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Quantitative combination effects between sulforaphane and 3,3'-diindolylmethane on proliferation of human colon cancer cells *in vitro*

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Abbreviations:

SFN, Sulforaphane; DIM, 3,3'-diindolylmethane; ITC, isothiocyanate; IC_{50}, half-maximal inhibitory concentration; CI, combination index; *f*a, affected fraction; *f*u, unaffected fraction; *D*, concentration; PARP, poly (ADP-ribose) polymerase

Abstract

Isothiocyanates and indoles derived from cruciferous vegetables possess growth-inhibiting and apoptosis-inducing activities in cancer cell lines *in vitro*. Isothiocyanates like sulforaphane (SFN) are cytotoxic, whereas indoles including indole-3-carbinol or its condensation product 3,3'-diindolylmethane (DIM) are acting by cytostatic mechanisms in human colon cancer cell lines. In the present study, we have investigated the impact of defined combinations of SFN and DIM (ratio 1:4, 1:2, 1:1, 2:1 and 4:1) on cell proliferation, cell cycle progression and apoptosis induction in cultured 40-16 colon carcinoma cells. Calculations of combination effects were based on the method of Chou and Talalay (1984), and were expressed as a combination index (CI) with CI<1, CI=1 or CI>1 representing synergism, additivity or antagonism, respectively. Interestingly, at a total drug concentration of 2.5 µM, all combinations of SFN and DIM were antagonistic. With increasing concentrations, the antagonistic effect gradually turned into a synergistic interaction at the highest combined cytotoxic concentration of 40 µM. Cell cycle analyses with SFN:DIM ratios of 1:1, 1:2 and 1:4 and total concentrations between 10 and 25 µM confirmed antagonism at low, and additive effects at higher doses. SFN (10 µM) in combination with DIM (10 µM) resulted in strong G_2/M cell cycle arrest which was not observed with either compound alone. Our results indicate that cytotoxic concentrations of SFN:DIM combinations affect cell proliferation synergistically. At low total concentrations (below 20 µM), which are physiologically more relevant, the combined broccoli compounds showed antagonistic interactions in terms of cell growth inhibition. These data stress the need for elucidating mechanistic interactions for better predicting beneficial health effects of bioactive food components.

1. Introduction

Cruciferous vegetables, in particular those of the *Brassica* genus (broccoli, cabbage, cauliflower, radish, mustard etc.) possess cancer preventive potential *in vitro* and *in vivo* that has been attributed to their content in thioglucoside conjugates, namely glucosinolates [1,2]. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released upon physical damage of plant cells (e.g. during cutting or chewing), glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates (ITCs).

The ITC sulforaphane (SFN) is particularly abundant in broccoli (*Brassica oleracea* L. var. *italica*) as its corresponding glucosinolate glucoraphanin [3]. In 1992, Zhang *et al.* initially identified SFN as a principal inducer of phase II enzymes including NAD(P)H:quinone oxidoreductase in broccoli [4]. Subsequently, chemopreventive efficacy of SFN was demonstrated by inhibition of 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis, of azoxymethane-induced colonic aberrant crypt foci formation in rats [5,6] and of intestinal adenoma formation in $Apc^{Min/+}$ mice [7,8]. Apart from its modulatory effects on carcinogen metabolism, induction of cell cycle arrest and apoptosis in various cancer cell lines were identified as mechanisms underlying the chemopreventive activities of SFN [9-13].

Mature broccoli is also rich in the indole-based glucosinolate glucobrassicin. Cleavage by myrosinase results in an unstable ITC that is further converted to indole-3-carbinol and other indole derivatives [3]. 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 (DIM) as a major condensation product [14]. Based on this observation, it has been suggested that DIM rather than indole-3-carbinol is responsible for the physiological effects of dietary indole-3-carbinol observed *in vivo* [15,16].

Both indole-3-carbinol and DIM possess cancer protective effects in reproductive organs, which are supposed to be due to the induction of specific cytochrome P450 enzymes involved in estrogen metabolism [17-19]. Indole-3-carbinol and DIM were also found to suppress cell proliferation and to induce apoptosis in breast, prostate, cervical and colon cancer cell lines [10,20,21], partly mediated by cell cycle arrest in the G_1 phase [22-24].

Numerous bioactive plant constituents are concertedly consumed with a diet rich in fruits and vegetables that is supposed to reduce cancer risk. It has been suggested that synergistic effects of combined low doses of these phytochemicals may account for the observed health benefits [25]. Although intense research is being conducted to reveal molecular mechanisms of chemopreventive activities and to demonstrate chemopreventive efficacy of single pure plant constituents, only few studies have been undertaken to systematically quantitate combination effects of these compounds. With respect to glucosinolate cleavage products and other substances found in Brassica vegetables, El-Bayoumy *et al.* demonstrated that a mixture of 1,4-phenylenebis(methylene)selenocyanate, phenethyl isothiocyanate, indole-3-carbinole and *d*-limonene was significantly more potent in inhibiting lung tumor multiplicity in A/J mice than indole-3-carbinol alone, but not than either of the other single compounds [26]. A synergistic induction of phase II enzymes was shown in rats treated with indole-3-carbinol and crambene (derived from the glucosinolate progoitrin) [27,28]. In a later study the same group reported that only a relatively high, non-physiological dose of a combination of indole-3-carbinol and crambene was able to reduce aflatoxin B1-induced expression of the preneoplastic marker glutathione *S*-transferase π in rats [29].

In extension of these studies focusing on the impact of combined broccoli compounds on the initiation stage of carcinogenesis, we were interested in how later stages would be affected by a mixture of SFN and DIM, using cell proliferation as an example. Interestingly, ITCs and indole derivates exert their anti-proliferative effects - at least in part - by different mechanisms. Recently, we have demonstrated that ITCs like SFN are cytotoxic in human colon cancer cell lines, whereas indoles like indole-3-carbinol or DIM are acting by a cytostatic mechanism [30]. Moreover, indoles were shown to halt the cell cycle in G_1 [21], while ITCs induce cell cycle arrest in G_2/M phase [31].

Thus, the aim of the present report was to quantify combination effects between the broccoli compounds SFN and DIM as representatives for ITCs and indoles, respectively, with regard to cell growth inhibition, cytotoxicity and on cell cycle distribution in the human colon cancer cell line 40-16.

2. Materials and Methods

Chemicals

Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier [32]. 3,3'-Diindolylmethane (DIM, CAS no. 1968-05-4) was purchased from LKT Laboratories Inc. (Minnesota, USA). All cell culture material was obtained from Invitrogen (Eggenstein, Germany). Fetal bovine serum was provided by PAA Laboratories (Pasching, Austria). McCoy's 5A cell culture medium, sulforhodamin B (SRB), propidium iodide (PI) and RNase A were obtained from Sigma (Taufkirchen, Germany). All material required for flow cytometry was purchased from Becton Dickinson (Franklin Lakes, NJ USA). The antibody against PARP (#9542) was 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, Germany). Materials and equipment for gel electrophoresis were purchased from Bio-Rad (Munich, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Inhibition of cell proliferation

The human colon cancer cell line 40-16, derived from a random HCT116 clone, was generously provided by B. Vogelstein from Johns Hopkins Oncology Center (Baltimore, USA). Cells were cultured as described earlier [30].

For combination experiments 40-16 cells (2.5 x 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 SFN or DIM dissolved in DMSO (final DMSO concentration 0.5%) in a concentration range of 0.3 to 40 µM, respectively. Alternatively, cells were treated with the combination of SFN and DIM (molar ratios 1:4, 1:2, 1:1, 2:1 and 4:1). Eight serial two-fold dilutions of the following starting concentrations were applied: 10 µM SFN + 40 µM DIM (1:4), 20 µM SFN + 40 µM DIM (1:2), 20 µM SFN + 20 µM DIM (1:1), 40 µM SFN + 20 µM DIM (2:1) and 40 µM SFN + 10 µM DIM (4:1). Cells were treated with 0.5% DMSO as solvent control. After incubation for additional 24 h, 48 h 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.* [33]. Calculations of anti-proliferative activities were based on the ratio of absorbance

readings of treated cells to those of solvent controls. Absorbance readings were corrected for absorbance of cells present at the time of treatment (day 0 values) in order to distinguish between cytostatic and cytotoxic effects. Negative values indicate cytotoxicity. 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 Table Curve Windows version 1.0 software (Jandel Scientific, San Rafael, USA) with the equation for a logistic dose-response curve. The percentage of cell growth inhibition was alternatively expressed as affected fraction (*f*a), with values between 0 and 0.5 for cytostatic effects, and 0.5 to 1 for cytotoxic effects.

Calculation of combination effects

Combination effects of SFN and DIM on cell growth inhibition were calculated according to Chou and Talalay [34]. Briefly, for median-effect plots, log (*f*a/*f*u) was plotted against log (*D*), where *D* represents the concentration of each single compound alone or the mixture of both, and *f*a and *f*u stand for the affected (values between 0 and 1) and unaffected (1-*f*a) fraction, respectively, at each concentration *D*.

Using CalcuSyn software (Biosoft, Ferguson, MO USA), which is based upon the method by Chou and Talalay [34], a combination index (CI) was computed for every fraction affected. CI<1, CI=1 or CI>1 represent synergism, additivity or antagonism of SFN and DIM, respectively. For the generation of CI-effect plots, original data points were taken and simulation curves were calculated for each experiment using Table Curve Windows version 1.0 software (Jandel Scientific, San Rafael, USA). These simulation curves were also used for adjusting CI values to fixed total concentrations.

Cell Cycle Analysis

40-16 cells were plated in 150 mm tissue culture dishes (1.75×10^6 cells/35 ml) and treated as indicated in figure legends after overnight growth. Attached and floating cells were collected, washed with PBS, fixed in ice cold 70% ethanol and stored at –20 °C. After washing twice with PBS, cells

were incubated with PBS containing propidium iodide (50 µg/ml) and RNase A (100 µg/ml) for 30 min at 37 °C. Then they were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). After discrimination of doublets, the percentage of cells in each phase of the cell cycle was determined by setting markers with CELL QUEST PRO software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western Blot Analysis

40-16 cells were plated in 100 mm tissue culture dishes (2.5×10^5 cells/10 ml) and treated as indicated in the figure legend 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). The protein content was determined using the bicinchoninic acid method [35] after precipitation with cold 10% TCA. Total protein (ca. 15 µg/lane) was subjected to 10% acrylamide SDS-PAGE under standard conditions and electroblotted onto polyvinylidene difluoride membranes in 15% methanol, 25 mM Tris, and 192 mM glycine. The membranes were blocked with 5% nonfat milk in Tris-buffered saline (10 mM Tris, pH 7.4, 100 mM NaCl) containing 0.05% Tween 20 for 1.5 h at RT and incubated with anti-PARP antibody (1:1000) overnight at 4 °C. After washing, the membranes were incubated with secondary antibody (1:10 000) conjugated with horseradish peroxidase for 1 h at RT. The membranes were developed using a chemiluminescence system. Equal protein loading per lane was ensured by using an anti-β-actin antibody (1:10 000).

Statistical Analysis

Results are presented as means ± standard deviation of data originating from at least three independent experiments. For statistical evaluation one-way ANOVA and Tukey's studentized range test was applied. Values of $p < 0.05$ were considered as statistically significant.

3. Results

Anti-proliferative effects of SFN and DIM

Incubation of 40-16 human colon cancer cells with SFN or DIM (**Figure 1**) at a concentration range of 0.3 to 40 µM led to a dose-dependent inhibition of cell proliferation. IC_{50} values obtained after 24 h, 48 h and 72 h of treatment were 6.8 ± 0.4 µM, 6.6 ± 0.5 µM, and 8.2 ± 0.4 µM for SFN, and 11.6 ± 1.7 µM, 10.1 ± 1.9 µM, and 6.8 ± 0.9 µM for DIM, respectively. Interestingly, after an incubation time of 24 h, SFN was significantly more effective in suppressing cell growth than DIM ($p = 0.009$), whereas after 72 h, DIM had a lower IC_{50} value than SFN. Accordingly, SFN was less toxic after 72 h compared to 24 h and 48 h incubations, whereas DIM's effectiveness increased with elongated treatment times.

Comparison of growth inhibition profiles clearly indicated that SFN was cytotoxic at concentrations higher than 10 µM, demonstrated by *f*a values higher than 0.5 (as described in *Materials and Methods*). On the other hand, the dose-response curves obtained with DIM revealed a cytostatic profile, as shown in a representative dose-effect plot (**Figure 2**). These results are in congruence with our recently reported observations [30].

Anti-proliferative effects of combinations of SFN and DIM

To determine combination effects of SFN and DIM on cell proliferation, cells were treated with constant molar ratios of the two drugs (1:4, 1:2, 1:1, 2:1 and 4:1) using eight serial two-fold dilutions of each mixture for 24 h, 48 h and 72 h. As an example, a dose-effect curve of the 1:1 mixture after 48 h of incubation is included in **Figure 2**. Notably, at the two highest total concentrations (20 and 40 µM), the fraction *fa* affected by the mixture was lower than that of SFN, but higher than that of DIM. In contrast, total concentrations below 20 µM inhibited cell growth less than either compound alone.

Quantification of combination effects according to Chou and Talalay

The method by Chou and Talalay is a widely accepted approach to analyze synergistic, additive or antagonistic effects of two compounds [34]. The first step of the procedure is to assess mutual exclusivity of the test compounds by generating median-effect plots, with log (*f*a/*f*u) plotted against log (*D*) (see *Material and Methods*). Parallelism of the regression lines obtained for either compound alone and the mixture of both indicates that two drugs are mutually exclusive, *i.e.* they have the same

target or mode of action. If the lines are not in parallel, the tested compounds are mutually non-exclusive, *i.e.* they act independently or have different modes of action. As shown in **Figure 3**, the regression lines of SFN, DIM and the 1:1 mixture were not in parallel. We therefore concluded that SFN and DIM are mutually non-exclusive and inhibit cell proliferation by different mechanisms. Additional median-effect plots generated for all combination experiments with molar ratios of SFN and DIM of 4:1, 2:1, 1:1, 1:2, and 1:4, and for 24 h, 48 h, and 72 h of incubation, respectively, further confirmed these results (data not shown).

In order to determine whether SFN and DIM influenced each other in a synergistic, additive or antagonistic manner, we used the CalcuSyn software to determine combination indices (CI) for all combinations and incubation times described above. CI values lower and higher than 1 indicate synergism and antagonism, respectively, whereas additive effects result in a combination index of 1. A representative CI-effect plot for the 1:1 combination of SFN and DIM after 48 h of treatment is depicted in **Figure 4**. At low effects provoked by high dilutions of the SFN:DIM combination, CI values were between 1 and 3, indicating that cell proliferation was affected in an antagonistic manner. However, CI values decrease with increasing effects. At the highest effects observed with highest SFN:DIM concentrations, CI values were even below 1, demonstrating that cytotoxic concentrations of the 1:1 combination inhibit cell proliferation synergistically.

SFN and DIM are antagonistic at low and synergistic at high concentrations

Since the effects of SFN:DIM combinations could not be directly compared due to different total concentrations, we adjusted the obtained CI values to the fixed total concentrations of 2.5, 5, 10, 20 and 40 μM by generating simulation curves from the original results. Data for five SFN:DIM combinations and increasing incubation times are summarized in **Figure 5**. In agreement with the results described above, CI values computed for low combined drug concentrations were higher than 1, indicating antagonism in terms of cell growth inhibition. The highest CI value of 4.2 ± 2.2 was calculated for the 1:4 combination after 24 h of treatment. Increasing concentrations resulted in decreasing CI values. Combinations of SFN and DIM showed synergistic effects (CI < 1) only at the highest total drug concentration (40 μM), except for the 1:4 combination after 72 h of treatment.

Synergism was strongest when the cells were treated with the 1:2 mixture of SFN and DIM for 24 h. At a total concentration of 20 µM, the two compounds influenced each other either additively (e.g., CI = 1.0 for the 1:2 combination after 24 h) or antagonistically (e.g., CI = 1.6 for the 1:1 combination after 72 h).

Taken together, low to moderate total concentrations of SFN:DIM combinations (2.5 to 20 µM) appear to inhibit proliferation of 40-16 cells in an antagonistic manner, whereas at cytotoxic total concentrations of 40 µM or higher, the compounds act in a synergistic manner. Overall, depending on the total concentration, strongest antagonistic and synergistic effects were observed i) after short incubation times, and ii) when DIM was present in excess of SFN.

Cell cycle effects of SFN, DIM and their combinations

Previous studies have established that SFN and DIM both halt cell cycle progression in cultured tumor cells. Notably, while in many cancer cell lines SFN induces cell cycle arrest in G_2/M phase [31], DIM provokes a stop in G_1 [21]. We therefore addressed the question how SFN:DIM combinations would affect cell cycle progression of 40-16 cells. We performed cell cycle analyses with cells treated with either SFN (5 µM or 10 µM) or DIM (10 µM) alone, or mixtures thereof. We used the same fixed molar ratios as described for cytotoxicity experiments, with the exception of combinations with an excess of SFN (SFN:DIM 2:1 and 4:1). Preliminary experiments had indicated that SFN in excess of DIM completely abrogated DIM's effects on cell cycle progression. Cells were arrested in G_2/M phase similarly as with SFN alone (data not shown). Thus, we tested 5 µM SFN in combinations with increasing concentrations of DIM (5, 10 and 20 µM, molar ratios of 1:1, 1:2, and 1:4). Additionally, cells were treated with a 1:1 mixture of 10 µM of each compound.

After 24 h of treatment, DIM (10 µM) and the lower dose of SFN (5 µM) as well as the combination of 5 µM SFN + 5 µM DIM had no effect on the sub-G_1 fraction, whereas SFN at a 10 µM concentration significantly induced a sub-G_1 peak indicative of apoptosis induction (**Figure 6**). Addition of increasing concentrations of DIM to 5 µM of SFN resulted in increasing effects. Strongest apoptosis induction was observed with the combination containing 10 µM SFN + 10 µM DIM (23.9 ± 2.2% sub-G_1). Increases in sub-G_1 fractions were accompanied by significant reduction in S phase as a

further sign of cell growth inhibition. Neither treatment with the compounds alone nor with combinations influenced the fraction of cells in G_1 or in G_2/M phase, with the exception of the 10 µM SFN + 10 µM DIM mixture, which provoked a strong G_2/M arrest (52.8 ± 5.2% *vs.* 26.8 ± 2.3% in untreated control cells). Consequently, the fraction of cells in G_1 was significantly reduced (15.5 ± 5.1%) in comparison with the control (44.0 ± 3.1%).

After 48 h of treatment, SFN (5 µM) and DIM (10 µM) had weak, but non-significant effects on cell cycle distribution. Further, combining 5 µM SFN with 5 µM or 10 µM DIM did not lead to a significant enhancement of effects when compared with the untreated control or with 5 µM SFN or 10 µM DIM, respectively. This may point to an antagonistic interaction. SFN at a 10 µM concentration was more potent in inducing the percentage of cells in sub-G_1 (28.6 ± 5.8%) than 5 µM SFN + 20 µM DIM. Addition of 10 µM DIM to 10 µM SFN further increased the sub-G_1 peak induced by SFN (41.1 ± 6.5%). Concomitantly, the percentage of cells in G_1 and S phase was significantly reduced by these treatment regimens. As observed after 24 h, the combination of 10 µM SFN with 10 µM DIM caused a significant G_2/M cell cycle arrest.

Effects of SFN, DIM and their combinations on PARP cleavage

To confirm the apoptosis-inducing potential of SFN and DIM combinations, we analyzed cleavage of the DNA repair enzyme PARP [poly (ADP-ribose) polymerase] by Western blotting (**Figure 7**). After 24 h of treatment, SFN alone at a concentration of 10 µM, as well as the 10 µM SFN + 10 µM DIM mixture induced PARP cleavage, which is consistent with the increase of the sub-G_1 fraction observed with cell cycle analyses. Treatment with 5 µM SFN + 20 µM DIM for 24 h was not sufficient to trigger PARP cleavage.

After 48 h, PARP cleavage was markedly induced by SFN (10 µM) and the mixtures of 10 µM SFN + 10 µM DIM and 5 µM SFN + 20 µM DIM. The effect produced by the latter combination was approximately equivalent to the 10 µM SFN + 10 µM DIM mixture after 24 h, which is consistent with sub-G_1 peak induction.

4. Discussion

In the present study, we have demonstrated that combinations of SFN and DIM, two chemopreventive compounds derived from cruciferous vegetables like broccoli, inhibit the growth of cultured human colon cancer cells antagonistically at low concentrations, but synergistically at cytotoxic concentrations. First, we determined the anti-proliferative potential of SFN and DIM alone in the 40-16 human colon carcinoma cell line. IC_{50} values as low as 6.6 to 11.6 μM underline the strong ability of SFN and DIM to inhibit tumor cell growth. We confirmed that SFN is a cytotoxic agent, whereas DIM has a cytostatic profile of cell growth inhibition [30]. Also, SFN and DIM were found to be mutually non-exclusive, which is not surprising, since fundamental differences in the growth inhibition profiles of the two compounds as well as different effects on cell cycle distribution have been reported previously [21,30,31].

A dose-response curve obtained with a 1:1 mixture of SFN and DIM in comparison with the single compounds clearly indicated that at high total concentrations (20 μM SFN and 20 μM DIM), the mixture was more active than either compound alone. In contrast, at low total concentrations, the dose-response curve of the mixture was below those of SFN and DIM alone, suggesting antagonistic interaction. This observation prompted us to quantitate combination effects of SFN and DIM using the method of Chou and Talalay [34]. Results of these analyses indicated that combinatory interactions between SFN and DIM are strongly dose-dependent, in that low combined concentrations are antagonistic, whereas cytotoxic concentrations are synergistic, regardless of the ratio applied. A possible explanation for this phenomenon might be that at low concentrations, mechanisms other than cell cycle arrest or apoptosis induction play a more prominent role for chemopreventive potential. In a majority of *in vitro* studies on apoptosis induction, including our investigation in the 40-16 cell line, SFN or DIM had to be applied at concentrations of at least 10 μM to induce apoptosis [30,36,37]. In contrast, SFN at concentrations as low as 1 - 5 μM was very effective in inducing phase II detoxification enzymes [38-40], whereas DIM affected biotransformation enzymes only at concentrations higher than 20 μM [21,38,41]. Therefore, treatment with low doses of SFN might cause increased ‘detoxification’ of the compounds themselves or of reactive intermediates involved in inhibition of cell proliferation, resulting in the observed antagonistic effects.

In the present study, only relatively high concentrations of combinations of SFN and DIM were able to inhibit cell proliferation or induce cell death in a synergistic manner, whereas intermediate concentrations (SFN + DIM = 20 µM) were weakly antagonistic or additive. Since SFN and DIM alone induce G_2 and G_1 cell cycle arrest, respectively [21,31], antagonism could also be explained by these opposing effects. To address this question, we performed cell cycle analyses with mixtures of SFN and DIM in ratios of 1:4, 1:2 and 1:1, with total drug concentrations ranging from 10 to 25 µM. Due to the distribution of analyzed cells to four different fractions (sub-G_1, G_0/G_1, S, and G_2/M), flow cytometry data were not suitable to calculate combination indices. Therefore, influences on cell cycle phases had to be compared directly.

Again, effects of SFN, DIM and their combinations strongly depended on total drug concentrations tested. Most interesting effects were observed with SFN alone at a concentration of 10 µM and mixtures of either 5 µM SFN + 20 µM DIM or 10 µM SFN + 10 µM DIM, whereas DIM at a 10 µM concentration did not cause any change in cell cycle distribution. Notably, addition of 10 µM DIM to 10 µM SFN significantly arrested cells in G_2/M and enhanced the effect of SFN alone (10 µM) with respect to reduction of the percentage of cells in G_1 and S phase as well as induction of a sub-G_1 peak. Since the 10 µM SFN + 10 µM DIM combination affected all phases of the cell cycle, the net result may be inhibition of cell proliferation, but not necessarily a strong enhancement of cytotoxic effects. This was confirmed when cell death was analyzed by the detection of PARP cleavage with Western Blotting. Cell cycle effects of the 5 µM SFN + 20 µM DIM mixture resembled those of SFN at a 10 µM concentration, although PARP cleavage was only detectable after 48 h of treatment with the combination. These data indicate that a threshold concentration of around 10 µM of SFN is necessary to sensitize cells to cell death, but DIM addition might modulate cell growth inhibition by enhancing the G_2/M arresting potential of SFN. Numerous attempts have been made to elucidate the mechanism how SFN induces G_2/M arrest. Reactive oxygen species-mediated DNA damage as well as disruption of tubulin polymerization have been proposed [12,42]. Further investigations have to clarify how DIM interacts with these mechanisms. Since SFN has also been described to cause G_1 arrest, e.g. in the HT-29 colon cancer cell line [43], further investigations with other cells line will be required to confirm the general validity of these results.

In terms of synergistic or antagonistic interactions between SFN and DIM, data obtained from cell cycle and Western blot analyses are consistent with data of the cell growth experiments. Low concentrations of combinations seem to be antagonistic, whereas higher total concentrations seem to act rather additive or weakly synergistic, especially in the case of the 10 µM SFN + 10 µM DIM combination. Although the impact on cell cycle phases differed depending on the ratio of SFN and DIM, CI values obtained from cytotoxicity data were similar regardless of the applied ratio of the two drugs.

A dose-dependent switch from antagonistic to synergistic interaction between two compounds has been reported before. Khafif *et al.* observed synergism of the green tea compound (-)-epigallocatechin-3-gallate (EGCG) and the spice curcumin in inhibiting cell growth of differentially transformed human oral epithelial cell lines [44]. The authors described antagonistic interactions at low doses of the drug combination, which were selective for normal or less progressed cells, but a synergistic growth-inhibitory effect on malignant cells, implicating a protective effect for normal tissues. In PC-3 human prostate cancer cells, curcumin in combination with phenethyl isothiocyanate induced apoptosis in an additive manner [45]. In this study, experiments were limited to concentrations relevant for apoptosis induction; therefore combination effects at lower concentrations were not determined. The same group also investigated the combined inhibitory effects of curcumin and PEITC on the growth of human PC-3 prostate xenografts in immunodeficient mice. While PEITC or curcumin alone had little effect, the mixture of both significantly reduced the growth of PC-3 xenografts [46].

Broccoli extracts represent a natural combination of ITCs and indoles. In mature broccoli about 70% of the glucosinolates are indole-based. The main compound is glucobrassicin as the precursor of DIM. The rest consists of different other glucosinolates, especially glucoraphanin, the cleavage of which leads to the formation of SFN [47]. In a very recent study broccoli extracts induced G_2/M cell cycle arrest and apoptosis in cultured rat glial cells [48]. Although the authors did not quantify concentrations of glucosinolate cleavage products, in comparison with the results presented here it can be speculated that either SFN was present in excess or that its concentration was high enough to induce a halt of the cell cycle in G_2/M. Another report described mitochondria-mediated apoptosis and

S-/M phase arrest in human bladder carcinoma cells induced by broccoli sprout extract [49]. Broccoli sprouts primarily (> 90%) contain glucoraphanin and consequently SFN after conversion to ITCs [47]. Notably, comparison with synthetic SFN revealed that the anti-proliferative potency of the extract was almost identical to that of the single compound, suggesting that non-ITC substances in the extract may not interfere with growth-inhibitory activity of ITCs [49]. In future studies, combinations of SFN or DIM together with other chemopreventive compounds should be conducted to investigate potential synergistic effects. With this respect, DIM and paclitaxel were shown to synergistically induce apoptosis in HER2/Neu human breast cancer cells [50].

Although progress is made in understanding combinatory effects between bioactive compounds present in natural foods, more research efforts are needed in order to elucidate mechanistic interactions and dose-dependent differences in the outcomes of combination treatments. The concept of combination chemoprevention should be paid more attention as it holds great potential for targeted prevention or preventory treatment of malignancies while causing little side effects. Transfer of promising findings achieved with single food components to the consumption of whole foods should however be done with caution.

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Figure legends:

Fig. 1. Chemical structures of SFN and DIM.

Fig. 2. Profiles of cell growth inhibition. Growth curves of SFN (○), DIM (▼) and the 1:1 mixture of SFN and DIM (□), respectively, with cultured 40-16 cells. Inhibition of cell proliferation was determined by sulforhodamine B staining. Cells were treated with eight different concentrations of SFN and DIM alone (0.3 to 40 μM), a 1:1 mixture of SFN and DIM (0.15 to 20 μM of each drug) or 0.5% DMSO as a solvent control for 48 h. Mean values from three independent experiments (± SD) are expressed as affected fraction (*f*a) compared with control cells. Dose-dependent inhibition of proliferation is indicated by *f*a values between 0 and 0.5, whereas values above 0.5 represent cytotoxic effects.

Fig. 3. Median-effect plot of 40-16 cells treated for 48 h with SFN (○), DIM (▼), a 1:1 mixture of SFN and DIM (□), respectively, or 0.5% DMSO as a solvent control. Data were taken from one representative cell proliferation experiment. Log (*f*a/*f*u) was plotted against log (*D*), whereby *f*a and *f*u stand for affected fraction and unaffected fraction, respectively, and *D* stands for the concentration.

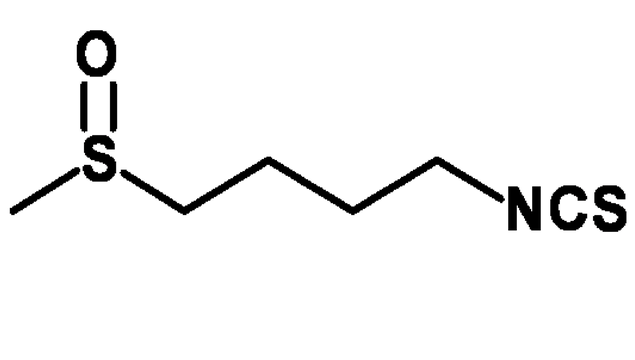
Fig. 4. CI-effect plot. CI (combination index) values obtained from cell proliferation experiments with 40-16 cells treated for 48 h with a 1:1 mixture of SFN and DIM or 0.5% DMSO as a solvent control were plotted against the effects (equivalent to affected fraction *f*a) mediated by five different concentrations (2.5, 5, 10, 20 and 40 μM total concentration) of this drug combination, respectively. Data are derived from three independent experiments.

Fig. 5. CI values of combinations of SFN and DIM. 40-16 cells were treated with combinations of SFN and DIM in ratios of 1:4, 1:2, 1:1, 2:1 and 4:1 or 0.5% DMSO as a solvent control for 24 h (**A**), 48 h (**B**) and 72 h (**C**). CI values were calculated as described in Materials and Methods. All original

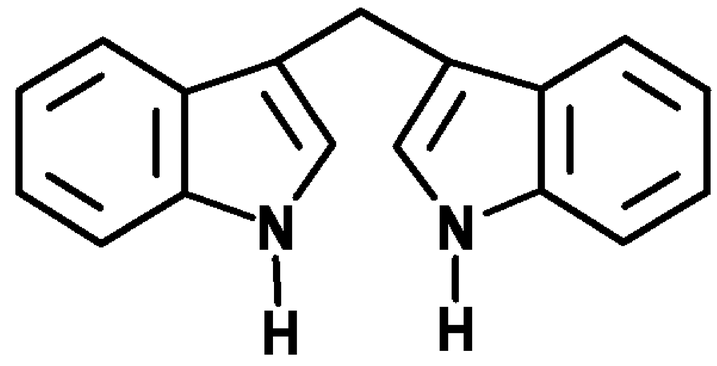
data were normalized to fixed total concentrations of 2.5, 5, 10, 20 and 40 µM as indicated. Data are means ± SD from three independent experiments (exception: n=2 for the 1:4 combination after 72 h).

Fig. 6. Cell cycle analyses. 40-16 cells were treated with SFN alone, DIM alone, combinations of SFN and DIM at ratios of 1:4, 1:2 and 1:1, or 0.5% DMSO as a solvent control for 24 h (**A**) and 48 h (**B**). The concentrations used are indicated in the figure. Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Bars represent the percentage of cells in sub-G_1, G_0/G_1, S and G_2/M cell cycle phases. Data are means ± SD from at least three independent experiments. *Means significantly ($p < 0.05$) different compared to respective control using one-way ANOVA und Tukey's studentized range test. ^{s}Means significantly ($p < 0.05$) different compared to SFN at a 5 µM concentration in the case of the 5 µM SFN + 20 µM DIM combination or to SFN at a 10 µM concentration in the case of the 10 µM SFN + 10 µM DIM combination, respectively. ^{d}Means significantly ($p < 0.05$) different compared to DIM at a 10 µM concentration.

Fig. 7. Detection of apoptosis induction by PARP cleavage. Immunoblotting for PARP using lysates from 40-16 cells treated with 0.2% DMSO (–), SFN, and DIM, alone and in combinations, respectively, for 24 h and 48 h as indicated. PARP cleavage was investigated by Western blotting using an antibody directed against full length PARP (116 kDa) and the cleavage product (89 kDa). Equal loading was confirmed by using an anti-β-actin antibody. One of two blots is shown.



Sulforaphane (SFN)



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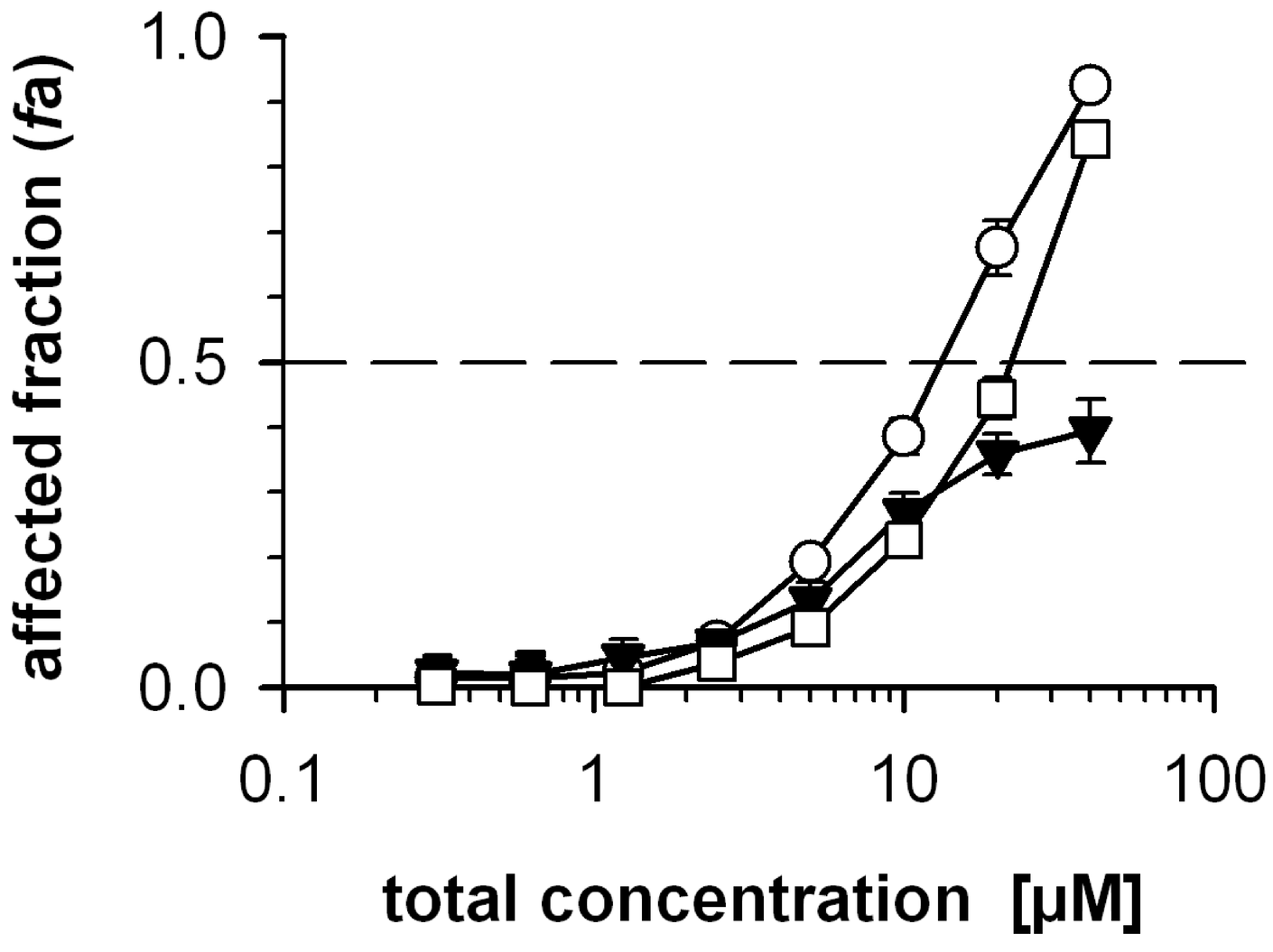
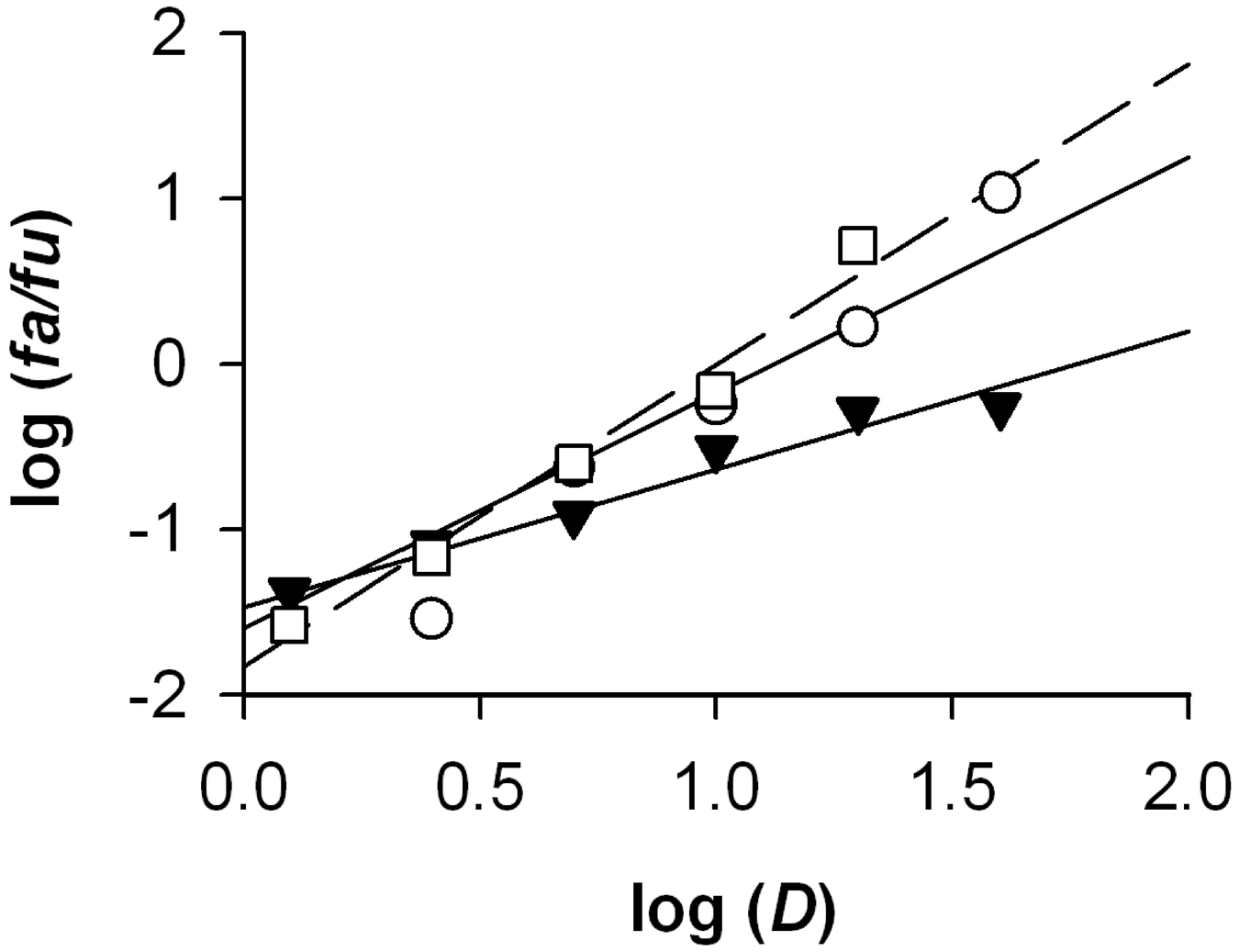
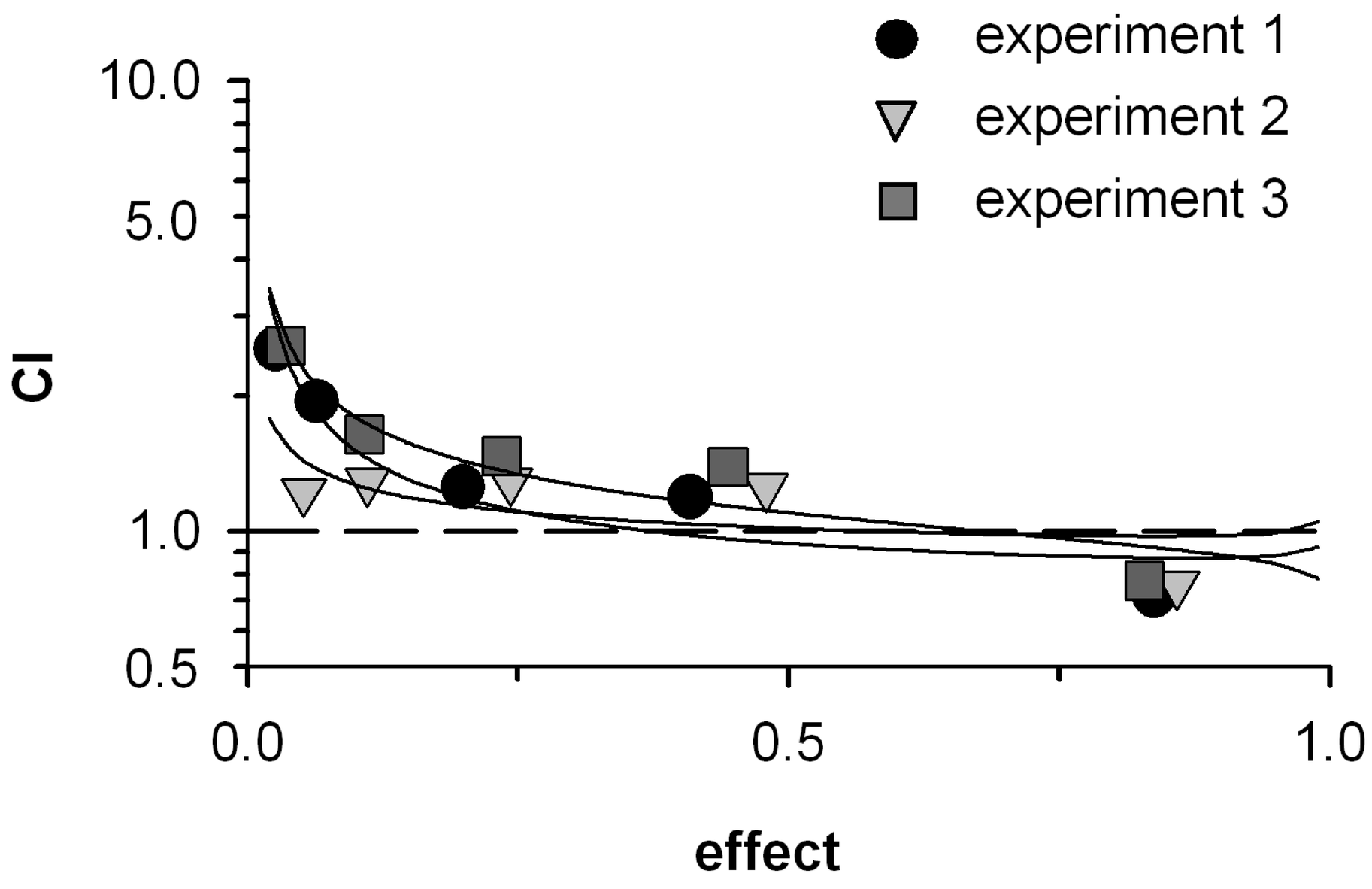
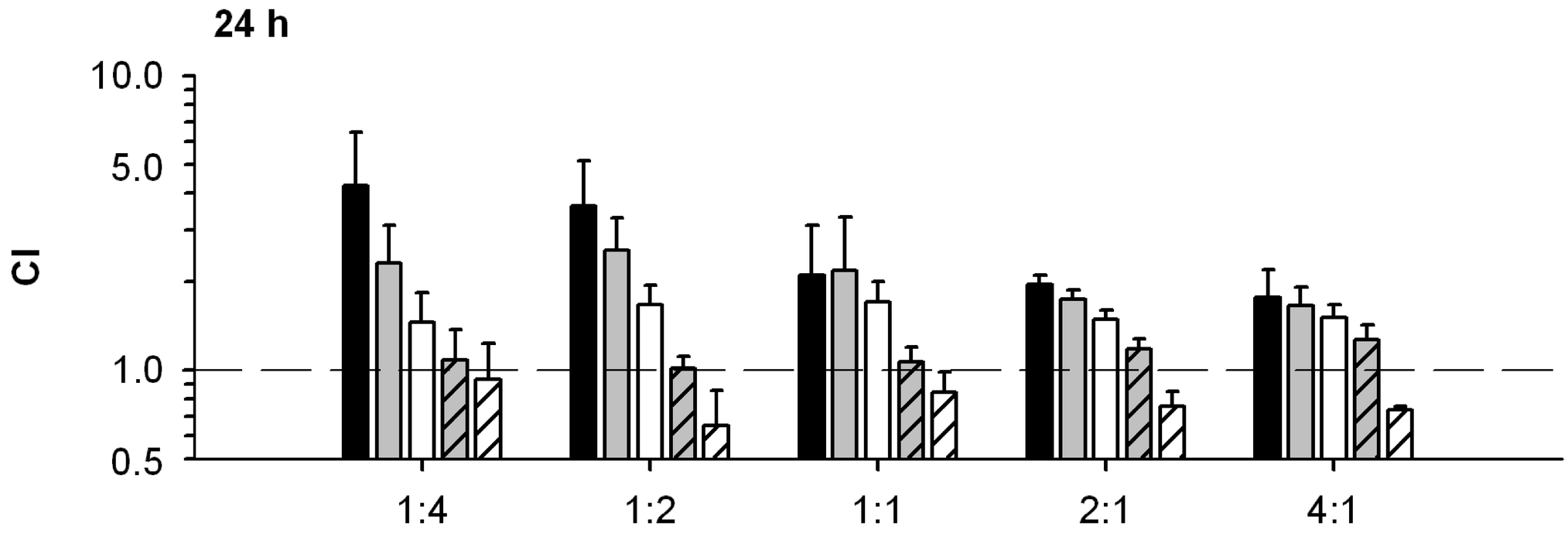

affected fraction (fa)
1.0
0.5
0.0
0.1
1
10
100
total concentration [µM]

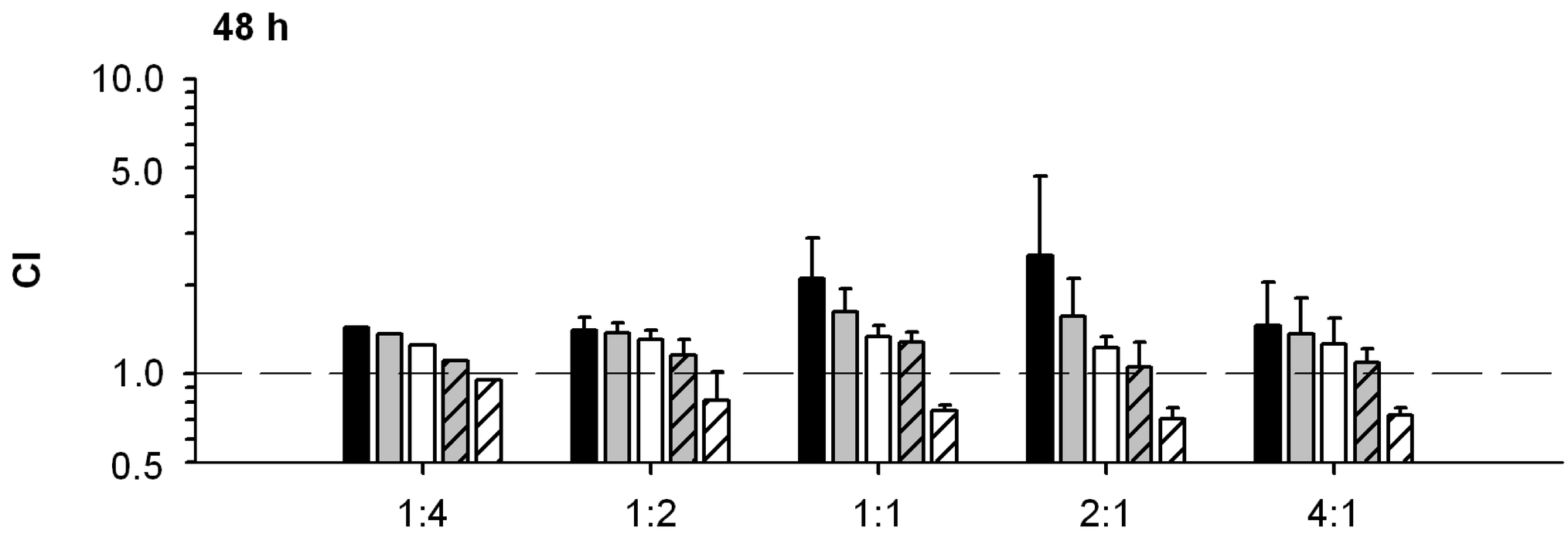

2
1
0
-1
-2
log (fa/fu)
0.0
0.5
1.0
1.5
2.0
log (D)


experiment 1
experiment 2
experiment 3
10.0
5.0
1.0
0.5
CI
0.0
0.5
1.0
effect

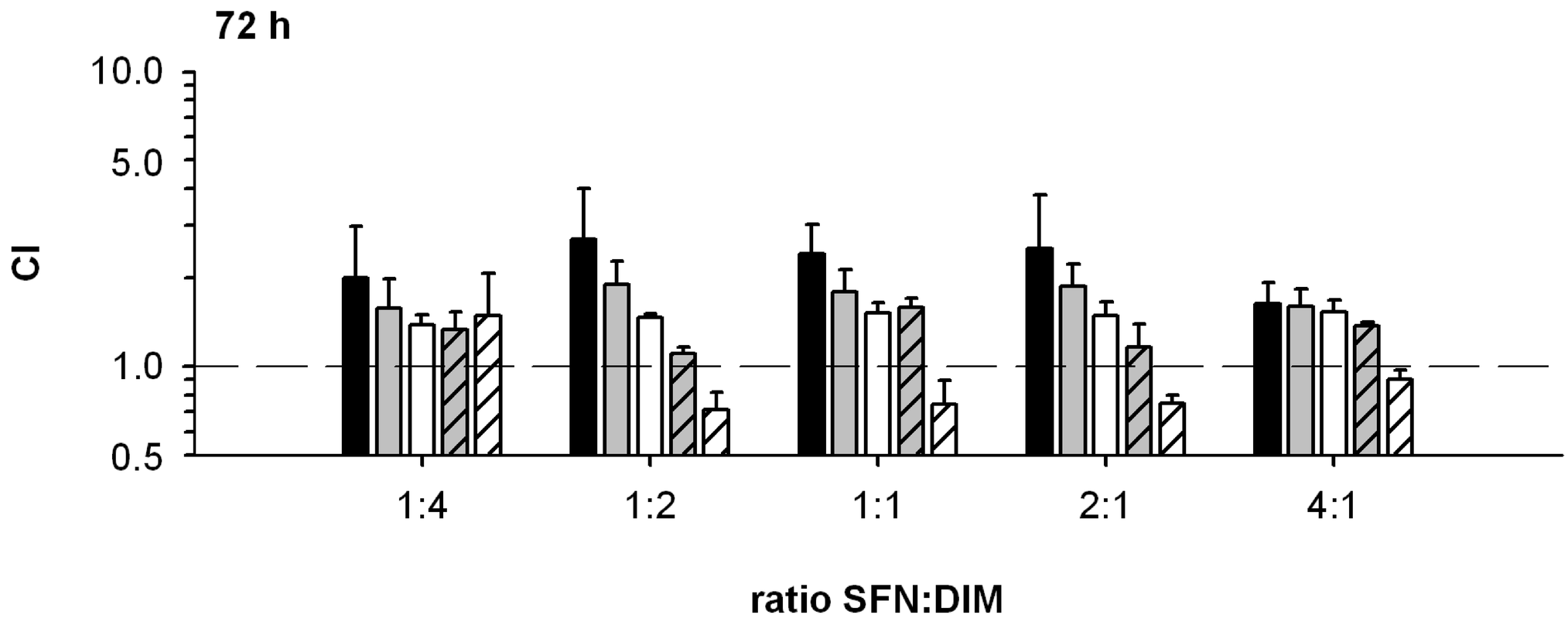
A

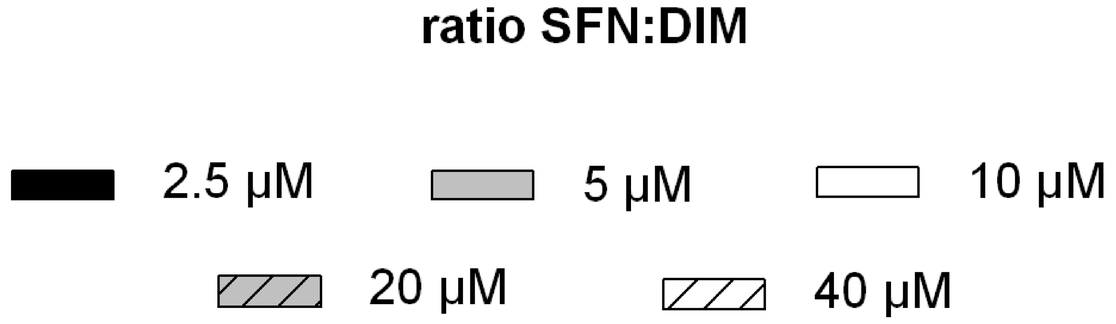

24 h
CI
10.0
5.0
1.0
0.5
1:4
1:2
1:1
2:1
4:1

B


48 h
CI
10.0
5.0
1.0
0.5
1:4
1:2
1:1
2:1
4:1

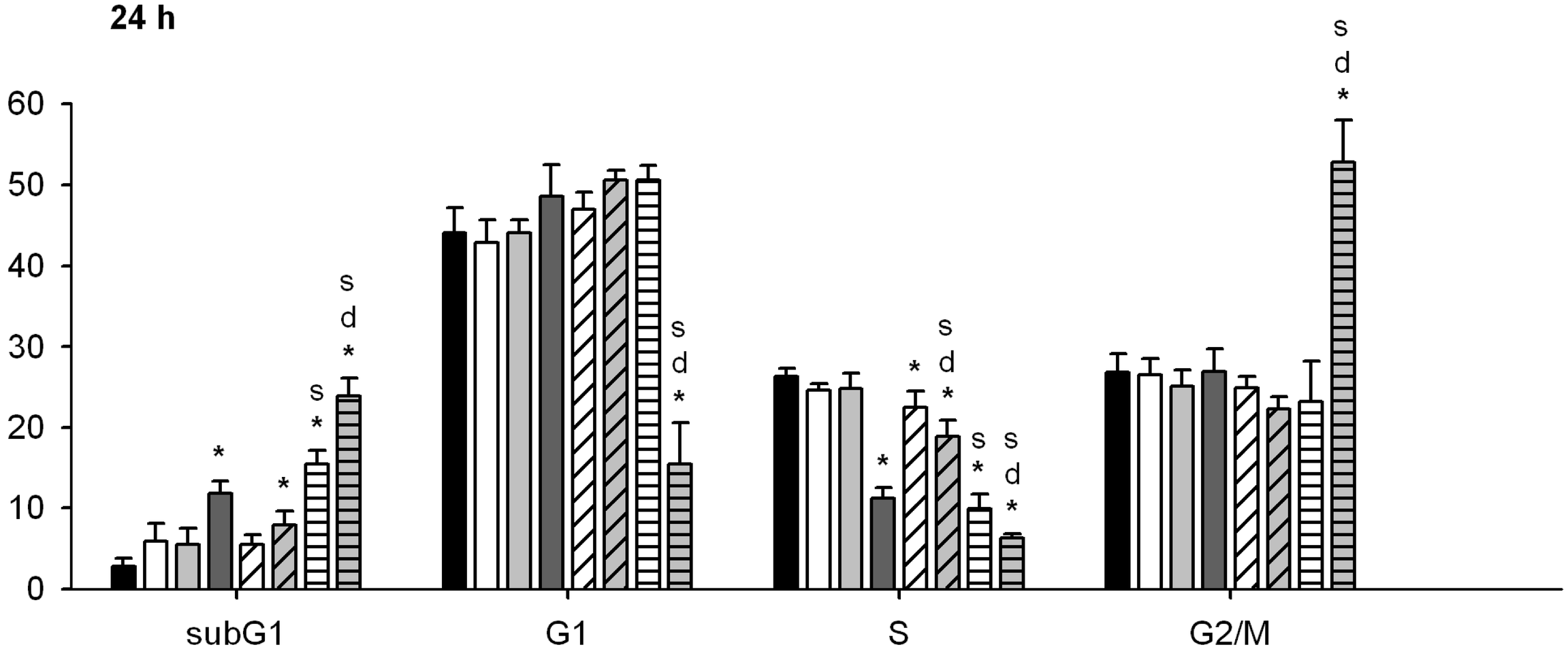
C


72 h
CI
10.0
5.0
1.0
0.5
1:4
1:2
1:1
2:1
4:1


ratio SFN:DIM
2.5 µM
5 µM
10 µM
20 µM
40 µM

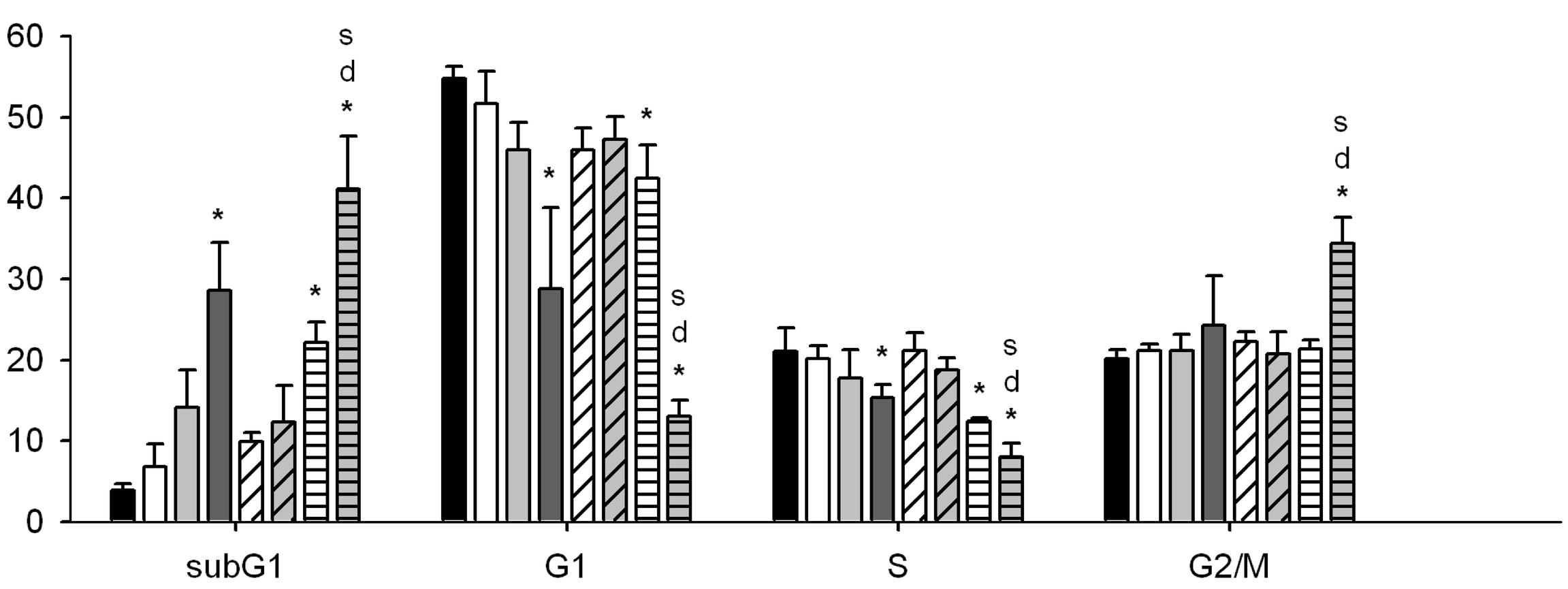
A

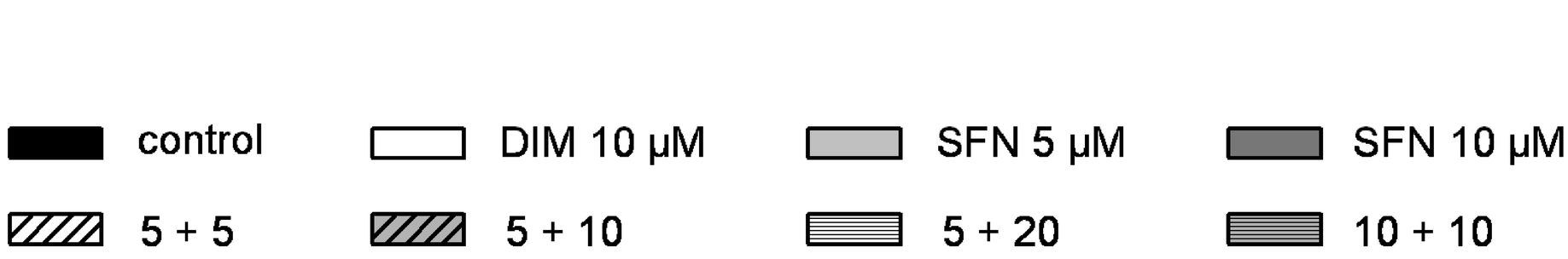
24 h

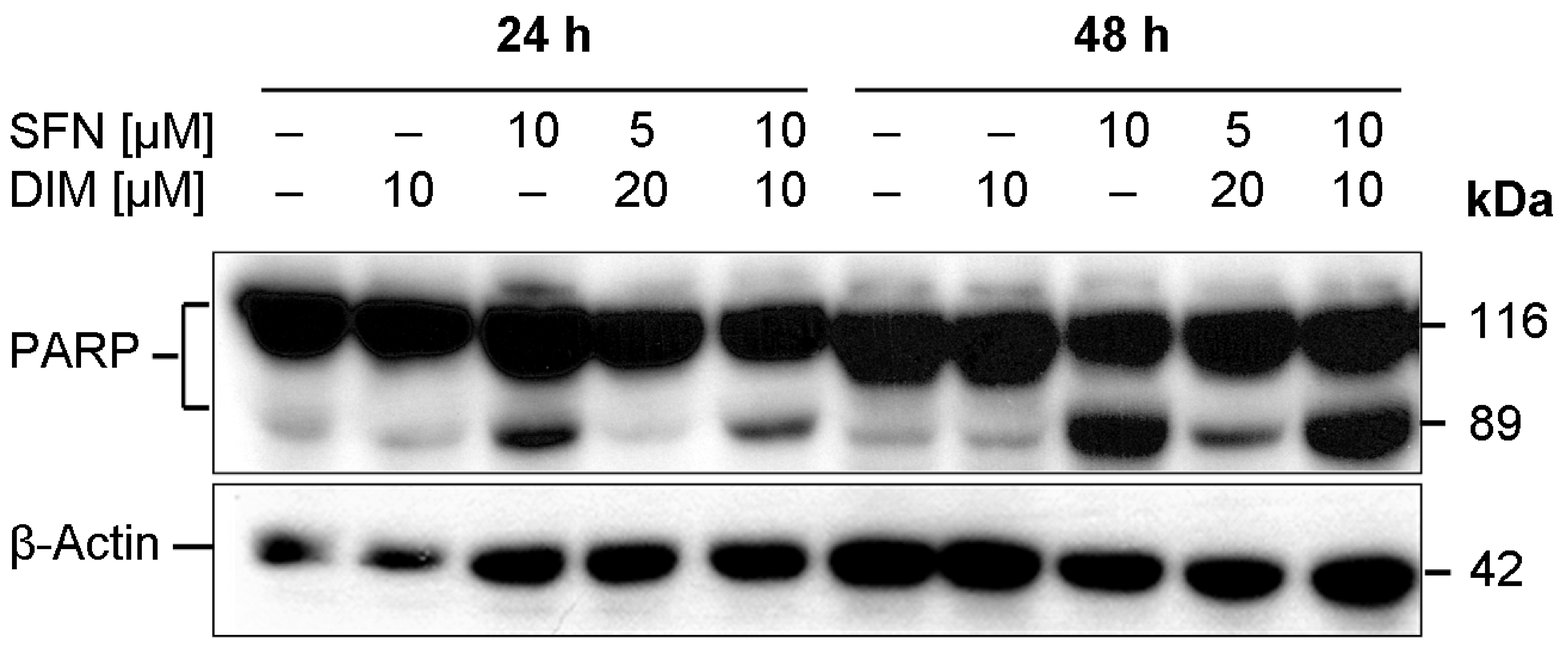

60
50
40
30
20
10
0
subG1
G1
S
G2/M
*
s
d

B

48 h


60
50
40
30
20
10
0
subG1
G1
S
G2/M
*
s
d


control
DIM 10 µM
SFN 5 µM
SFN 10 µM
5 + 5
5 + 10
5 + 20
10 + 10


24 h
48 h
SFN [µM] – – 10 5 10 – – 10 5 10
DIM [µM] – 10 – 20 10 – 10 – 20 10
kDa
PARP
116
89
β-Actin
42