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Isothiocyanates as novel cytotoxic and cytostatic agents: molecular pathway on human transformed and non-transformed cells

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Abstract

Cancer chemoprevention is a new approach in the management of cancer. Traditional cytotoxic chemotherapeutic approaches cannot cure most advanced solid malignancies. Chemoprevention can be defined as the use of non-cytotoxic drugs and natural agents to block the progression to invasive cancer. Recently, isothiocyanates, natural products found in the diet of humans, has been shown to function as cancer chemopreventive agents. They are strong inhibitors of phase I enzymes and inducers of phase II enzymes. They can also induce apoptosis and modulate cell-cycle progression of highly proliferating cancer cells. This commentary will review the mechanism of apoptosis and growth inhibition mediated by different isothiocyanates. Particular attention will be given to the effects of the new isothiocyanate 4-(methylthio)butylisothiocyanate (MTBITC). Since selective targeting and low toxicity for normal host tissues are fundamental requisites for proposed chemopreventive agents, we will also review the effects of different isothiocyanates on non-transformed human cells.

Keywords: Isothiocyanates; Apoptosis; Cell cycle; Cancer cells; Non-transformed cells; Selectivity

1. Introduction

According to the multistep carcinogenesis concept, cancer develops in a series of steps, with accumulation of molecular changes progressing through preinvasive histological changes to invasive disease [1]. The earliest events of this process are mutations, deletions, or polysomy at the genomic level, which are successively translated into cellular morphological changes or tissue structural changes [2]. Uncontrolled proliferation, invasion, and metastasis are additional events necessary to induce phenotypic, then physiological, modifications in the tissue.

Cancer chemoprevention encompasses the concepts of inhibition, reversal, and retardation of the cancer process through the use of specific natural or synthetic chemical agents to reverse, suppress, or prevent progression to invasive cancer [3].

Epidemiological studies indicate that vegetables and fruit can prevent a variety of human cancers [4] through the action of antioxidants such as carotenoids, vitamin E, etc., suggesting that such agents may be able to protect cells from the mutagenic action of reactive oxygen species [5]. The pioneering works of Wattenberg [6], Talalay et al. [7], and Conney et al. [8] have shown that dietary chemicals can prevent chemical carcinogenesis in the laboratory and experimental animals.

A group of vegetables with considerable anticarcinogenic properties are the cruciferous vegetables. *Cruciferae* comprise a large number of vegetables (such as red cabbage, white cabbage, cauliflower, Brussels sprouts and broccoli) that are among the most frequently consumed

Abbreviations: CDK, cyclin-dependent kinase; GSH, glutathione; GST, glutathione-S-transferase; MTBITC, 4-(methylthio)butylisothiocyanate; PI, propidium iodide

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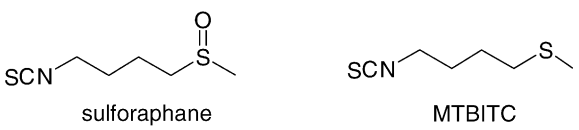
worldwide [9]. They contain a specific group of constituents, the glucosinolates, which have a common structure comprising a β-D-thioglucose group, a sulfonated oxime moiety and variable side chains derived from methionine, tryptophane, phenylalanine, or branched-chain amino acids [10]. Glucosinolates remain chemically stable within the cytoplasm until brought into contact with myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1), which is released upon disintegration of the cellular plant structure or by intestinal bacteria [11]. Upon hydrolysis, glucosinolates yield equimolar quantities of glucose, aglycone and sulfate, but the instability of the aglycones leads to further reactions, the main products of which are thiocyanates, nitriles and isothiocyanates. The pattern of reaction products varies with the structure of the side chain and the reaction condition.

Over the last decades, strong efforts have been made to identify active compounds in cruciferous vegetables and to understand the molecular mechanisms, which cause their protective effects. There is now ample evidence from animal models to show that certain isothiocyanates and their conjugates can inhibit cytochrome P450 enzymes, which activate nitrosamines to alkylating carcinogens responsible for induction of lung tumors [12,13]. There has also been much recent interest in isothiocyanates that can induce phase II enzymes. An important example is sulforaphane, an isothiocyanate present at high levels in broccoli, which induces phase II enzymes [14], but other isothiocyanates derived from common brassica vegetables may well exert similar biological activity [15]. Overall, the most demonstrated principles of chemoprevention by cruciferous vegetables are therefore induction of phase II enzymes and inhibition of phase I enzymes.

Recent evidence suggests that isothiocyanates might be involved in different, but much less well-characterized mechanisms, such as induction of cell-cycle arrest and apoptosis in human cancer cells in vitro. Due to the vast number of isothiocyanates studied in the field of chemoprevention, we will limit this commentary to the studies performed on sulforaphane (one of the most promising isothiocyanate) (Fig. 1a), and on the new isothiocyanate MTBITC (Fig. 1b), obtained by hydrolysis of glucoerucin, the 4-methylthiobutylglucosinolate present in rocket (*Eruca sativa* Miller).



(a) (b)

Fig. 1. Chemical structure of sulforaphane (a) and MTBITC [4-(methylthio)butylisothiocyanate] (b).

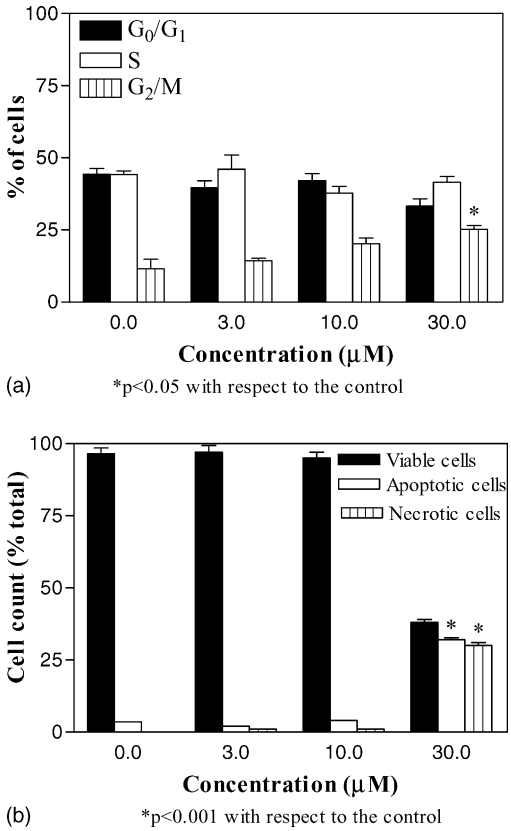


Fig. 2. Effects of sulforaphane on Jurkat cell-cycle (a) and apoptosis induction (b). At 48 h, samples were analyzed by FACStar^{+} flow cytometry. Results are expressed as percentages of total cell counts. Data are means ± S.E. (deviation bars, except when smaller than the symbol size) of three independent experiments.

1.1. Sulforaphane

In order to study the effects of sulforaphane on transformed cell lines, Jurkat T-lymphoblastoid cells were treated with different concentrations of sulforaphane (3, 10, and 30 μM).

Measurements of the effects of sulforaphane on cell-cycle progression and on apoptosis induction were performed by flow cytometry. The effects of sulforaphane were time- and dose-dependent. In fact, concentrations greater than 3 μM were necessary to alter cell-cycle progression with maximal accumulation of G_2/M cells observed at 30 μM and following 48 h of exposure to sulforaphane (Fig. 2a). A remarkable fraction of cells characterized by decreased DNA stainability observed in the DNA content distribution as a distinct peak below that of the G_1 population (“sub-G_1-cells”) suggested the induction of apoptosis by sulforaphane, which was confirmed by using Annexin V/PI assay. Apoptosis was observed at the highest dose of sulforaphane and at late stages during the treatment (at 24 and 48 h). In fact, after 8 h of treatment of Jurkat cells with sulforaphane, the fraction of Annexin $V^{positive}$ and $PI^{negative}$ cells – i.e. apoptotic cells – was 5%. It increased to 16% after 24 h and 32% after 48 h (Fig. 2b).

The appearance of necrotic cells, revealed by PI uptake (Annexin $V^{positive}$–$PI^{positive}$), presented the same kinetics. In fact, the highest fraction of necrotic cells was observed following 48 h of exposure to 30 μM sulforaphane (Fig. 2b). Moreover, cells treated with sulforaphane 30 μM for 48 h showed raised expression of p53 (1281.0 versus 185.0 in controls, $P < 0.01$) and bax (896.0 versus 293.5 in controls, $P < 0.01$). bcl-2 protein level was only slightly affected (166.0 versus 280.5 in controls, $P =$ n.s.) [16].

Since selective targeting and low toxicity for normal host tissues are fundamental requisites for proposed chemopreventive agents, sulforaphane was studied on non-transformed T-lymphocytes. The effects of sulforaphane on non-transformed T-lymphocytes were similar to those recorded on Jurkat T-leukemia cells. The most marked effect was the suppression of phytohemagglutinin-driven proliferation of normal T-cells, through an arrest in the G_1 phase of the cell cycle, particularly marked after treatment with sulforaphane 30 μM for 72 h (Fig. 3a). The analysis of cell-cycle-regulatory molecules operative in the G_1 phase of the cell cycle, specifically, cyclins D2 and D3, and CDK4 and CDK6, indicated that sulforaphane treatment of the cells resulted in a significant down-modulation of cyclin D3, whereas the expression of cyclin D2, CDK4 and CDK6 was more mildly attenuated [17]. In particular, treatment with sulforaphane greatly decreased the expression of cyclin D3 at 24, 48 and 72 h. The maximum effect was registered at 24 h, where the expression of cyclin D3 was decreased by 60% (890.0 versus 356.0; $P < 0.001$). Moreover, a progressive dose-related increase in the fraction of apoptotic cells was observed after treatment with sulforaphane 10 and 30 μM for 72 h (Fig. 3b). Interestingly, the highest concentration of sulforaphane also induced necrosis (Fig. 3b). Cells treated with sulforaphane showed raised p53 expression (292.5 versus 151.5 in controls, $P < 0.001$), which however did not reach the seven-fold increase we previously recorded in Jurkat leukemia cells. As in Jurkat cells, bcl-2 levels showed a slight, non-significant decrease (835.0 versus 761.5 in controls). On the other hand, bax levels rose only slightly (202.0 versus 196.5, $P =$ n.s.), as compared with the highly significant three-fold increase previously found in Jurkat cells. These observations suggest that induction of *p53* expression might be an important step during sulforaphane-induced apoptosis.

Taken together, these findings indicate that sulforaphane is active not only in transformed lymphocytes but also in their normal counterpart.

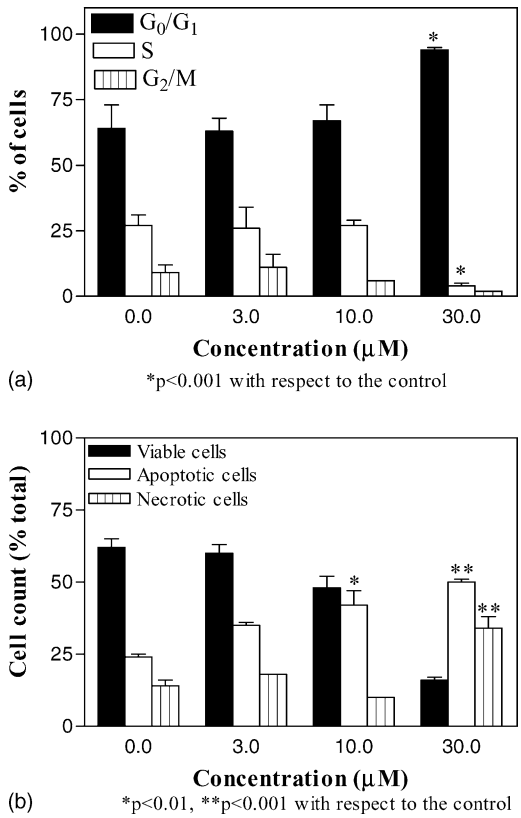


Fig. 3. Effects of sulforaphane on cell-cycle (a) and apoptosis induction (b) of human non-transformed T-lymphocytes. At 72 h, samples were analyzed by FACStar$^+$ flow cytometry. Data are means ± S.E. (deviation bars, except when smaller than the symbol size) of three independent experiments.

1.2. MTBITC

The effects of MTBITC on Jurkat T-leukemia cells were similar to those obtained by treating the cells with sulforaphane [18]. In fact, MTBITC altered cycle progression of Jurkat cells mainly through an inhibition of G_2/M phase of cell cycle (Fig. 4a). A significant increase of cells in G_2/M phase was observed following only 8 h of exposure. A more marked increase in the proportion of G_2/M cells and a loss of S-phase cells were also apparent following 24 h of treatment. However, G_2/M inhibition did not persist after 48 h of treatment (Fig. 4a).

Treatment with MTBITC greatly decreased the expression of cyclin B1 at 8 and 24 h, with a maximum effect registered at 24 h, where the expression of cyclin B1 was decreased by about 63% [18]. At 48 h, the levels of cyclin B1 are similar between untreated and treated cells, because of the lack of persistence of G_2 block. The expression of CDK1 was not affected [18].

Apoptosis induction registered at 48 h time point could account for the lack of G_2/M arrest. In fact, a significant induction of apoptosis was observed at the highest dose of MTBITC (10 μM) and at late stages (48 h) during the treatment of Jurkat cells (25% versus 3% in the control, $P < 0.01$) (Fig. 4b). Treatment for 48 h with different doses of MTBITC also increased the fraction of necrotic cells (Fig. 4b). The induction of apoptosis was associated with an increase of p53 and bax, but not bcl-2, protein expression [18]. In fact, treated cells showed raised p53 (1558.0 versus 185.0 in controls, $P < 0.001$) and bax (621.5 versus 293.5 in controls, $P < 0.001$) protein levels. On the other

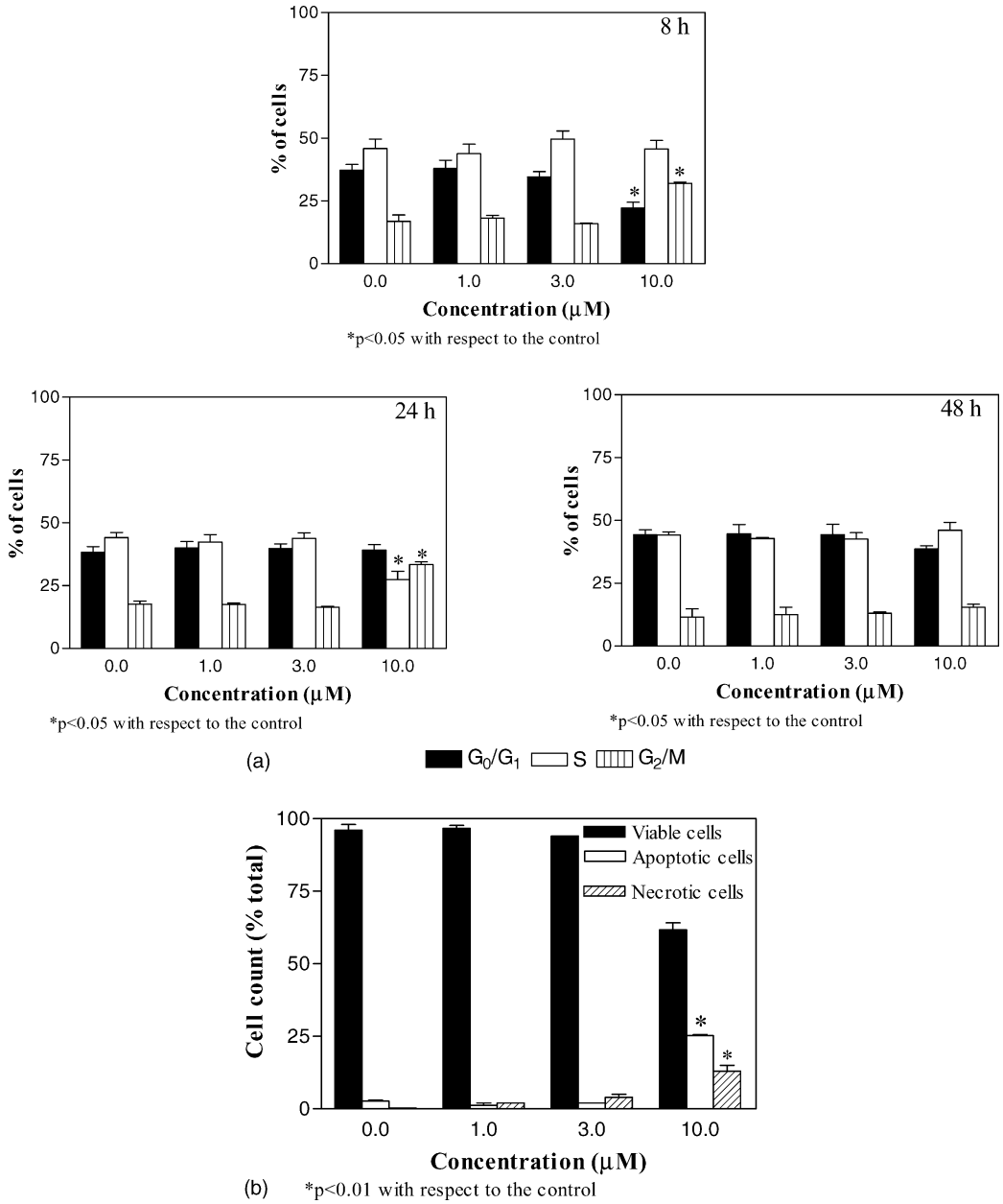


Fig. 4. Effects of MTBITC on Jurkat cell-cycle (a) and apoptosis induction (b). At 48 h, samples were analyzed by FACStar^{+} flow cytometry. Results are expressed as percentages of total cell counts. Data are means ± S.E. (deviation bars, except when smaller than the symbol size) of three independent experiments.

hand, bcl-2 levels showed a slight, non-significant decrease (242.0 versus 280.5 in controls).

The behavior of MTBITC on normal T-lymphocytes was completely different from that we recorded for sulforaphane. At all time points, normal T-lymphocytes reached the same growth status as untreated samples, regardless of the concentration of the drug [18]. In fact, similar percentages of cells in the different phases of cell cycle between untreated and MTBITC-treated cells were observed, as shown in Fig. 5a for treatment with the highest concentration of MTBITC and the most prolonged time of exposure tested.

When normal lymphocyte cultures were treated with MTBITC, also the proportion of apoptotic cells after staining with Annexin V and PI was similar between untreated and treated samples (Fig. 5b).

Collectively taken, these results suggest that MTBITC selectively affected cell-cycle progression and apoptosis induction of transformed T-cells. The different effects of MTBITC may only be quantitative, occurring in the range of concentrations used. However, differences in membrane composition between normal and cancer cells in general have been described [19] and it is possible that these changes may affect the sensitivity of the cells to MTBITC.

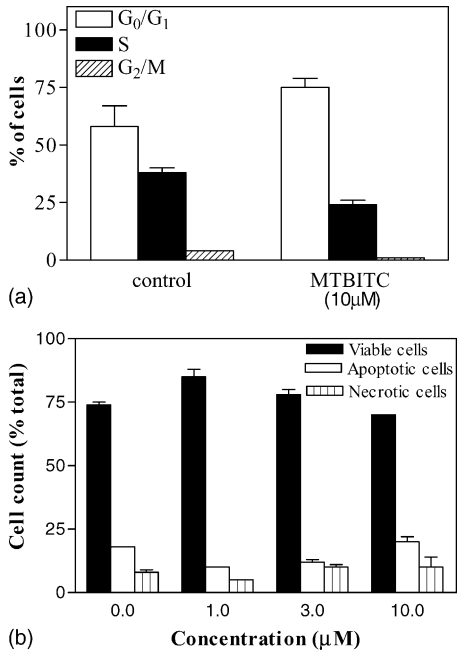


Fig. 5. Effects of MTBITC on cell-cycle (a) and apoptosis induction (b) of human non-transformed T-lymphocytes. At 72 h, samples were analyzed by FACStar^{+} flow cytometry. Data are means ± S.E. (deviation bars, except when smaller than the symbol size) of three independent experiments.

The effectiveness of active compounds in cancer cells could be founded on a specific energy-dependent pathway of drug incorporation as appears to be true for aloe emodin [20]. Furthermore, MTBITC could also target other important molecular events, at least partially explaining its differential effects in normal and transformed lymphocytes. One possibility regards Fas expression. Sen et al. [21] have reported that whereas normal human peripheral blood lymphocytes are only 35% positive for the Fas receptor, Jurkat cells are 100% positive and express 10-fold greater density of Fas receptors.

We could exclude that the differences in the activity of MTBITC in normal cells are dependent on minor differences in intracellular uptake. In fact, it is known that GSH may be involved in the uptake of isothiocyanates, occurring mainly in the form of GSH-conjugated dithiocarbamates, with GST catalyzing the conjugation reaction [22]. The uptake of isothiocyanates is therefore related to intracellular GSH levels, which are known to be rather similar in normal human lymphocytes and Jurkat cells (29.7 and 33.2 nmol/mg whole cell lysate, respectively) [23].

2. Conclusions

The data presented above suggest that not all isothiocyanates are suitable for the use as chemopreventive agents. Only a few studies have investigated whether isothiocyanates can exhibit selective cytotoxic activity against transformed human tumor cells, a property that has been sought in synthetic anticancer drugs. A specific inhibition of the growth of transformed cells by allyl isothiocyanate was hypothesized only according to the decreased sensitivity to cytotoxic effects in detransformed HT29 human colon carcinoma cells in vitro [24]. Although detransformed cells do express a variety of markers of differentiation that are typical of the normal phenotype, they remain immortalized tumor cells [24]. Moreover, sulforaphane did not exert any cytotoxic effect on intestinal-differentiated $CaCo_2$ cells, although it was very effective on undifferentiated $CaCo_2$ and HT29 cells [25]. However, $CaCo_2$ and HT29 are two human colon cancer cell lines, therefore very different with respect to non-transformed cells. A selective antitumor effects was recently suggested for phenethyl isothiocyanate and allyl isothiocyanate only on the basis of their median toxic concentration values on normal proliferating lymphocytes, that were 10–20-fold higher than those recorded in human leukemia cells [26].

By using transformed T-cells and their normal counterpart we demonstrated that sulforaphane did not have a selective cytotoxic activity against transformed cells. Although in vitro studies do not necessarily predict in vivo outcomes, our findings raise important questions regarding the suitability of sulforaphane for cancer chemoprevention.

MTBITC holds great promise for future development as a chemopreventive agent. The reasons for the different behavior of MTBITC with respect to that recorded for sulforaphane remain to be elucidated. By looking at the chemical structures of MTBITC and sulforaphane (Fig. 1), we can see that the only difference is the oxidation status of sulfur. As above mentioned, GSH was the principal driving force for accumulation of isothiocyanates, while cellular GST further enhances such accumulation. Many isothiocyanates can elevate GSH levels and induce GST. Exposure of cells to isothiocyanates results in rapid uptake and accumulation of isothiocyanates through the GSH conjugation reaction, which is catalyzed by GST; such accumulation then leads to an elevation of cellular GSH and GST, which in turn causes more rapid and higher accumulation of isothiocyanates in cells [22]. However, the degree of such synergism may depend on specific isothiocyanates. For example, such synergism may be significant for sulforaphane because increases of cellular GSH level and GST activity resulted in increases of both initial uptake and long-term accumulation levels of sulforaphane [22]. Moreover, sulforaphane was previously found to be the most potent inducer among several dozen of isothiocyanates tested, and the presence of oxygen on sulfur enhances potency [27]. In contrast, such synergism may be limited for other isothiocyanates, such as allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate [22]. No information is currently available on MTBITC in this context. It is therefore possible but speculative that part of these effects is mediated by GSH and GST.

In conclusion, our findings underline the need of more in-depth studies of the toxicity profiles of different

isothiocyanates. In fact, any chemopreventive use of isothiocyanates would have to be very carefully examined, as dietary supplementation with single, putative anticarcinogenic compounds is not warranted without extensive investigation into their possible harmful effects.

Acknowledgments

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