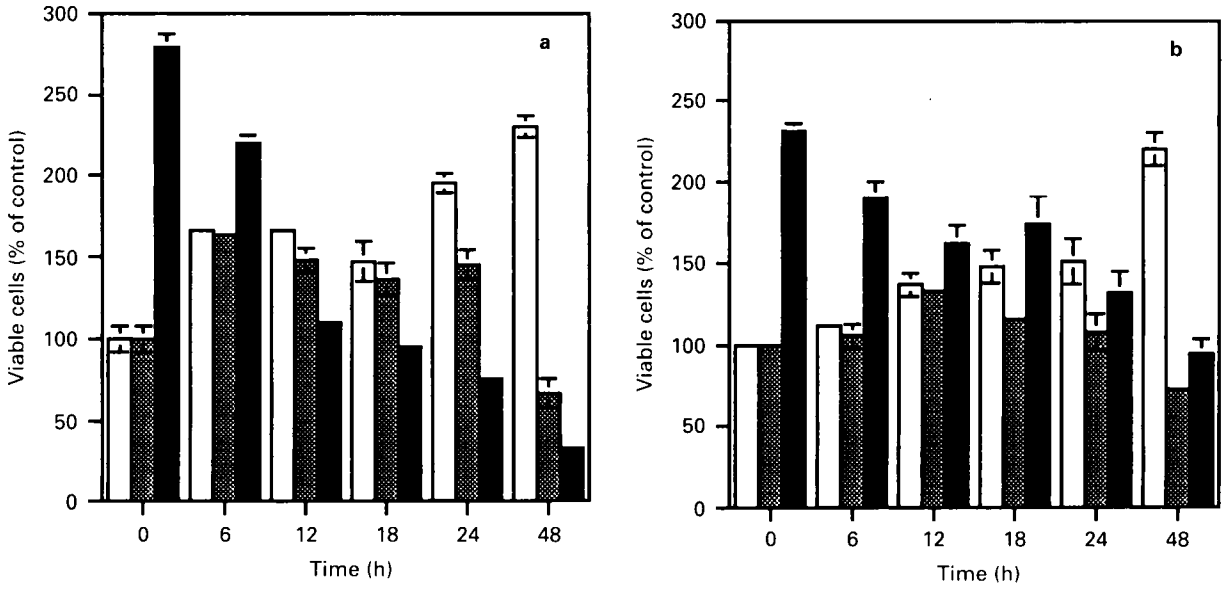

Figure 3. Kinetics and reversibility of the effects of DIM and sulforaphane on HT29 cell viability. At the beginning of the experiment, HT29 cells were allowed to grow in 1% FCS-containing medium in the presence of 0.2% ethanol ( ) (A and B), DIM 10 μM (A) of sulforaphane 15 μM (B) for 0 (control), 6, 12, 18, 24 or 48 h. At each time of incubation one half of cultured cells was used to estimate cellular viability ( ), the other half of cells was rinsed with a DIM or sulforaphane-free medium and then cultured for another 30 h in a fresh medium containing 1% FCS alone ( ). Cell viability was then determined as described in Materials and methods. Results are expressed as percent of control cells (number of viable cells at time 0 of the experiment) and are the mean ± SEM of three separate experiments. (a) A significant difference compared to untreated cells at $p<0.05$. (b) A significant difference compared to DIM- or sulforaphane-treated cells at $p<0.05$.

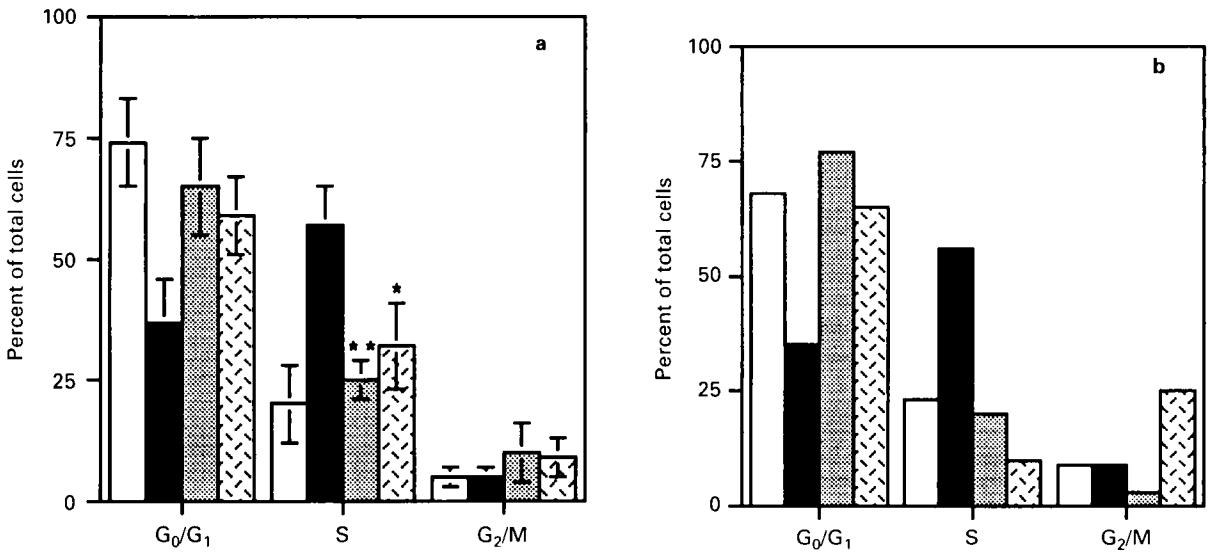

Figure 4. Effects of DIM and sulforaphane on HT29 cell cycle distribution. At time zero of the experiment, quiescent HT29 cells were either maintained in serum-free medium ( ) or stimulated to grow in the presence of 1% FCS and simultaneously treated with 0.2% ethanol (control) ( ) or 10 μM ( ) or 15 μM ( ) sulforaphane during 15 h (a) and 48 h (b). Cell cycle distribution was then analyzed by flow cytometry after labeling with propidium iodide as described in Materials and methods. Results in (a) are expressed as percent of total viable cells and are the mean ± SEM of five separate experiments. $^*p<0.05$; $^{**}p<0.01$ compared to control cells ( ). Results in (b) represent one typical experiment.

death is not reversible after 24 h of treatment (Figure 3b).

Effect of DIM and sulforaphane on differentiated CaCo2 cells

Finally, the effect of DIM and sulforaphane on differentiated cells was checked using differentiated CaCo2 cells (Figure 5). A first trial led to the conclusion that, as was observed for undifferentiated HT29 cells, DIM and sulforaphane were also effective on undifferentiated CaCo2 cells (data not shown). However, it is noteworthy that a 20 μM concentration of DIM is needed to decrease undifferentiated CaCo2 cells viability by 50%, revealing that CaCo2 cells are less sensitive than HT29 cells. Concerning experiments on differentiated cells, CaCo2 cells were allowed to reach confluence in a 10% FCS-containing medium. Starting from confluence, the cells were maintained in culture for 2–3 weeks to ensure a higher degree of differentiation (13). The value of alkaline phosphatase activity measured in these cells confirmed their differentiated state (data not shown). Therefore, they were treated with 0.2% ethanol (control), 10 μM DIM or 15 μM sulforaphane in 1% FCS-containing medium, and the percent of viable cells was estimated after 24 and 48 h of incubation. Interestingly, our results (Figure 5) clearly show that DIM did not affect CaCo2 cell viability in the range of concentrations tested and even when added at up to 100 μM (not shown). The viability of differentiated CaCo2 cells was not altered by sulforaphane until 30 μM; however, 50 μM sulforaphane decreased the percentage of viable cells by approximatively 70% compared to untreated cells.

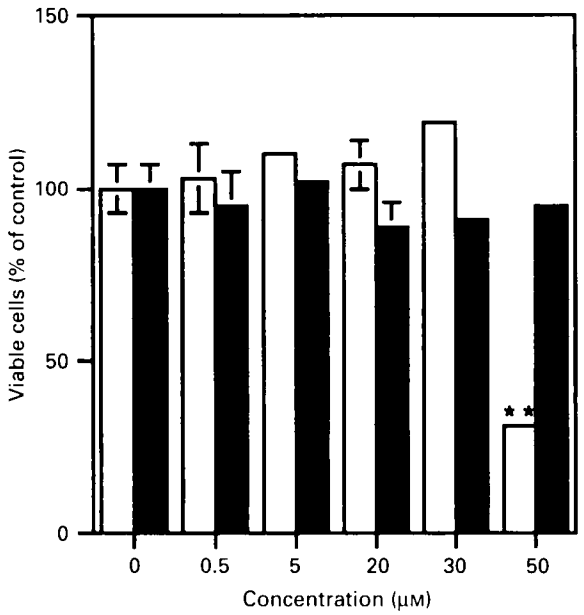

Figure 5. Effect of DIM and sulforaphane on CaCo2 intestinal-like differential cells. Post-confluent and differentiated CaCo2 cells were cultivated in 1% FCS-containing medium in the presence of DIM (■) or sulforaphane (□) at the indicated concentrations during 48 h. Cellular viability was then analyzed using the MTT assay as described in Materials and methods. Results are expressed as percent of control cells (i.e. 0.2% ethanol-treated cells) and are the mean ± SEM of three separate experiments (**$p < 0.01$).

Discussion

A number of previous experimental and epidemiological studies have demonstrated a decreased risk of colon cancer with a high consumption of vegetables. The glucosinolates hydrolysis products have been attracting increasing attention since a number of experimental protocols suggested that some of them could be involved in the anticarcinogenic properties of cruciferous vegetables.[7] Especially I3C, the major indole derivative occurring from glucobrassicin hydrolysis, has been shown to possess high bioactivities on numerous animal cells and tissues *in vivo* and *in vitro*.[10,21] However, in some *in vivo* studies the anticancer activity of I3C was attributed to the main product of its acid condensation, i.e. DIM.[22,23] Moreover, recently, Ge *et al.*[11] have shown that DIM induced apoptosis in mouse mammary cancer cells (MCF7 cells) *in vitro*. Isothiocyanates, the most common degradation products of aryl and alkyl glucosinolates, have also been tested as chemopreventive agents in many works. More than 20 compounds have been assessed and some of them have been shown to inhibit chemical carcinogenesis in animal models and cancer cell growth *in vitro*.[8,24] Sulforaphane has previously been predicted to exert chemoprotective activity since it is a potent monofunctional inducer of phase II enzymes in cultured cells as well as in mouse tissues; moreover, it is a potent inhibitor of CYP2E1 and it has been shown to block mammary tumor development in rats treated with 9,10-dimethyl-1,2-benzanthracene.[24–26]

Our results described for the first time the effect of glucosinolate-derived products on intestinal-derived cancer cells in culture. Our results showed that glucobrassicin by itself revealed no anticancerous properties when tested on human colonic adenocarcinoma HT29 cells. This result is in agreement with other studies showing that various intact glucosinolates were without effect on different cancer cell lines.[12] Among its hydrolytic breakdown products, IAA

and ICA did not exert any significant effect on HT29 cells viability. Surprisingly, I3C affected colon cancer cell growth or viability only at 100 μM. In contrast, low concentrations of DIM exerted a dose-dependent cytotoxic effect on HT29 colon cancer cells, preceded by DNA synthesis inhibition. This effect was not reversible after a 15 h period of treatment. Our results are consistent with the hypothesis of a potent antitumoral role of DIM rather than of I3C. Moreover, in our study it was observed for the first time that sulforaphane exerted a strong cytotoxic effect on colon cancer cells (HT29). This effect was significant at 15 μM and irreversible when the treatment exceeds 24 h. However, this isothiocyanate inhibited DNA synthesis in a reversible manner when added on quiescent cells (data not shown), whatever the duration of the treatment, suggesting that, comparatively with DIM, the cytotoxic effects of sulforaphane involve different pathways.

Moreover, it is particularly interesting to note that up to 30 μM DIM and sulforaphane did not exert any cytotoxic effect on intestinal-differentiated CaCo2 cells, although both products were very effective at these low concentrations on undifferentiated CaCo2 and HT29 cells. The selective toxicity of DIM and sulforaphane observed against cancer cells might be due to differences between normal and malignant cell membrane composition.[27] Isothiocyanates have been reported to exert cytotoxic effects via damage to the plasma membrane and to other intercellular membrane systems in hepatocytes.[28] Selective toxicity of other dietary factors against transformed cells has already been reported.[29]

Although our results need to be confirmed in other intestinal cancerous and normal cells, it is tempting to propose that the presence of such selective compounds in the diet may prevent the development of tumors by interfering with the growth of preneoplastic lesions while having little effect on normal cells. The cumulative effects of these inhibitions may contribute to the chemopreventive properties of the parent foodstuffs observed in epidemiological studies. Moreover, the chemical structure of DIM and sulforaphane could help to provide a basis for the further design of specific compounds pharmacologically active in cancer adjuvant chemotherapy.

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