Antiproliferative and cancer chemopreventive activity of phytoalexins: focus on indole phytoalexins from crucifers

Minireview

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Phytoalexins are produced by plants after exposure to physical, biological or chemical stress and a specific group of these metabolites represent indole phytoalexins produced by important plants of the family Cruciferae. With respect to the epidemiologically proven cancer chemopreventive properties of brassica vegetables, antiproliferative and anticarcinogenic activities of indole phytoalexins have been studied. Several indole phytoalexins (i.e. brassinin, spirobrassinin, brassilexin, camalexin, 1-methoxyspirobrassinin, 1-methoxyspirobrassinol and methoxyspirobrassinol methyl ether) have been found to possess significant antiproliferative activity against various cancer cells and this activity is supposed to be associated with the modulation of activity of transcription factors regulating cell cycle, differentiation and apoptosis. Indole phytoalexins (i.e. cyclobrassinin, spirobrassinin, brassinin) also exhibited cancer chemopreventive activity in models of mammary and skin carcinogenesis. Understanding the molecular and cellular mechanism of action of such drugs and their structure-activity relationships is necessary for development new derivatives with more favourable profile of antiproliferative and chemopreventive activities.

Key words: Phytoalexins, chemoprevention, carcinogenesis, brassinin, spirobrassinin, camalexin.

Phytoalexins are antimicrobial compounds produced by plants as secondary metabolites in response to several forms of stress, including fungal infection [3]. Accumulation of phytoalexins around infection sites is considered as one of the most important antimicrobial defense mechanisms of the plant. By now, phytoalexins have been isolated from over 30 different plant families since K. O. Müller first introduced the phytoalexin concept in 1940 [41].

Among phytoalexins, resveratrol (trans-3,4',5-trihydroxystilbene), a polyphenolic phytoalexin found in grapes and grape products such as wine has attracted significant interest due to its anticancer and anti-inflammatory effects [25, 47]. These findings are consistent with epidemiological studies that defined the so-called "French paradox" [43, 45] as the association of reduced mortality from coronary heart disease and breast cancer with increased red wine consumption [21, 43]. Resveratrol has been shown to possess cancer chemopreventive activity by inhibiting cellular events associated with tumor initiation, promotion, and progression [8, 25, 36]; by inhibiting ribonucleotide reductase [17]; and by inhibiting the signaling pathway associated with the activation of NF-κB and NF-κB-dependent genes such as MCP-1 that would normally promote inflammation, protect against apoptosis, and potentiate cell growth [30]. Pertinent to cancer prevention, resveratrol also suppresses the expression of inducible nitric oxide synthase [51] and cyclooxygenase-2 [48], and possesses both, estrogenic and antiestrogenic properties as a mixed agonist/antagonist [6] which contribute to its antioncogenic mechanism. Moreover, resveratrol has been shown to inhibit cell proliferation of some cancer cells *in vitro* [5, 13, 36, 51]. Antiproliferative effect of resveratrol against A431 human epidermoid carcinoma cells has been shown to be based on a dose- and time-dependent downregulation of hyperphosphorylated protein pRb ac-

companied by downregulation of protein expression of all five E2F (1–5) family members of transcription factors that results in a stoppage of the cell cycle progression at the $G_1 \rightarrow S$ phase transition thereby leading to a G_0/G_1 arrest and subsequent apoptotic cell death [1].

Some phytoalexins from crucifers have also been shown to exhibit significant anticancer activity by inhibition of proliferation of cancer cells and/or by suppression of the process of carcinogenesis, in addition to their antimicrobial (antifungal and antibacterial) activity. Cruciferous crops (*Cruciferae*, syn. *Brassicaceae*) are cultivated worldwide and constitute an extremely important group of plants. Enormous quantities of vegetable crucifers, such as broccoli (*Brassica oleracea* var. *botrytis*), cauliflower (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), and a variety of cabbages (*B. oleracea*) are consumed annualy. Moreover, oilseed crucifers (*Brassica spp.*) represent the third largest source of edible vegetable oils, whereas brown mustard seeds (*B. juncea*) together with white mustard seeds (*Sinapis alba*) and wasabi (*Wasabiae japonica*) are well-known condiments [28]. The worldwide impact of cruciferous crops is best assessed by the tremendous number and variety of scientific articles published annually.

Phytoalexins from crucifers were first reported in 1986 [49] and since then, close to 30 cruciferous phytoalexins have been isolated and their structures elucidated (Fig. 1, compounds 1-25).

The unique structural feature of majority of these compounds is the presence of indole ring and a side chain or another heterocycle, containing a nitrogen atom and one or two sulfur atoms. Most interestingly, crucifers appear to be the only plant family producing sulfur-containing phytoalexins. Several of these phytoalexins are produced by more than one species of crucifers and their production can be induced by diverse pathogens and/or abiotic factors [41] (Tab. 1).

Despite their related biogenetic origin, the cruciferous phytoalexins have rather different structures, which would suggest substantially different biological activities.

Antiproliferative effect of cruciferous phytoalexins

Cytotoxic effect of indole phytoalexins brassinin (1), spirobrassinin (12) and cyclobrassinin (8) was tested on mouse

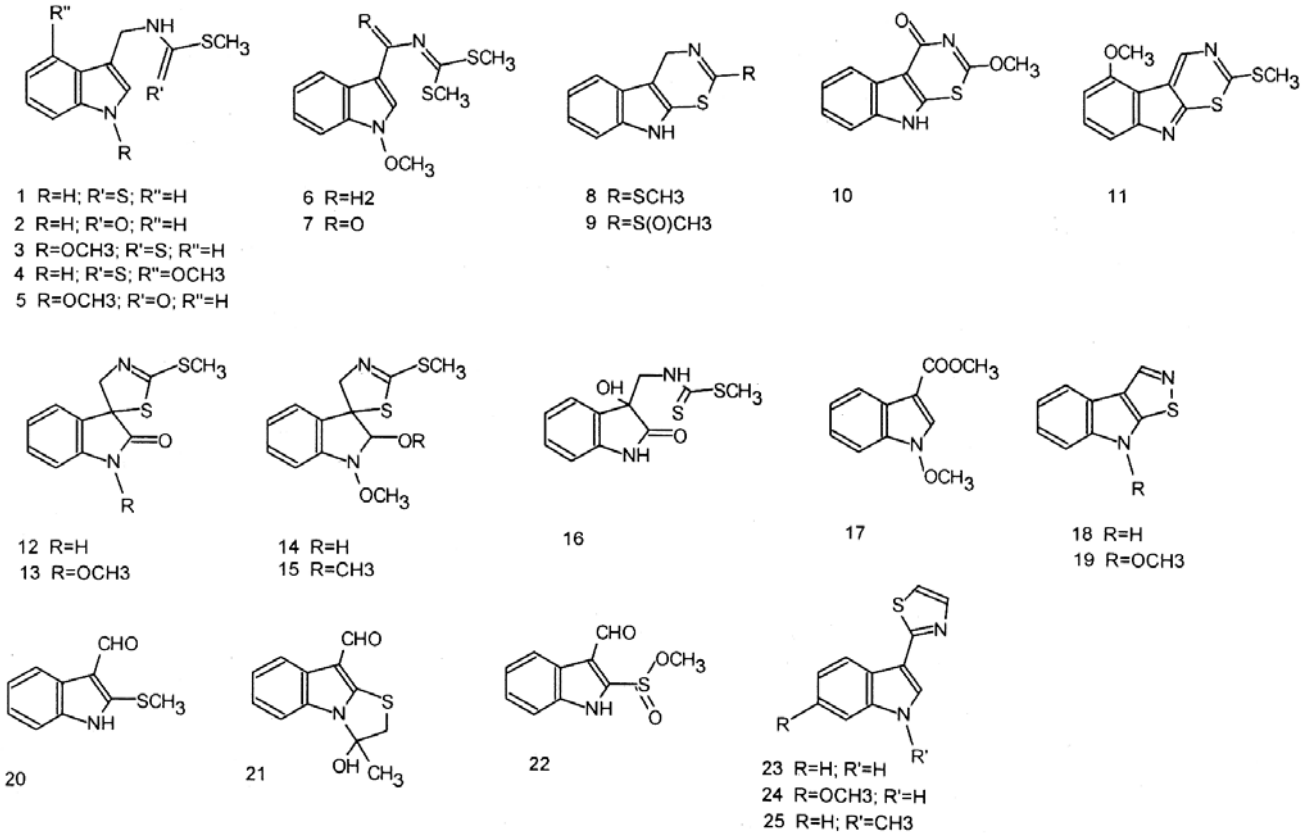

Figure 1. Structures of cruciferous phytoalexins: 1 brassinin, 2 brassitin, 3 1-methoxybrassinin, 4 4-methoxybrassinin, 5 1-methoxybrassitin, 6 1-methoxybrassenin A, 7 1-methoxybrassenin B, 8 cyclobrassinin, 9 cyclobrassinin sulfoxide, 10 cyclobrassinone, 11 dehydro-4-methoxycyclobrassinin, 12 spirobrassinin, 13 1-methoxyspirobrassinin, 14 1-methoxyspirobrassinol, 15 1-methoxyspirobrassinol methyl ether, 16 dioxibrassinin, 17 methyl 1-methoxyindole-3-carboxylate, 18 brassilexin, 19 sinalexin, 20 brassicanal A, 21 brassicanal B, 22 brassicanal C, 23 camalexin, 24 6-methoxycamalexin, 25 1-methylcamalexin.

Table 1. Some phytoalexins from crucifers, inducing agent, and antimicrobial activity

Phytoalexin	Plant species (inducing agent)	Antimicrobial activity
Brassinin (1)	*B. napus* (abiotic-$CuCl_2$) *B. oleracea* (*Pseudomonas cichorii*) *B. rapa* (UV, *P. cichorii*) *R. sativus* (*P. cichorii*)	*Bipolaris leersiae*, *Pyricularia oryzae*, *Phoma lingam*
Cyclobrassinin (8)	*B. carinata* (abiotic-$CuCl_2$) *B. juncea* (abiotic-$CuCl_2$) *B. napus* (*P. lingam*) *B. nigra* (abiotic-$CuCl_2$) *B. oleracea* (*P. cichorii*) *B. rapa* (UV, *P. cichorii*) *C. cucumerinum*	*B. leersiae*, *P. oryzae*, *P. lingam*, *Pythium ultimum*, *Rhyzoctonia solani*, *F. nivale*,
Spirobrassinin (12)	*B. carinata* (*P. lingam*) *B. juncea* (*P. lingam*) *B. napus* (*P. lingam*) *B. oleracea* (UV, *P. cichorii*) *B. rapa* (UV, *P. cichorii*) *R. sativus (P. cichorii)*	*P. oryzae*, *P. lingam*, *C. cucumerinum*
Camalexin (23)	*Capsella bursa-pastoralis* (*Alternaria brassicae*) *Arabis lyrate* (*P. syringae*) *Arabidopsis thaliana* (*P. syringae*)	*E. coli*, *B. subtilis*, *A. brassicae* *L. monocytogenes*, *S. cerevisiae*, *P. cichorii*, *P. lingam*, *P. syringae*, *R. solani*, *X. campestris*

leukemia L1210 and melanoma B16 cells using the MTT assay after 24 h of cultivation. The highest cytotoxic effect was induced by brassinin, which at concentration 100 μmol reduced the cell growth of L1210 and B16 by 35% of the solvent control and at concentration 10 μmol brassinin reduced cancer cell proliferation by 15% (L1210) and 9% (B16). Spirobrassinin was less efficient against both cell lines and at concentration 100 μmol reduced the cell growth of L1210 and B16 by 12% of the solvent control, whereas at concentration 10 μmol spirobrassinin had no effect on the proliferating activity of both cell lines. Cyclobrassinin did not exhibit antiproliferative activity against L1210 and B16 cell lines [46].

Cyclobrassinin (8), brassilexin (18), 5-methoxybrassilexin and homocyclobrassinin were evaluated as growth inhibitors with cultured KB cells, and it was found, that brassilexin was the most effective, inhibiting KB cell growth at a concentration of 8 $\mu g.ml^{-1}$ (45.9 μmol), while cyclobrassinin was less effective [50].

Antiproliferative activity of camalexin (23) was evaluated against the human breast cancer cell line SKBr3 which over-expresses topoisomerase IIα and is well characterized with respect to estrogen and epidermal growth factor receptor and sensitivity to chemotherapeutic drugs [24]. Camalexin exhibited marked antiproliferative activity against SKBr3 cells (IC_{50}=2.7 μmol) and was more potent than cisplatin (IC_{50}=7.4 μmol) and melphalan (IC_{50}=13.0 μmol). However, antiproliferative activity of camalexin against SKBr3 was significantly less in comparison with agents such as mitoxantrone (IC_{50}=0.016 μmol), etoposide (IC_{50}=0.60 μmol), and amsacrine (IC_{50}=0.16 μmol) which are known to act via inhibition of topoisomerase II [39]. Thus, antiproliferative effect of camalexin presumably does not result from an inhibition of topoisomerase II, in spite of its close structural resemblance with natural thiazolyl indolequinone BE 10988 (Fig. 2, compound 26) which has anticancer properties and acts as an inhibitor of topoisomerase II [40].

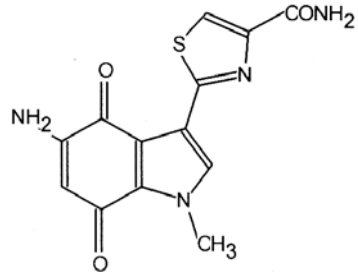

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Figure 2. Structure of thiazolyl indolequinone BE 10988 (compound 26).

According to preliminary results of MTT assay, 1-methoxyspirobrassinin (13), 1-methoxyspirobrassinol (14) and 1-methoxyspirobrassinol methyl ether (15) exhibit a reasonable antiproliferative activity against estrogen receptor(ER)-negative human breast cancer cell line MDA-MB 231 at concentration 1 μmol and against T-ALL (Jurkat) cell line at concentration 10 μmol. By contrast, all agents mentioned above stimulate growth of ER-positive human breast cancer cells MCF-7 at concentration 10 μmol [Mezencev, Mojžiš, Pilátová, unpublished data].

The mechanism of antiproliferative effect of indole phytoalexins is not known yet. Brassinin, spirobrassinin as well as their analogues belong to the group of dithiocarbamates, which also express strong anticancer effect and some of them operate via modulation of heat shock response and nuclear factors activity (NF-κB) [12]. Dithiocarbamates (DTCs) are potent antioxidant agents that can switch the expression of genes dependent on the activation of the transcription factors AP-1 and NF-κB [2].

Nuclear factor-kappa B (NF-κB) is a p50–p65 heterodimer which plays a pivotal role in immune response and cell growth and recent studies have shown that the factor NF-κB is also a regulator of apoptosis. Activation of NF-κB by certain apoptotic stimuli (TNF, ionizing radiation, certain chemotherapeutic compounds) has been found to protect

cells from apoptosis, which is induced by these same stimuli. In addition, inhibition of NF-κB activation potentiates apoptosis induced by these reagents. Resistance to cancer therapies appears to be mediated by resistance to apoptosis, and thus these apoptotic stimuli are less effective due to simultaneous activation of NF-κB. Therefore, new approaches to cancer therapy that inhibit nuclear translocation of NF-κB and thus suppress its transcriptional activity may prove to be highly effective in the treatment of tumors [53].

Some DTCs (prolinedithiocarbamate, diethyldithiocarbamate, and ammonium dithiocarbamate) induce p21/KIP1/CIP1 expression in p53-dependent pathway leading to G_1/S arrest of human hepatoma Hep G2 cells [29].

DTCs also trigger the expression of myeloid differentiation antigens, as well as other changes associated to the differentiation and inhibit proliferation of human promonocytic leukemic cell lines U-937 through mechanism that involves activation of the AP-1 transcription factor [2]. The effects of DTCs on myeloid cell differentiation supports a therapeutical potential of these agents in bone marrow-derived malignancies.

Structure of spirobrassinin is similar to structural elements of other known anticancer agents, such as pentacyclic oxindole alkaloids found in *Uncaria tomentosa* (cat's claw) from region of upper Andes and Peru, which is wildly used in folk medicine as an anti-inflammatory, contraceptive and anti-cancer remedy [27]. Pentacyclic oxindole alkaloids from *U. tomentosa* (except from mitraphylline) exhibit antiproliferative effect on HL60 and U-937 leukemic cell lines without inhibiting the growth of normal human blood progenitor cells. The most potent activity of this kind has been demonstrated by uncarine F (Fig. 3, compound 27), with

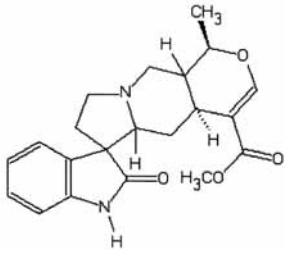

Figure 3. Structure of uncarine F (compound 27).

IC_{50} = 21.7–29 μmol. The mechanism of this effect may involve inhibition of DNA polymerase activity exhibited by *U. tomentosa* extracts in *in vitro* studies [15].

Cancer chemopreventive activity of cruciferous phytoalexins

Chemoprevention of cancer is a new pharmacological approach to arrest or reverse the process of carcinogenesis, and thus to prevent cancer, as contrasted with classical chemotherapy for treatment of an existing disease [44]. In recent decades, considerable attention has been directed towards cancer prevention by natural products [20]. Epidemiological evidence has suggested that the intake of green and yellow vegetables is inversely related to risk of cancers of variety of tissues including colon and breast [54]. Vegetables most often associated with reduction of cancer risk are from the family of Cruciferae and especially from the genus *Brassica*, e.g. cauliflower, cabbage, Brussels sprouts and broccoli [9]. In connection with these facts, possible role of indole phytoalexins has become interesting in exploring the cancer chemopreventive potential of crucifers.

The chemopreventive effect of brassinin (1), cyclobrassinin (8) and 2-methylbrassinin was evaluated in the model of mammary carcinogenesis based on 7,12-dimethylbenz[a]anthracene (DMBA)-induced precancerous lesions in mouse mammary gland organ culture [31]. There appears to be a good correlation between the chemopreventive activity in this assay and *in vivo* carcinogenesis, and agents which show chemopreventive activity in the organ culture often show chemopreventive activity in N-methyl-N-nitrosourea or DMBA-induced mammary carcinogenesis in Sprague-Dawley rats [34, 35]. Results showed that brassinin, and cyclobrassinin were comparably active in inhibiting the formation of preneoplastic mammary lesions in culture; both compounds inhibited the incidence of mammary lesions in a dose dependent manner and at concentrations 10 μmol there was 80.0% (brassinin) and 90.9% (cyclobrassinin) reduction in the number of mammary glands with lesions as compared to control glands incubated with vehicle. However, 2-methylbrassinin was not significantly active in this process, which together with the fact that cyclobrassinin is a biologically derived product of the oxidative cyclization of brassinin leads to possible conclusion, that oxidative cyclization of brassinin may be an effective metabolic activation step [32].

Brassinin exhibited dose-dependent inhibition of DMBA-induced mouse skin tumors that were promoted by treatment with TPA (12-O-tetradecanoylphorbol-13-acetate) in CD-1 mice. In this two-stage skin carcinogenesis assay brassinin inhibited DMBA-induced and TPA-promoted skin carcinogenesis when present only during the TPA treatment phase and additional protection was not afforded when brassinin was present during both the initiation and promotion phases of experiment. These data support inhibition of carcinogenesis during the promotion phase but do not exclude inhibition during the initiation phase [32].

Following evaluation of cancer chemopreventive activity in the model of mammary carcinogenesis revealed significant inhibition of formation of DMBA-induced mammary lesions ($p<0.05$) for cyclobrassinin (91.0%), spirobrassinin (76.3%), brassinin (73.0%) and N-ethyl-2,3-dihydrobrassi-

nin (66.3%) in comparison with the solvent control at concentration of tested drugs 10 mg.dm^{-3}. At the same concentration, inhibition was not statistically significant for 1-methoxybrassinin (44.5%) spirohomobrassinin (22.3%), 2-methylspirobrassinin (7.7%) and 2-methylbrassinin (0%), while several methyl substituted analogues of brassinin (4-methylbrassinin, 5-methylbrassinin, 7-methylbrassinin) and 5-chlorobrassinin exhibited toxicity to the mammary glands and therefore not deemed effective [33].

The mechanism of chemopreventive activity of cruciferous indole phytoalexins is still an open question. According to WATTENBERG there are three main categories of cancer chemopreventive agents: (i) agents that prevent carcinogen formation; (ii) blocking agents (anti-initiators) that are effective when administered prior to or simultaneously with the carcinogen and that may either inhibit the metabolic activation of pro-carcinogens or enhance detoxification and scavenge the ultimate carcinogens prior to their action; and (iii) suppressing agents (anti-promoters) that are also effective when given subsequent to the administration of carcinogen during tumor promotion [55]. As brassinin and cyclobrassinin have induced 4- and 29-fold increase of a phase II detoxification enzyme NAD(P)H:quinone oxidoreductase activity (QR) in mammary gland organ culture, it appears that in this concept brassinin and cyclobrassinin belong to the group of anti-initiators. On the other hand, in the two stage skin carcinogenesis model brassinin acted as an anti-promoter showing inhibition of promotional stage of carcinogenesis [32].

Cruciferous indole phytoalexins are structurally and biogenetically related to indole glucosinolates such as glucobrassicin that are also sulfur-containing metabolites produced by crucifers and thought to be part of the crucifer constitutive defenses [38, 41]. Glucosinolates undergo enzymatic hydrolysis by myrosinase on crushing of the plant tissues to give isothiocyanates, nitriles, alcohols, and so on [16], and some of the hydrolysis products are also able to influence phase I and phase II biotransformation enzyme activities, thereby influencing several processes related to chemical carcinogenesis [52]. Organic isothiocyanates block the production of experimental tumors induced in rodents by diverse carcinogens (polycyclic aromatic hydrocarbons, azo dyes, ethionine, N-2-fluorenylacetamide, and nitrosamines) in liver, lung, mammary gland, forestomach, and esophagus [56]. Their anticarcinogenic effects appear to be mediated by tandem and cooperating mechanisms: (i) suppression of pro-carcinogen activation by cytochromes P450, probably by a combination of down-regulation of enzyme levels and direct inhibition of their catalytic activities, which thereby lower the levels of formed ultimate carcinogens; and (ii) induction of phase II enzymes such as glutathione transferases and NAD(P)H: quinone reductase, which detoxify any residual electrophilic metabolites generated by phase I enzymes and thereby destroy their ability to damage DNA [56]. The isothiocyanate sulforaphane [$CH_3S(O)(CH_2)_4N{=}C{=}S$] isolated from broccoli sprouts displays strong anti-cancer activities in a rat mammary tumor model by activating phase II enzymes [14] and isothiocyanates from cruciferous vegetables have been shown to increase the excretions of carcinogens also in humans [23]. Sulforamate, an aliphatic analogue of brassinin derived from sulforaphane was shown to be an inducer of NAD(P)H:quinone oxidoreductase in murine Hepa 1c1c7 cell culture and also a potent inhibitor of preneoplastic lesion formation in carcinogen-treated mouse mammary glands in organ culture [19]. Indole-3-carbinol (I3C), which is structurally similar and biogenetically closely related to brassinin, has been shown to be inhibitory at the initiation stage of carcinogenesis [42]. Dietary I3C functions as a potent inducer of 2-hydroxylation of estradiol in rodents [7] and humans [37], thus increasing the antiproliferative metabolite 2-hydroxyestrone and decreasing 16α-hydroxyestrone, carcinogenic metabolite of estradiol. This change in estrogen metabolism may be the reason, at least in part, why I3C inhibits mammary tumorigenesis in various mouse models [7, 22]. Other possible anticancer effects of I3C are suggested by the observation that I3C inhibits expression of cyclin-dependent kinase 6 and induces a G_1 cell cycle arrest [10] or apoptosis [18, 26]. In spite of its chemopreventive activity, I3C have been shown to promote liver carcinogenesis in trouts and lung carcinogenesis in mice. Indole-3-carbinol administration is known to induce phase I specific enzymes in addition to phase II enzymes, and thus it can stimulate metabolic activation of pro-carcinogens [4, 11].

Conclusions

In spite of the fact that certain indole phytoalexins from crucifers have been shown to exhibit significant antiproliferative activity against various cancer cells, knowledge concerning antiproliferative activity of cruciferous indole phytoalexins is still very limited and further experiments should be performed in order to find the most active natural compounds and their analogues against particular cancer cell lines. From current status of knowledge it is possible to suppose, that antiproliferative activity of indole phytoalexins results from modulation of activity of transcription factors regulating cell cycle, cell differentiation and apoptosis rather than from direct interaction with DNA [46].

The development of new and better drugs capable to inhibit the development of invasive cancer remains a principal need since cancer chemoprevention has been established as a new promising strategy [44]. Toward this goal, it is essential to understand the molecular and cellular mechanism of action of such drugs, as well as to find their structure-activity and quantitative structure-activity relationships. In this context, better understanding of mechan-

ism of significant cancer chemopreventive activity of cruciferous phytoalexins as well as rational synthesis of their derivatives with more favorable profile of activity could be of great value.

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