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Comparison of growth inhibition profiles and mechanisms of apoptosis induction in human colon cancer cell lines by isothiocyanates and indoles from *Brassicaceae*

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Abstract

The isothiocyanates sulforaphane and PEITC (β-phenethyl isothiocyanate) as well as the indoles indole-3-carbinol and its condensation product 3,3′-diindolylmethane are known to inhibit cancer cell proliferation and induce apoptosis. In this study, we compared the cell growth inhibitory potential of the four compounds on the p53 wild type human colon cancer cell line 40-16 ($p53^{+/+}$) and its p53 knockout derivative 379.2 ($p53^{-/-}$) (both derived from HCT116). Using sulforhodamin B staining to assess cell proliferation, we found that the isothiocyanates were strongly cytotoxic, whereas the indoles inhibited cell growth in a cytostatic manner. Half-maximal inhibitory concentrations of all four compounds in both cell lines ranged from 5–15 μM after 24, 48 and 72 h of treatment. Apoptosis induction was analyzed by immunoblotting of poly(ADP-ribose)polymerase (PARP). Treatment with sulforaphane (15 μM), PEITC (10 μM), indole-3-carbinol (10 μM) and 3,3′-diindolylmethane (10 μM) induced PARP cleavage after 24 and 48 h in both 40-16 and the 379.2 cell lines, suggestive of a p53-independent mechanism of apoptosis induction. In cultured 40-16 cells, activation of caspase-9 and -7 detected by Western blotting indicated involvement of the mitochondrial pathway. We detected time- and concentration-dependent changes in protein expression of anti-apoptotic Bcl-x_L as well as pro-apoptotic Bax and Bak proteins. Of note is that for sulforaphane only, ratios of pro- to anti-apoptotic Bcl-2 family protein levels directly correlated with apoptosis induction measured by PARP cleavage. Taken together, we demonstrated that the glucosinolate breakdown products investigated in this study have distinct profiles of cell growth inhibition, potential to induce p53-independent apoptosis and to modulate Bcl-2 family protein expression in human colon cancer cell lines.

Keywords: Isothiocyanates; Indoles; Apoptosis; Caspases; Colon cancer; Bcl-2 family proteins

1. Introduction

Colorectal cancer is among the most frequent types of cancers in the world and the second most leading cause of cancer-related death in industrialized countries [1]. Numerous epidemiological studies have reported a strong association between a high intake of fruits and vegetables and lower risk of developing colorec-

Abbreviations: GNS, gluconasturtiin; GRA, glucoraphanin; IC_{50}, half-maximal inhibitory concentration; Myr, myrosinase; PARP, poly(ADP-ribose)polymerase; PEITC, β-phenethyl isothiocyanate

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tal cancer [2,3]. Vegetables of the *Brassicaceae* family, in particular those of the *Brassica* genus (broccoli, cabbage, cauliflower, radish, mustard, etc.) have received much attention, because they are reported to have anticancer activity in vitro and in vivo [4,5]. Thioglucoside conjugates, namely glucosinolates, are responsible for the chemopreventive activity of cruciferous vegetables. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released on physical damage of plant cells (e.g. during cutting or chewing), glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates [6]. A modulation of carcinogen biotransformation enzymes, i.e. inhibition of phase I cytochrome P-450 (CYPs) and induction of phase II detoxification enzymes have been identified as possible mechanisms relevant for the cancer-preventive properties of glucosinolate cleavage products (reviewed in refs. [7,8]). Moreover, these compounds have been shown to possess growth-inhibiting and apoptosis-inducing activities in various cancer cell lines in vitro [9].

Apoptosis (programmed cell death) is a natural form of cell death, which can be induced either by an "extrinsic" pathway mediated via activation of death receptors such as CD95 (Fas, APO-1), or by an "intrinsic" mitochondria-mediated pathway [10]. Both pathways lead to activation of caspases, i.e. cysteinyl proteases which are responsible for the execution of cell death by cleaving cellular substrates. Proteins of the Bcl-2 family, which comprise pro-apoptotic (e.g. Bax, Bak) as well as anti-apoptotic (e.g. Bcl-2, Bcl-x_L) members, are major regulators of the intrinsic pathway. They form homo- and heterooligomers, which act directly at the outer mitochondrial membrane. The ratio of pro- to anti-apoptotic oligomers has been suggested to play an important role in determining commitment to cell death [11]. Breakdown of the mitochondrial membrane potential leads to a release of pro-apoptotic factors such as cytochrome *c* from the intermembrane space into the cytosol, and to formation of the so-called apoptosome, which includes apoptotic protease activating factor-1 (Apaf-1), dATP and procaspase-9. Subsequent activation of the initiator caspase-9 in the apoptosome leads to cleavage of effector caspases such as caspase-3 and -7 [10]. During apoptosis, the DNA repair enzyme poly(ADP-ribose)polymerase (PARP) (116 kDa) is cleaved by caspase-3 and -7 into a 89 kDa and a 24 kDa fragment. PARP cleavage represents a hallmark of late apoptosis [12].

Sulforaphane is one of the best investigated isothiocyanates, which is particularly abundant in broccoli (*Brassica oleracea* L. var. *italica*) in the form of its corresponding glucosinolate glucoraphanin [6]. In 1992, Zhang et al. initially identified sulforaphane as a principal inducer of phase II enzymes including NAD(P)H:quinone oxidoreductase in broccoli [13]. Subsequently, several studies demonstrated chemopreventive potential of sulforaphane, i.e. by inhibition of 7,12-dimethylbenz[*a*]anthracene-induced mammary tumors, of azoxymethane-induced colonic aberrant crypt foci formation in rats [14,15] and of benzo[*a*]pyrene-induced forestomach cancer in mice [16]. In addition to its modulatory effects on carcinogen metabolism, induction of apoptosis in various cancer cell lines was identified as a potential mechanism underlying the chemopreventive activities of sulforaphane (reviewed in refs. [9,17–19]). For example, Gamet-Payrastre et al. reported that sulforaphane treatment for up to 72 h at a concentration of 15 μM induced cell cycle arrest in G_2/M-phase followed by apoptosis induction in cultured HT29 colon carcinoma cells [20].

β-Phenethyl isothiocyanate (PEITC) is found as its corresponding glucosinolate gluconasturtiin in considerable quantities in water cress (*Nasturtium officinale* L.) [6]. Like sulforaphane, PEITC is able to inhibit CYPs and to induce phase II detoxification enzymes [8]. In animal studies, PEITC was shown to inhibit 4-(methylnitrosamino)-1-(3-pyridyl)-1-butone-induced pulmonary neoplasia in rats and mice [9,21], and azoxymethane-induced colonic aberrant crypt foci formation in rats [14]. During the progression phase of carcinogenesis, PEITC was able to inhibit proliferation and to induce apoptosis in leukemia and colon, prostate, lung, and liver cancer cell lines [8,9,17,22]. The mechanisms by which PEITC induces programmed cell death are only partially understood. Activation of c-Jun NH_2-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) have been suggested to play a role, as well as mitochondrial- and death receptor-mediated pathways (reviewed in ref. [9]).

Indole-3-carbinol is the main hydrolysis product of the glucosinolate glucobrassicin, of which the corresponding isothiocyanate is unstable [6]. Indole-3-carbinol is especially known for its cancer-protective effects in reproductive organs, both in vitro and in vivo [23–25]. Under low pH conditions, as in the stomach, several condensation reactions of indole-3-carbinol occur which result in the formation of 3,3′-diindolylmethane as a major condensation product [26]. Therefore, it has been suggested that 3,3′-diindolylmethane and not indole-3-carbinol is responsible for the physiological effects of dietary indole-3-carbinol in vivo [27,28]. Both indole-3-carbinol and 3,3′-diindolylmethane were found to suppress cell proliferation and induce apoptosis in breast, prostate, cervical and colon cancer cell lines (refs. [17,28,30,31]

and literature cited therein). Mechanisms responsible for growth inhibition and apoptosis induction have been shown to involve cell cycle arrest in the G_1 phase [17,30].

In the present study, we compared profiles of cell growth inhibition of sulforaphane, PEITC, indole-3-carbinole and 3,3′-diindolylmethane in two human colon cancer cell lines by investigating their effects on cell proliferation in a concentration- and time-dependent manner. We further examined apoptosis-inducing potential by detection of PARP cleavage. Mechanistic investigations were focused on the mitochondrial pathway of apoptosis induction, i.e. by activation of caspases-9 and -7 and changes in the expression of Bcl-2 family proteins.

2. Materials and methods

2.1. Chemicals

Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier [32]. 3,3′-Diindolylmethane (CAS no. 1968-05-4) was purchased from LKT Laboratories Inc. (Minnesota, USA). Phenethyl isothiocyanate (PEITC, CAS no. 2257-9-2) and indole-3-carbinol (CAS no. 700-06-1) were from Sigma (Taufkirchen, Germany). Glucoraphanin and gluconasturtiin were isolated according to a previously reported procedure [33]. The enzyme myrosinase (EC 3.2.3.147) was isolated from mustard seeds (*Sinapis alba* L.) and purified according to Pessina et al. [34]. The stock solution used in the present study had a specific activity of ∼60 units/mg of soluble protein. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 μmol/min of sinigrin at pH 6.5 and 37 °C. All cell culture materials were obtained from Invitrogen (Eggenstein, Germany). Fetal bovine serum was provided by Pan (Aidenbach, Germany). McCoy's 5A cell culture medium and sulforhodamin B (SRB) were purchased from Sigma (Taufkirchen). Antibodies against PARP (#9542), Caspase-7 (#9492), Caspase-9 (#9502) as well as Bcl-x_L (#2762), Bax (#2772) and Bak (#3792) were obtained from Cell Signaling Technology (Beverly, USA). Anti-mouse- and anti-rabbit-secondary antibodies were obtained from Santa Cruz (Heidelberg, Germany). β-Actin antibody AC-15 (A5441) was purchased from Sigma (Taufkirchen). Materials and equipment for gel electrophoresis were from Bio-Rad (Munich, Germany). All other chemicals were from Sigma (Taufkirchen).

2.2. Inhibition of cell proliferation

The human colon cancer cell lines 40-16 p53 wild type ($p53^{+/+}$) and its p53 knockout derivative 379.2 ($p53^{-/-}$) were provided by B. Vogelstein from Johns Hopkins Oncology Center (Baltimore, USA). We chose this particular pair of cell lines in order to investigate the role of the tumor suppressor protein p53 in our mechanistic studies. Cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified environment of 5% CO_2 in air.

Cultured 40-16 or 379.2 cells (2.5×10^4 cells/ml in McCoy's 5A medium) were plated in 96-well plates (200 μl/well). After overnight growth, cell culture medium was changed and cells were treated with the test compounds dissolved in DMSO (final DMSO concentration 0.5%) in a concentration range of 0.4–50 μM in duplicates. Cells were treated with 0.5% DMSO as solvent control. After treatment for an additional 24, 48 or 72 h, respectively, the medium was discarded and cells were fixed using 50 μl of 10% aqueous trichloro acetic acid (TCA) for 30 min at 4 °C. Sulforhodamin B staining was performed as described by Skehan et al. [35]. Calculation of anti-proliferative activity was based on the ratio of absorbance readings of treated cells to those of solvent controls (set as 100%) after correction for absorbance of cells present at the time of treatment (day 0 values). Negative values indicate cytotoxic effects. In these cases, day 0 values of the control were used to calculate the percentage of cytotoxicity. IC_{50} values (half maximal inhibitory concentrations) were computed from the results of eight serial two-fold dilutions of test compound tested in duplicate from at least three independent experiments, using TableCurve Windows Version 1.0 software (Jandel Scientific, San Rafael, USA) with the equation for a logistic dose–response curve.

2.3. Western blot analysis

40-16 or 379.2 cells were plated in 100 mm tissue culture dishes (5×10^5 cells/10 ml) and treated as indicated in the figure legends after overnight growth. Attached and floating cells were collected, lysed and homogenized in SDS lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromphenol blue). Protein content was determined using the bicinchoninic acid (BCA) method [36] after precipitation with cold 10% TCA. Total protein (ca. 30 μg/lane) was subjected to SDS-PAGE (10% acrylamide for PARP and 12% for caspases and Bcl-2 proteins) under standard conditions and electroblotted onto polyvinylidene difluoride membranes in 15% methanol, 25 mM Tris and 192 mM glycine. Equal protein loading per lane was ensured by using an anti-β-actin antibody. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (10 mM Tris, pH 7.4, 100 mM NaCl) containing 0.01% Tween 20 for 1 h at RT and incubated with primary antibody overnight at 4 °C. After washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase and streptavidin-horseradish peroxidase for 1 h at RT. Membranes were developed using a chemiluminescence system. For quantitation of protein expression, band densities were semi-quantitatively measured using TINA software Version 2.09a (Raytest Isotopenmessgeräte GmbH, Staubenhardt, Germany). Values were normalized to β-actin expression, background staining was subtracted, and values were expressed in relation to control values, which were set as 1.0 for each time point.

2.4. Statistical analysis

Results are presented as mean ± standard deviation of data originating from at least three independent experiments. For statistical evaluation Student's *t*-test was applied. Values of $p < 0.05$ were considered as statistically significant.

3. Results

3.1. Anti-proliferative effects of sulforaphane, PEITC, indole-3-carbinol and 3,3′-diindolylmethane

To investigate the effect of the test compounds on cell growth, we utilized the 40-16 human colon cancer cell line established from a random HCT116 clone, which is deficient in the mismatch repair protein hMLH but expresses wild type p53 [37]. In addition, we used the respective p53 knockout cell line 379.2 ($p53^{-/-}$) generated from the 40-16 cell line [38]. Incubation with sulforaphane, PEITC, indole-3-carbinol or 3,3′-diindolylmethane (Fig. 1) at a concentration range of 0.4–50 μM led to a dose-dependent inhibition of cell proliferation. IC_{50} values obtained after 24, 48 and 72 h of treatment in cultured 40-16 ($p53^{+/+}$) and 379.2 ($p53^{-/-}$) cells are summarized in Table 1, and ranged from 5.1 to 14.9 μM. With the exception of 3,3′-diindolylmethane, which became more inhibitory in 379.2 cells with longer incubation times, the compounds were as effective at inhibiting cell proliferation after 24 h of incubation as after 72 h. Interestingly, 379.2 cells seemed to be as sensitive towards the treatment as the wild type cell line, suggesting that all four test compounds act independently of p53.

In addition to the synthetic forms of the isothiocyanates sulforaphane and PEITC, we generated the two compounds in situ by adding myrosinase (Myr) to the respective glucosinolate precursors, glucoraphanin (GRA) and gluconasturtiin (GNS). As can be seen from the IC_{50} values in Table 1, mixtures of the glucosinolates with Myr showed equal dose-dependent growth inhibition and cytotoxicity compared to the synthetic compounds. GRA and GNS alone did not show anti-proliferative effects after 72 h of treatment (Fig. 2A). Myrosinase alone had no effect on cell growth (data not shown).

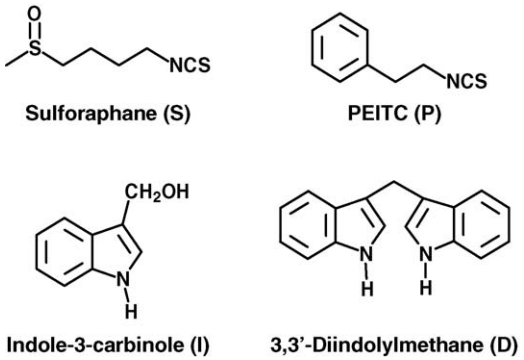

Fig. 1. Chemical structures of sulforaphane, β-phenethyl isothiocyanate (PEITC), indole-3-carbinol and 3,3′-diindolylmethane.

Further comparison of the profiles of cell growth inhibition revealed clear differences between the isothiocyanates sulforaphane and PEITC on the one hand, and the indoles indole-3-carbinol and 3,3′-diindolylmethane on the other hand. All compounds significantly inhibited cell growth at concentrations above 3.1 μM. Sulforaphane and PEITC displayed cytotoxic effects at concentrations higher than 12.5 μM, detected by a reduction in cell numbers (i.e. absorbance values after SRB staining) to values below the initial baseline (Fig. 2B).

Table 1
Summary of anti-proliferative potential

	Cell line					
	40-16 IC_{50} ± S.D. (μM)[a]			379.2 IC_{50} ± S.D. (μM)[a]		
	24[b]	48[b]	72[b]	24[b]	48[b]	72[b]
Sulforaphane	7.7 ± 1.4	6.6 ± 1.3	5.7 ± 1.6	6.1 ± 1.6	6.8 ± 2.8	6.0 ± 2.6
GRA + Myr	7.4 ± 0.7	5.6 ± 0.7	6.3 ± 1.7	n.d.	n.d.	n.d.
PEITC	9.6 ± 3.1	10.0 ± 1.4	8.2 ± 3.2	7.1 ± 1.4	8.7 ± 1.3	10.0 ± 2.4
GNS + Myr	7.2; 10.8[c]	7.6; 9.5[c]	8.6 ± 3.2	n.d.	n.d.	n.d.
Indole-3-carbinol	10.0 ± 3.1	8.0 ± 1.8	6.5 ± 0.7	6.0; 15.2[c]	5.1[c]	8.5 ± 2.2
3,3′-Diindolylmethane	14.5 ± 1.8	11.2 ± 3.6	10.0 ± 4.1	14.9 ± 3.9	11.1 ± 7.0	8.7 ± 2.2[*]

n.d., not determined.
[a] Halfmaximal inhibitory concentrations (mean ± standard deviation with $n \geq 3$ except for those indicated).
[b] Incubation time (h).
[c] $n \leq 2$.
[*] Means significantly ($p < 0.05$) different between 24 and 72 h of treatment.

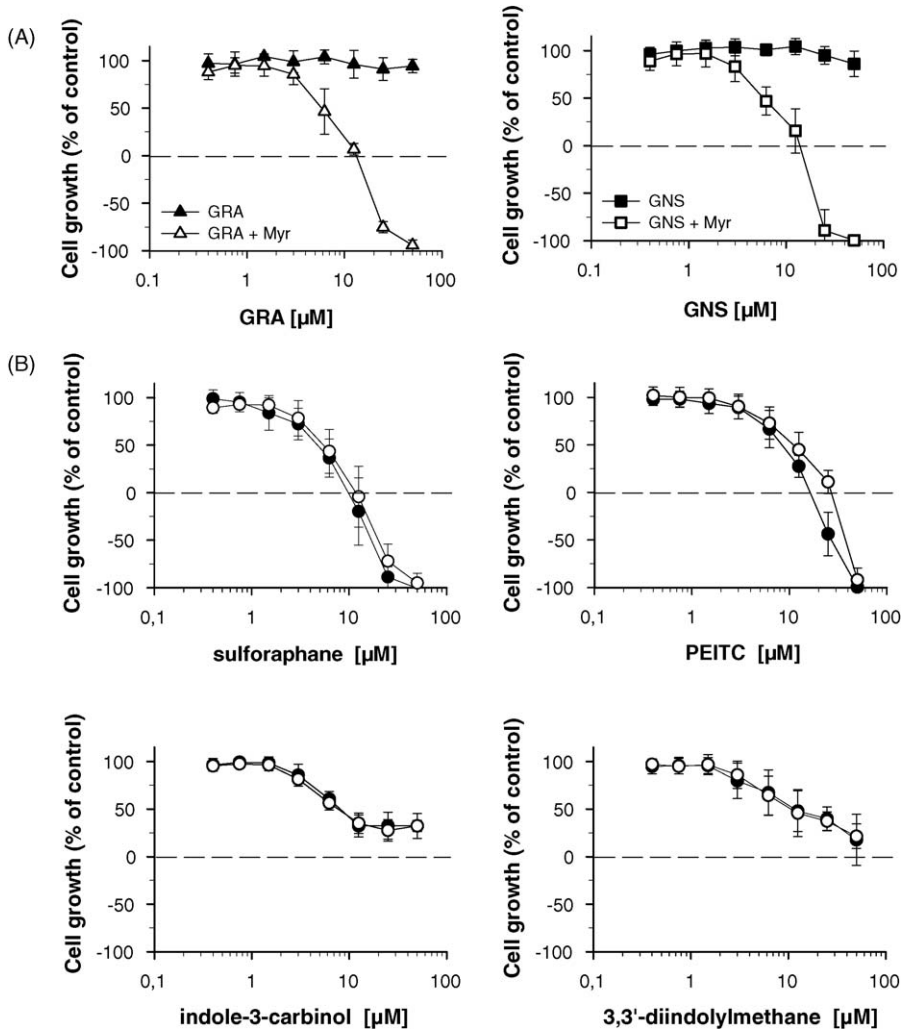

Fig. 2. Profiles of cell growth inhibition. (A) In situ generation of sulforaphane and PEITC. Effects of the glucosinolates GRA and GNS, respectively, on proliferation of 40-16 cells were tested in the presence or absence of myrosinase (Myr; 43.75 mU/ml). (B) Growth curves of sulforaphane, PEITC, indole-3-carbinol and 3,3′-diindolylmethane, respectively, with cultured 40-16 ($p53^{+/+}$) (●) and 379.2 ($p53^{-/-}$) (◯) cells. Inhibition of cell proliferation was determined by sulforhodamine B staining. Cells were treated with eight different concentrations of the test compounds (0.4–50 μM) or 0.5% DMSO as a solvent control for 72 h. Mean values from at least three independent experiments (±S.D.) are expressed as percentage of the values from control cells. Dose-dependent inhibition of proliferation is shown by values above the baseline, whereas values below the baseline represent cytotoxic effects.

In contrast, growth inhibition curves of cells treated with indole-3-carbinol or 3,3′-diindolylmethane showed a cytostatic profile (Fig. 2B). Even at a concentration of 50 μM, cell numbers were still above the baseline, indicating inhibition of cell proliferation, but with no indication of cytotoxicity.

3.2. PARP cleavage as a measure of apoptosis induction

Only a few studies have directly compared the intensity and mechanisms of apoptosis induction by the two main groups of glucosinolate breakdown products [39,40]. Since in nature indoles and isothiocyanates are often found in concert, parallel investigations of the compounds and comparison of the effects are of special interest.

We investigated apoptosis induction by analyzing the cleavage of PARP by immunoblotting (Fig. 3). For Western blot experiments, 40-16 and 379.2 cells were incubated in the presence of test compounds for 10, 24 and 48 h at a concentration range of 10–20 μM as indicated. All four compounds induced PARP cleavage after 24 and 48 h of treatment in 40-16 ($p53^{+/+}$) (Fig. 3A) as well as in

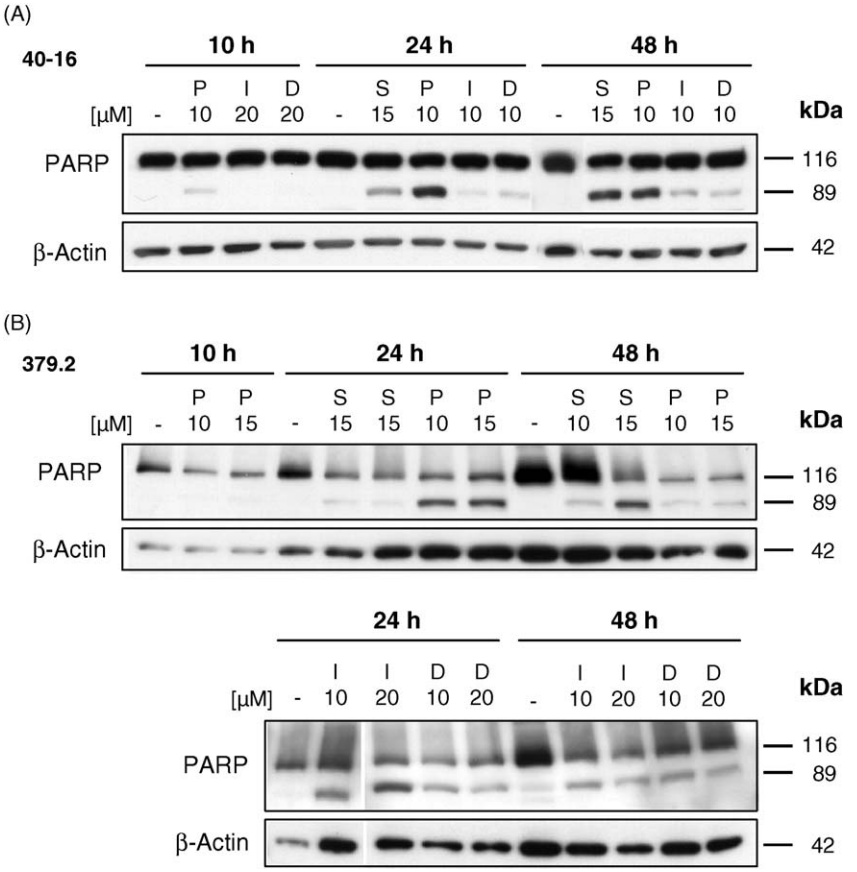

Fig. 3. PARP cleavage. Immunoblotting for PARP using lysates from 40-16 ($p53^{+/+}$) (A) and 379.2 ($p53^{-/-}$) (B) cells treated with sulforaphane (S), PEITC (P), indole-3-carbinol (I), 3,3′-diindolylmethane (D) or 0.2% DMSO (−) as indicated for 10, 24 and 48 h, respectively. PARP cleavage was investigated using Western blot analyses using an antibody directed against full length PARP (116 kDa) and the cleavage product (89 kDa). Equal loading was assessed by using an anti-β-actin antibody.

379.2 ($p53^{-/-}$) cells (Fig. 3B). PARP cleavage induced by 15 μM sulforaphane was detected earlier in 40-16 (24 h) than in 379.2 cells (48 h). PEITC (10 μM) strongly induced apoptosis in the wild type cell line after 24 and 48 h, whereas in the p53 (−/−) cell line, detection of the PARP cleavage product was weaker after 48 h than after 24 h. These results indicated that apoptosis induction by all four compounds appears to be independent of a functional p53 protein. Overall, in the 40-16 cell line the two indoles had a weaker apoptosis inducing effect than the isothiocyanates. This observation is consistent with the different anti-proliferative profiles of the two groups of compounds described above.

3.3. Activation of caspases-9 and -7

To investigate the involvement of the mitochondrial pathway in apoptosis induction, we performed immunoblotting of caspase-9. For the following mechanistic investigations, experiments were performed only with the wild type cell line 40-16 ($p53^{+/+}$), since p53 did not seem to play a role in the effects exerted by the test compounds. Treatment of 40-16 cells with sulforaphane, PEITC, indole-3-carbinol and 3,3′-diindolylmethane, respectively, led to a down-regulation of procaspase-9 (Pro-C9) (Fig. 4), which was already visible after 10 h, but more pronounced after 17 and 24 h of incubation. Activated caspase-9 (C9), represented by a 37 kDa cleavage product of the procaspase, appeared after 17 h of treatment. Interestingly, the kinetics of caspase-9 activation differed between the four compounds tested. PEITC at a concentration of 10 μM showed a rapid effect with a marked activation after 17 h, which declined again after 24 h. In contrast, sulforaphane at a concentration of 15 μM only weakly activated caspase-9 cleavage after 17 and 24 h, but had a stronger effect after 32 h. We included a 32 h time point for sulforaphane because we had

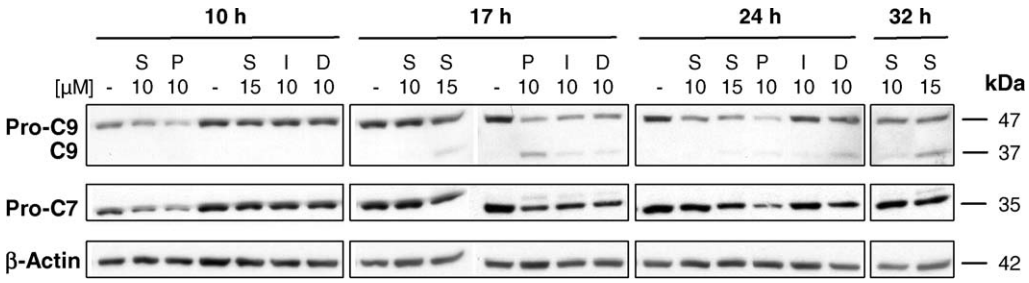

Fig. 4. Caspase activation. Immunoblotting for caspase-9 and -7 using lysates from 40-16 cells treated with sulforaphane (S), PEITC (P), indole-3-carbinol (I), 3,3′-diindolylmethane (D) or 0.2% DMSO (−) as indicated for 10, 17, 24 and 32 h, respectively. Cleavage of caspase-9 was investigated using a polyclonal antibody directed against procaspase-9 (47 kDa) and cleaved caspase-9 (37 kDa). Equal loading was assessed by using an anti-β-actin antibody.

observed delayed effects of this compound in preliminary experiments. Effect of the indoles on caspase-9 cleavage was weaker than that of the isothiocyanates. Treating 40-16 cells with 10 μM indole-3-carbinol and 3,3′-diindolylmethane led to a distinct down-regulation of procaspase-9 expression after 17 h, but the effect was weaker after 24 h. Nevertheless, activated caspase-9 could be detected after 24 h incubation with 3,3′-diindolylmethane.

As a downstream effect of caspase-9 activation we were interested in the expression of caspase-7, which is cleaved directly by caspase-9, but also by other caspases [41]. The isothiocyanates and indoles induced down-regulation of procaspase-7 (Pro-C7) after 10 h, which was more pronounced after 17 h (Fig. 4). After treatment periods of 24 and 32 h, effects of the indoles and sulforaphane, respectively, declined, whereas procaspase-7 cleavage became even more intense after treatment with PEITC. These results suggest an important role of caspase-7 in PEITC-induced apoptosis, which appears to be mediated via caspase-9 activation. These data point towards an involvement of the mitochondria-mediated pathway in apoptosis induction by the four agents tested.

3.4. Expression profiles of Bcl-2 family proteins

We were further interested in whether the compounds had an impact on the expression of Bcl-2 family proteins. All four test compounds induced a strong down-regulation of Bcl-x_L protein expression after 10, 24 and 48 h of treatment (Fig. 5). Interestingly, effects of the isothiocyanates persisted with time, whereas after treatment with the indoles, Bcl-x_L protein levels decreased after 24 h (ratio in comparison with the control = 0.6) but increased again after 48 h (ratios of 0.7 and 0.8, respectively).

Next, we analyzed the expression profiles of the pro-apoptotic family members Bax and Bak. Sulforaphane and PEITC induced a marked decrease in Bax expression after 24 h of treatment (ratios of 0.6), whereas after 48 h of treatment, Bax protein levels increased again (ratios 0.9 and 0.8, respectively) (Fig. 6A). Sulforaphane had little effect on Bak protein levels; after 48 h of incubation, a slight up-regulation could be observed (ratio 1.2 in comparison to the control). In contrast, PEITC dose-dependently induced a down-regulation of Bak with similar kinetics as detected for Bax: the stronger effect was seen after 24 h (ratio 0.5 at 15 μM) and recov-

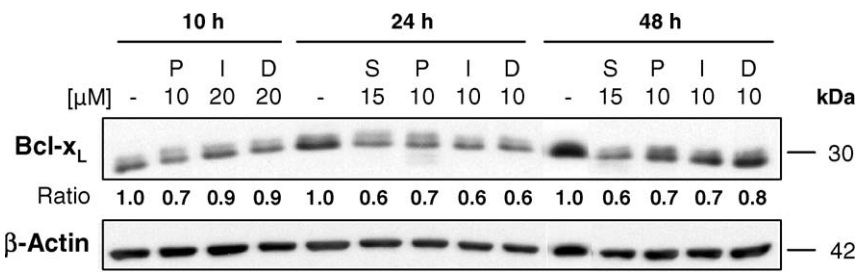

Fig. 5. Protein expression of Bcl-x_L. Immunoblotting for Bcl-x_L using lysates from 40-16 cells treated with sulforaphane (S), PEITC (P), indole-3-carbinol (I), 3,3′-diindolylmethane (D) or 0.2% DMSO (–) as indicated for 10, 24 and 48 h, respectively. Relative expression values were calculated as described in Section 2. Equal loading was assessed by using an anti-β-actin antibody.

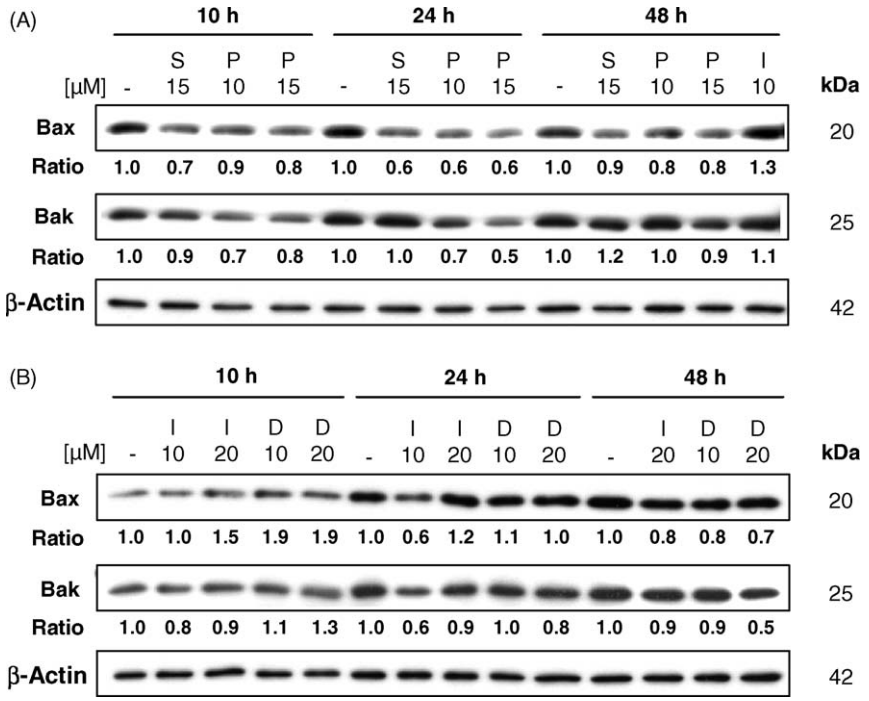

Fig. 6. Protein expression of Bax and Bak. Immunoblotting for Bax and Bak using lysates from 40-16 cells treated with sulforaphane (S), PEITC (P) (A), indole-3-carbinol (I), 3,3′-diindolylmethane (D) (B) or 0.2% DMSO (−) as indicated for 10, 24 and 48 h, respectively. Relative expression values were calculated as described in Section 2. Equal loading was assessed by using an anti-β-actin antibody.

ery could be observed after 48 h (ratio 0.9 at 15 μM) (Fig. 6A). The indoles had a different effect on Bax and Bak protein expression (Fig. 6B). At a concentration of 10 μM, indole-3-carbinol induced a down-regulation at 24 h (ratio 0.6), which reverted into an up-regulation after 48 h (ratio 1.3) (Fig. 6A). In contrast, at a 20 μM concentration, indole-3-carbinol resulted in an initial up-regulation of Bax after 10 h (ratio 1.5), which declined after 24 h (ratio 1.2) and turned into a slight down-regulation after 48 h (ratio 0.8). Indole-3-carbinol also had dose-dependent effects on Bak expression. At a concentration of 10 μM, Bax expression was down-regulated after 24 h (ratio 0.6) and recovered after 48 h (ratio 0.9), while a concentration of 20 μM appeared to have little effect on Bak protein levels. For 3,3′-diindolylmethane, results were more consistent. Expression of Bax was initially strongly up-regulated by 3,3′-diindolylmethane after 10 h (ratio 1.9), unchanged after 24 h (ratio 1.0) and down-regulated after 48 h (ratio 0.8). With 3,3′-diindolylmethane at a concentration of 20 μM and to a lesser extent also at a concentration of 10 μM, Bak levels were only slightly up-regulated after 10 h (ratio 1.3), but changed into a down-regulation after 24 and 48 h (ratios of 0.8 and 0.5, respectively). These results suggest that all four test compounds appear to have distinct effects with different kinetics on the expression of Bcl-2 family members.

3.5. Ratio of pro- and anti-apoptotic Bcl-2 family protein expression

Normalized expression levels generated in Figs. 5 and 6 were used to calculate ratios of Bax to Bcl-x_L, as well as of Bak to Bcl-x_L expression (summarized in Table 2). Interestingly, although the test compounds mostly induced a marked down-regulation of the pro-apoptotic proteins Bax and Bak, the ratios between Bax and Bak to Bcl-x_L were partially changed in favor of pro-apoptotic factors. In particular, sulforaphane treatment strongly increased the Bak:Bcl-x_L ratio in a time-dependent manner by a factor of 2.0 after 48 h of treatment, which was consistent with its effect on PARP cleavage as an indication of apoptosis induction (Fig. 3). A relation between the Bax and Bak to Bcl-x_L ratio and apoptosis induction was not so obvious for PEITC treatment, because it had little influence on the ratios, but rapidly induced apoptosis in 40-16 cells after 10 h of incubation (Fig. 3). Indole-3-carbinol (20 μM) treatment for 10 h increased the Bax:Bcl-x_L ratio 1.7-fold, whereas the Bak:Bcl-x_L ratio was not

Table 2
Relative ratios of Bax:Bcl-x_L and Bak:Bcl-x_L protein expression in 40-16 cells

	Ratio					
	Bax:Bcl-x_L[a]			Bak:Bcl-x_L[a]		
	10[b]	24[b]	48[b]	10[b]	24[b]	48[b]
Sulforaphane (15 μM)	n.d.	1.0	1.5	n.d.	1.7	2.0
PEITC (10 μM)	1.3	0.9	1.1	1.0	1.1	1.4
Indole-3-carbinol (10 μM)	1.7[c]	1.2	1.8	1.1[c]	1.1	1.5
3,3′-Diindolylmethane (10 μM)	2.0[c]	1.8	1.0	1.4[c]	1.7	1.1

n.d., not determined.
[a] Relative expression values were obtained as described in Section 2. Ratios were calculated relative to the controls at the respective time points, which were set as 1.0.
[b] Incubation time (h).
[c] Derived from incubations with 20 μM of the respective compound.

changed. These effects were not sufficient to commit cells to apoptosis. When 40-16 cells were treated with 10 μM indole-3-carbinol for 48 h, ratios of Bax:Bcl-x_L and Bak:Bcl-x_L were elevated by 1.8- and 1.5-fold, respectively. At this concentration and time point, apoptosis induction was detectable by PARP cleavage (Fig. 3). 3,3′-Diindolylmethane exerted stronger effects after 10 and 24 h of treatment. The Bax:Bcl-x_L ratio was elevated 2-fold after 10 h (20 μM) and 1.8-fold after 24 h (10 μM). Bak:Bcl-xL was increased by 1.7-fold after 24 h of treatment (10 μM). Similar to the effects of indole-3-carbinol, these changes were not sufficient to induce apoptosis. After 48 h of incubation, the ratios of Bax:Bcl-x_L and Bak:Bcl-x_L returned to control levels.

4. Discussion

Isothiocyanates and indole derivates found in *Brassica* vegetables such as broccoli are considered to be cancer chemopreventive agents, which act at several stages of carcinogenesis. They are known to inhibit the growth of cancer cells and to induce apoptosis, but the mechanisms are still only partially understood. Consequently, the present study was undertaken to shed more light on the mechanisms by which two isothiocyanates, sulforaphane and PEITC, and two indoles, indole-3-carbinol and its condensation product 3,3′-diindolylmethane, exert their anti-proliferative properties in cultured human colon cancer cells.

First, we determined the cell growth inhibitory effects of the test compounds in the 40-16 colon cancer cell line expressing the wild type tumor suppressor protein p53, in comparison with its p53 knockout derivative 379.2. Many chemotherapeutic agents act via p53, therefore, expression of functional p53 may be an important decisive factor regarding the response of tumor cells to these agents. Virtually every human tumor type bears either a mutation, a deletion or functional inactivation of p53, rendering the tumor cells often resistant towards chemotherapeutic treatment [42]. Therefore, substances which are able to induce apoptosis in cancer cells independent of p53 are of special interest. All four *Brassicaceae*-derived test compounds inhibited cell growth and induced PARP cleavage to a similar extent in the p53 knockout cell line as in the wild type cell line, indicting that the observed effects were induced by mechanisms independent of functional p53.

These observations are in accordance with other studies. Sulforaphane was reported to induce apoptosis in the human colon cancer cell line HT29, which bears a mutated p53 [20], and also in mouse fibroblasts with varying p53 status [43]. For PEITC, apoptosis induction was initially reported to be p53-dependent [44]. More recent studies disproved this finding by demonstrating apoptosis induction in p53-deficient human leukemia and prostate cancer cells [9,45]. Indole-3-carbinol and 3,3′-diindolylmethane were also found to induce programmed cell death in a p53-independent manner in prostate, breast and osteosarcoma cell lines [30,46,47]. These observations underline the potential of glucosinolate cleavage products as promising anti-cancer agents.

With respect to profiles of cell growth inhibition, we observed that the isothiocyanates acted as cytotoxic agents, whereas the indole derivatives were cytostatic. So far, only three reports have directly compared the anti-proliferative properties of indoles and isothiocyanates [39,40,48]. In two of these studies, human colon cancer cell lines were utilized: HT29 and Caco-2 [48], as well as LS-174, Caco-2 and HCEC [39]. Interestingly, the authors of both papers observed very weak anti-proliferative activity of indole-3-carbinol ($IC_{50} > 100$ μM after 24 h of treatment), but

stronger effects of 3,3′-diindolylmethane, sulforaphane and PEITC (PEITC was only used in the study by Bonnesen et al. [40]). These findings are in contrast to our results, because we determined comparable IC_{50} values (5–15 μM) for all four compounds after 24, 48 and 72 h of treatment. However, the authors of the above-cited studies did not differentiate between cytostatic and cytotoxic effects.

The cytostatic profile of growth inhibition exerted by the indoles leads to the suggestion that these compounds may not have the ability to induce apoptosis. Nevertheless, we demonstrated apoptosis induction in 40-16 ($p53^{+/+}$) and in 379.2 ($p53^{-/-}$) human colon cancer cells by both compounds at concentrations as low as 10 μM. Thus, it is possible that a portion of proliferating cells are partially resistant towards the apoptosis-inducing effects by these agents. It has been reported that indole-3-carbinol and 3,3′-diindolylmethane induce G_1 cell cycle arrest in human breast [25,29] and prostate cancer cell lines [30]. This may be a possible effect in 40-16 colon cancer cells.

In contrast to the results obtained for the indoles, the isothiocyanates were cytotoxic at concentrations higher than 12.5 μM. We detected via PARP cleavage apoptosis induction by sulforaphane (15 μM) and PEITC (10 μM) after 24 h of incubation, suggesting that the observed cytotoxic effect is due to induction of programmed cell death. At higher concentrations, toxicity could also be due to necrosis, as described in other cell lines [39,49]. Interestingly, necrosis induction in cancer cells has not been described for indoles so far. This may be an alternative explanation for the lack in cytotoxicity that we observed in this study.

To gain more information about the apoptosis inducing capacity of the compounds tested, we focused on the mitochondrial pathway and analyzed caspase-9 and -7 activation as well as the expression of Bcl-2 family proteins as the main regulators. A generally accepted mechanism which contributes to apoptosis induction induced by several anticancer compounds is the down-regulation of anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-x_L on the one hand, and the up-regulation of pro-apoptotic Bcl-2-family members like Bax and Bak on the other hand. This has been previously shown for sulforaphane-induced apoptosis in human prostate cancer cells [9], for PEITC-induced apoptosis in mouse prostate cancer cells [50] and for indole-3-carbinol- and 3,3′-diindolylmethane-induced apoptosis in human breast cancer cells [30,51].

In the study described here, treatment of 40-16 cells with 15 μM sulforaphane for 48 h led to a two-fold increase in the ratio of Bak to Bcl-x_L, and a 1.5-fold increase in the ratio of Bax to Bcl-x_L compared to control. Because we detected PARP cleavage after 48 h of sulforaphane treatment, apoptosis induction could be – partly – a consequence of the superiority of Bax and Bak oligomerisation in relation to Bcl-x_L at the outer mitochondrial membrane.

For PEITC, we observed only slight changes in the Bax:Bcl-x_L and the Bak:Bcl-x_L ratios. Nevertheless, we detected a strong and rapid (after 10 h) PARP cleavage by PEITC, which leads to the conclusion that Bcl-2 protein expression may play only a minor role in apoptosis induction by PEITC. Noteworthy, Bcl-2-family proteins are not only regulated by protein expression but also by translocation, phosphorylation and conformational changes. PEITC was reported to induce a conformational change of Bax and translocation of Bax to the mitochondria in the human hepatoma cell line HepG2 [22]. Interestingly in this respect, a recent report by Tang and Zhang showed that PEITC treatment at a 15 μM concentration within 3 h induced apoptosis in cultured human bladder cancer UM-UC-4 cells by directly damaging both the outer and inner mitochondrial membranes, which was followed by a collapse of the mitochondrial transmembrane potential, release of cytochrome *c* and activation of caspase-9 [52]. Since these effects occurred very rapidly after treatment of cells or isolated mitochondria with the isothiocyanate, it can be speculated that they are independent of changes in the expression of Bcl-2 family members. These mechanisms could also be effective in the 40-16 cell line, and their involvement in the rapid apoptosis induction by PEITC will be further investigated.

Indole-3-carbinol and 3,3′-diindolylmethane (20 μM) caused a strong up-regulation in the ratios of pro-apoptotic Bcl-2 family members in relation to Bcl-x_L after 10 h of treatment. These effects did not lead directly to apoptosis induction, because we could not observe PARP cleavage after a short incubation period of 10 h. Thus, an elevated ratio of pro- to anti-apoptotic Bcl-2 family alone is not sufficient to induce apoptosis. Taking into consideration the cytostatic cell growth inhibition profiles observed with the indole derivatives, it may be concluded that additional mechanisms, e.g. cell cycle arrest, as observed in breast and prostate cancer cell lines [30], initially block the response of the cells to undergo apoptosis.

In conclusion, we have introduced new aspects of cell growth inhibition potential and mechanisms of four well-known naturally occurring glucosinolate cleavage products. By direct comparison of growth inhibition profiles of colon cancer cells we demonstrate substantial differences between the isothiocyanates sulforaphane

and PEITC on the one hand, and the indoles indole-3-carbinol and 3,3′-diindolylmethane on the other hand. The isothiocyanates were strongly cytotoxic, whereas the indoles acted in a rather cytostatic manner. Concurrently, we found that at comparable doses, sulforaphane and PEITC are more efficient inducers of apoptosis than the indoles. Moreover, apoptosis induction of all four compounds was p53-independent, which makes them even more attractive as anticancer drugs. The mitochondria appear to be involved in the cell death program induced by all four compounds, indicated by caspase-9 activation and changes in the ratios of pro- to anti-apoptotic Bcl-2-family proteins. Future studies should focus on the mechanisms behind the differences of the anti-proliferative properties of isothiocyanates and indoles. Because these compounds occur naturally in the plant in varying quantities, potential combination effects should also be investigated.

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