

# Serotonin Receptors, Novel Targets of Sulforaphane Identified by Proteomic Analysis in Caco-2 Cells

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## Abstract

**Cancer chemopreventive activity of sulforaphane has been predominantly associated with its ability to induce phase II detoxification enzymes. In the present study, novel targets of sulforaphane were identified and characterized using a proteomics approach. Two-dimensional gel electrophoresis and mass spectrometry were used to produce protein profiles of human colon adenocarcinoma Caco-2 cells treated with 5  $\mu$ mol/L sulforaphane for 48 h and control cells (0.05% DMSO). Gel comparisons showed the down-regulation to undetectable level of the serotonin receptor 5-HT<sub>3</sub> after sulforaphane treatment. In addition, Aldo-keto reductase and D-dopachrome decarboxylase were also differentially expressed in control and treated cell extracts. To elucidate two-dimensional gel findings, the neurotransmitter receptors 5-HT<sub>3A</sub>, 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, and the serotonin reuptake transporter were analyzed using Western blotting. Results showed a decrease of neurotransmitter receptors in a dose-dependent manner after sulforaphane treatment. Moreover, after exposure of Caco-2 cells to sulforaphane, nicotinic acetylcholine receptor protein level was increased. These findings suggested a potential effect of sulforaphane on serotonin release. Activation of neurotransmitter receptors followed by initiation of cyclic AMP signaling might be crucial events in colon carcinoma progression. Thus, the effect of sulforaphane may help to elucidate signaling pathways serotonin-mediated in colon cancer and lead to development of potential novel therapeutic agents. [Cancer Res 2008;68(13):5487–91]**

## Introduction

Sulforaphane, an isothiocyanate found in cruciferous vegetables, has been widely studied as a chemopreventive agent (1). The most studied role of sulforaphane in chemoprevention is its ability to induce phase II detoxification enzymes. Experimental evidence suggests that sulforaphane is effective against many carcinogen-induced tumors through induction of phase II detoxification enzymes. Data from *in vivo* and *in vitro* studies showed that sulforaphane activates NF-E2 p45-related factor-2 transcription factor in binding antioxidant response elements in the promoter regions of target genes, thereby increasing cellular defenses against oxidative stress (2, 3). The anticarcinogenic activities of sulforaphane also include inhibition of phase I enzymes, protection against heterocyclic amine-induced DNA adduct formation, induction of apoptosis, and arrest of cell cycle progression (4–6). Recent studies suggest that sulforaphane suppresses lipopolysac-

charide-induced cyclooxygenase-2 (COX-2) expression through the modulation of multiple targets in COX-2 gene promoter (7). COX-2 is a key enzyme catalyzing the rate-limiting step in the biosynthesis of prostaglandins from arachidonic acid. Accumulating evidence shows the overexpression of COX-2 in several epithelial carcinomas including bladder, breast, lung, esophagus, and colon (8, 9). Pathogenesis of colorectal cancer consists of a series of molecular and cellular events that become visible with adenomatous polyps formation (10). The current research is focused on assessing whether an increase in nutritional intake of sulforaphane may provide beneficial effects for colon cancer prevention. These levels are achievable, as data from a human intervention study showed that 2 hours after consumption of 100 grams standard and high-glucosinolate broccoli, plasma concentrations of sulforaphane and its metabolites reached 2.2 and 7.3  $\mu$ mol/L, respectively (11). The purpose of this investigation was to identify and characterize novel targets of sulforaphane in human colon adenocarcinoma Caco-2 cells using relatively low concentrations of sulforaphane (5  $\mu$ mol/L). In the present study, the novel effect of sulforaphane as modulator of neurotransmitter receptors was reported.

## Materials and Methods

**Materials.** Human colon adenocarcinoma Caco-2 cell line was obtained from the European Collection of Cell Culture. Cell culture medium and supplements were purchased from Invitrogen. Sulforaphane (4-methylsulfinylbutyl isothiocyanate; purity, 97%) was purchased from LKT laboratories (Alexis Biochemicals). Reagents for two-dimensional gel electrophoresis were obtained from GE Healthcare, Bio-Rad, and Genomic Solutions (Proteomic Solutions). Antibodies for Western blotting were purchased from Abcam, Dako, and Santa Cruz. All other chemicals were purchased from Sigma and Roche Applied Science.

**Cell culture and treatments.** Cells were grown in DMEM. The cell culture medium was supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine. Cell growth for proteomics studies was performed using 10-cm dishes under 5% CO<sub>2</sub> in air at 37°C. Cells at passage 40 to 55 were treated with sulforaphane (5  $\mu$ mol/L) for 48 h when they reached 50% of confluence. Sulforaphane was dissolved in DMSO. Control cells were treated with equal volume of DMSO (0.05%). For Western blotting studies, cells ( $2 \times 10^4$  per cm<sup>2</sup>) were seeded in 6-well plates and treated with sulforaphane (5, 10, and 20  $\mu$ mol/L) for 48 h.

**Protein extraction.** Cells were washed twice with ice-cold PBS and then incubated for 30 min in the NP40 buffer [20 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 10% Glycerol, and 1% NP40] with addition of one tablet (in 10 mL buffer) of complete mini protease inhibitor cocktail (Roche) just before use. Cells were harvested by scraping and the homogenate was centrifuged at 13,000 *g* at 4°C for 15 min. The supernatants were collected and protein concentrations were determined using Bradford assay (Sigma) according to the manufacturer's instructions. Aliquots of samples were stored at –80°C.

**Two-dimensional gel electrophoresis.** Protein extracts were desalted and concentrated to 4 mg/mL using a Biomax 5NMW Membrane Ultrafree 0.5 centrifugal filter (Millipore) at 12,000 *g* and 4°C before the two-dimensional gel procedure. For each sample, two biological and three technical replicate gels were run.

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For Isoelectric focusing (IEF), the samples were prepared by mixing 200  $\mu\text{g}$  protein with rehydration solution containing 7 mol/L urea, 2 mol/L thiourea, 2% (w/v) 3-[[3-(cholamidopropyl)dimethylammonio]-1-propane-sulfonate hydrate, 18.2 mmol/L DTT, 2% pH 3-11 NL IPG buffer (GE Healthcare), and a trace of bromophenol blue (final volume, 440  $\mu\text{L}$ ). This was applied to a 24-cm Immobiline DryStrip pH 3-11 NL (GE Healthcare), and the strips allowed to rehydrate overnight at 20°C. IEF was then performed on the IPGphor unit (GE Healthcare) for a total 44,800 V hours at 20°C using the following conditions: Step and Hold at 500 V for 500 V hours, Gradient to 1,000 V over 800 V hours, Gradient to 8,000 V over 13,500 V hours, and Step and Hold at 8,000 V for 30,000 V hours. After IEF, the focused strips were frozen at -70°C until required.

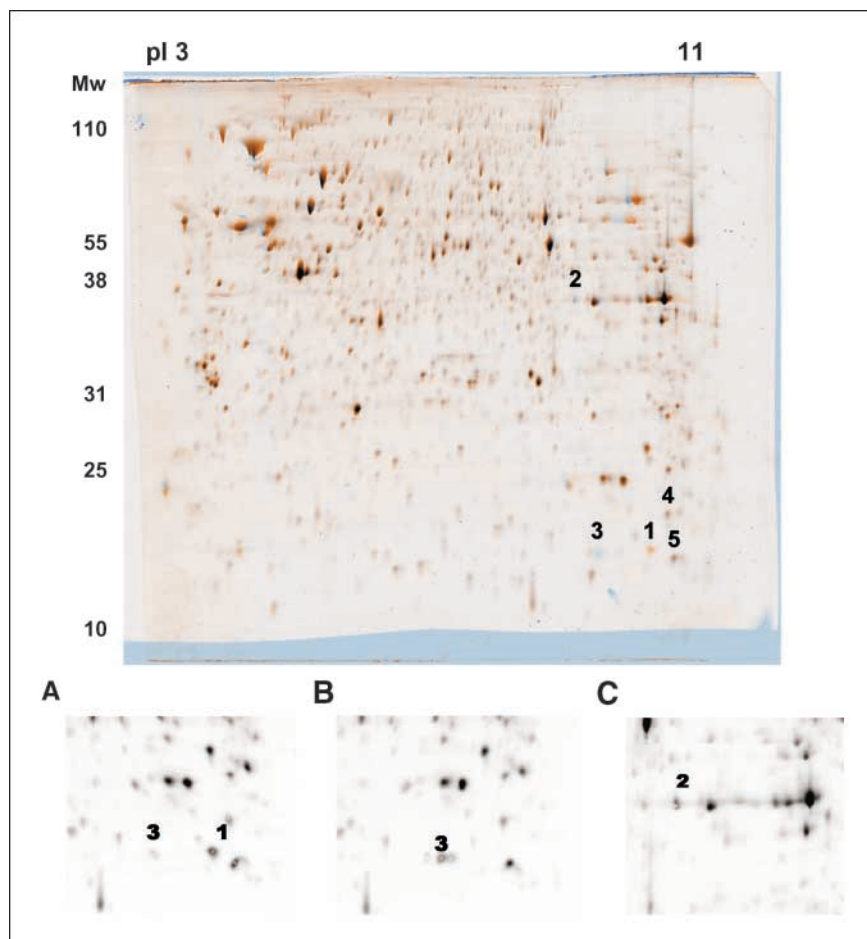
Before the second dimension, the focused strips were conditioned in filtered (0.45  $\mu\text{m}$ ) equilibration buffer, prepared as 5% SDS and 0.01% bromophenol blue in 0.122 mol/L Tris acetate (Tris Acetate Equilibration Buffer; Genomic Solutions), modified with 5 mg/mL final volume SDS, 360 mg/mL urea, and 300 mg/mL 99% glycerol. To reduce and alkylate cysteines, the strips were treated first with 8 mg/mL DTT in the equilibration buffer (9 mL; 30 min with gentle shaking) before being transferred into 25 mg/mL iodoacetamide in the equilibration buffer (9 mL; 30 min with gentle shaking). The equilibrated strips were layered onto home-cast 10% (v/v) Duracryl gels (26  $\times$  21 cm; 1-mm thick) in the Investigator 2nd Dimension Running System (Genomic Solutions), and electrophoresis was carried out using a limiting power of 20 W per gel in a discontinuous buffer system [3 L cathode buffer (200 mmol/L Tris base, 200 mmol/L Tricine, and 14 mmol/L SDS) and 10.5 L anode buffer (25 mmol/L Tris/acetate buffer pH 8.3)].

**Gel imaging and analysis.** After electrophoresis, the gels were stained with Sypro-Ruby (Bio-Rad) according to the manufacturer's instructions and imaged on the Pharos FX+ Imaging System (Bio-Rad) at 100- $\mu\text{m}$  resolution using a 532-nm excitation laser and a 605-nm emission filter. Images were

saved as 16 bit TIF files and analyzed by ProteomWeaver version 3.1 software (Definiens). Automatic matching with control gels was performed with base-paired normalization to compare the spot volume data.

**Protein identification.** Protein spots with altered levels of expression were excised from the gel using the ProPick excision robot (Genomic Solution), and in-gel trypsin-digested using a ProGest Protein Digester (Genomic Solutions). Gel plugs were first conditioned with two 20-min incubations in 200 mmol/L ammonium bicarbonate (ABC) in 50% acetonitrile (50  $\mu\text{L}$ ) followed by 10-min incubations with acetonitrile (50  $\mu\text{L}$ ). The gel plugs were then conditioned for 15 min with 25 mmol/L ABC (50  $\mu\text{L}$ ) followed by 10 min in acetonitrile (50  $\mu\text{L}$ ). A final 5-min incubation of acetonitrile (50  $\mu\text{L}$ ) preceded trypsin digestion at 37°C (3 h) using 50 ng (5  $\mu\text{L}$  each well) sequencing-grade porcine trypsin (Promega) dissolved in 25 mmol/L ABC. Digestion was stopped after 3 h, and peptides were extracted with formic acid (10%; 5  $\mu\text{L}$  each tube). Tryptic digests were analyzed using an Ultraflex MALDI-ToF (matrix-assisted laser desorption/ionization time-of-flight) instrument (Bruker Ltd.) at the Institute of Food Research/John Innes Centre Joint Proteomics facility.

Proteins were identified from the peptide mass peak list by the Protein Mass Fingerprint technique using an offline copy of Mascot search tool (Matrix Science). SPTrEMBL was selected as sequence database; the taxonomy group searched was Homo sapiens; trypsin was selected as the reagent used for protein digestion with one allowed missed cleavage; the mass tolerance was 50 ppm; peptide masses were stated to be monoisotopic; carbamidomethyl modification of cysteine (as a fixed modification); and methionine oxidation (as a variable modification) were permitted. The results give a Probability Based Mowse Score, equal to  $-10\text{XLog}(P)$ , where  $P$  is the probability that the observed match is a random event. Protein scores of  $>61$  are considered statistically significant ( $P < 0.05$ ) under the selected variables.



**Figure 1.** Automatic matching between sulforaphane-treated (5  $\mu\text{mol/L}$ ; 48 h) and control gels. Coordinates for first-dimension ( $pI$ ) and second-dimension ( $Mw$ ) separation have been shown on the map. The spot numbers indicate the identified protein listed in Table 1. *Blue spots*, protein spots from the sulforaphane-treated gel. *Red spot*, protein spots from control gel. Boxes highlight the spot picking from specific areas of change on the individual gels: control (A) and sulforaphane-treated samples (B and C).

**Table 1.** Characteristics of proteins identified from sulforaphane-treated and control gels (spot number 1, 2, and 3 are differentially expressed)

Spot number	pI	Mw kDa	Protein name	Accession number Swiss-Prot	Score*	Coverage (%)
1 <sup>†</sup>	6.95	25.30	Serotonin receptor	Q9UEP2	51	26
2 <sup>‡</sup>	7.13	37.11	AKR	P52895	111	45
3 <sup>†</sup>	7.25	12.69	D-dopachrome decarboxylase	P30046	48	40
3 <sup>‡</sup>	6.71	12.81	D-dopachrome decarboxylase	P30046	47	40
4 <sup>†</sup>	8.44	15.21	Profilin-1	P07737	187	65
4 <sup>‡</sup>	8.44	15.21	Profilin-1	P07737	167	65
5 <sup>†</sup>	8.98	10.29	Chaperonin 10-related protein	Q9UNM1	86	54
5 <sup>‡</sup>	8.98	10.29	Chaperonin 10-related protein	Q9UNM1	79	54

\* $P < 0.05$  for protein scores of  $>61$ .

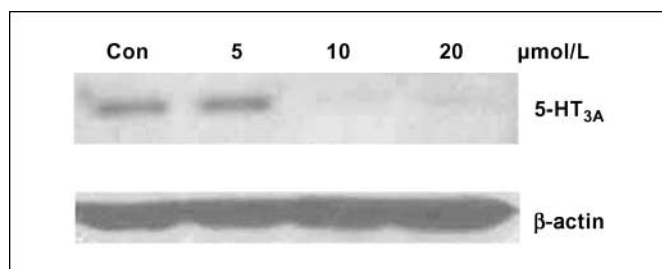
<sup>†</sup> Spot picked from control gel.

<sup>‡</sup> Spot picked from gel under sulforaphane treatment.

**Western blotting.** Samples (60  $\mu$ g of total cell protein extracts) were separated under reducing conditions on 10% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad) with a semidry transfer cell (Trans-Blot; Bio-Rad). Transfer was run at 15 V for 40 min. Membranes were blocked for 1 h at room temperature with Marvel fat-free milk powder (5%, w/v), Tween 20 (0.05%, v/v) in PBS. Antigenic bands were detected by exposing the membranes to primary antibodies (Abcam) overnight at 4°C [1:500 dilution for rabbit polyclonal antibodies to 5-HT<sub>3A</sub> and 5-HT<sub>1A</sub> receptors, and mouse monoclonal (ST51-2) to serotonin transporter (SERT); 1:1,000 dilution for rabbit polyclonal antibody to 5-HT<sub>2C</sub> receptor; 1:5,000 for goat polyclonal antibody to aldo-keto reductase (AKR) and rabbit polyclonal to Nicotinic Acetylcholine Receptor (nAChR)] followed by incubation with secondary antibodies [1:10,000, horseradish peroxidase (HRP)-conjugated goat anti-rabbit or HRP-conjugated rabbit anti-goat; 1:1,000, polyclonal goat anti-mouse]. Signals were detected using an enhanced chemiluminescence kit (GE Healthcare) according to manufacturer's instructions and  $\beta$ -actin level was determined as loading control.

## Results

Figure 1 shows the automatic matching between the two-dimensional gel of Caco-2 cells under sulforaphane treatment and the control. Boxes show the spots picking from the gels where significantly different protein expression was found. To validate protein identification analyses, two identically expressed spots were picked from control and sulforaphane-treated gels and successfully identified as Profilin-1 and Chaperonin 10. Characteristics of identified proteins are listed in Table 1 where protein scores



**Figure 2.** Effect of sulforaphane on 5-HT<sub>3A</sub> subunit serotonin receptor. The Caco-2 cells were treated with 5, 10, and 20  $\mu$ mol/L of sulforaphane for 48 h. Whole cell lysates (60  $\mu$ g of protein per lane) were used for Western blot analysis to detect expression of 5-HT<sub>3A</sub> with rabbit polyclonal antibody. The blot shows the down-regulation of 5-HT<sub>3A</sub> subunit serotonin receptor (25 kDa) compared with control sample (*first lane*)  $\beta$ -actin level was determined by stripping and reprobing the membrane.

of  $>61$  are considered statistically significant ( $P < 0.05$ ). Analyses of proteins differentially expressed showed as follows:

1. A spot was down-regulated to undetectable level after sulforaphane treatment. This spot was picked from control gels and identified as serotonin receptor 5-HT<sub>3</sub>. To confirm the two-dimensional gel findings, the effect of sulforaphane was determined by Western blot analysis using the antibody for 5-HT<sub>3A</sub> subunit. As shown in Fig. 2, 5-HT<sub>3A</sub> (25 kDa) decreased in a dose-dependent manner, compared with control cells, after 48 hours of exposure to sulforaphane (5  $\mu$ mol/L).
2. In sulforaphane-treated gels, a higher intensity protein spot was identified as AKR. Western blotting was used to study the effect of sulforaphane on AKR in Caco-2 cells treated with 5, 10, and 20  $\mu$ mol/L sulforaphane for 48 h. Results showed a dose-dependent increase of AKR (35 kDa; Fig. 3), thereby confirming the two-dimensional gel analyses data.
3. In sulforaphane-treated gels, another protein spot identified as D-dopachrome decarboxylase showed a shift in isoelectric point. Using the NetPhos 2.0 Server (Denmark) to analyze the sequence of D-dopachrome decarboxylase, two phosphorylation sites were predicted with high confidence scores at Ser<sup>76</sup> (score, 0.954) and Ser<sup>78</sup> (score, 0.993) that may account for the shift on the two-dimensional gel.

As shown in Fig. 4, the effect of sulforaphane on neurotransmitter receptors was further investigated using Western blot analysis. Serotonin receptors 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, and SERT were analyzed in Caco-2 cells treated with 5, 10, and 20  $\mu$ mol/L sulforaphane for 48 h. Results showed a decrease of these proteins in a dose-dependent manner after sulforaphane treatment. In contrast, Western blot analyses showed a dose-dependent increase of nAChR expression after exposure to sulforaphane (Fig. 4D).

## Discussion

Sulforaphane is a potent cancer preventive agent, but its multiple mechanisms of action are not fully understood. In the present study, we showed for the first time that sulforaphane down-regulates the expression of three serotonin receptors in colon cancer Caco-2 cells. In addition, a decrease of SERT protein expression level was found in Caco-2 cells after treatment with

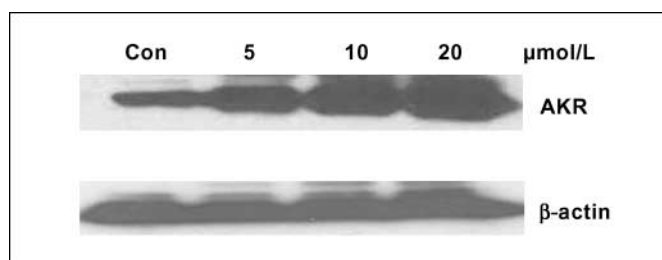
sulforaphane. These results suggested a potential effect on serotonin release. Serotonin is synthesized from the enterochromaffin cells of the gastrointestinal tract to regulate gastrointestinal motility and epithelial function (12–14). Actions and responses between serotonin and a wide number of 5-HT receptors induce contraction of smooth muscle in the colon, secretion of chloride ions and emesis (15, 16), and cellular responses mediated by G proteins (17). Release of serotonin is regulated by complex neuronal and humoral inputs and also by stimulatory receptors (nicotinic acetylcholine,  $\beta$ -adrenergic, and muscarine) and inhibitory receptors ( $\alpha$ -adrenergic,  $\gamma$ -aminobutyric acid, histamine  $H_3$ -receptors, receptors for vasoactive intestinal polypeptide, and somatostatin; ref. 18). To investigate potential interactions of sulforaphane with these stimulatory receptors, we found a dose-dependent up-regulation of the nAChR in Caco-2 cells after sulforaphane treatment. Up-regulation of nAChR may be due to its constant turning off by sulforaphane, as a decrease in neurotransmitters activity is believed to cause up-regulation of receptors (19).

The increased protein level of the stimulatory nAChR may also explain the down-regulation of serotonin reuptake by SERT and, finally, the down-regulation of serotonin receptors in response to signal for serotonin release.

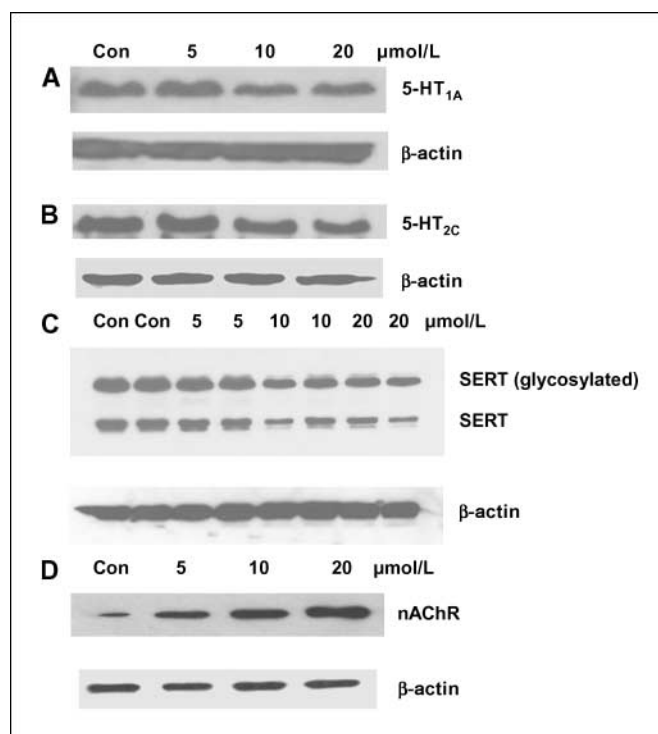
The majority of serotonin receptors are G-protein-coupled receptors (GPCR; ref. 17), exception being the 5-HT<sub>3</sub> receptor, which is a ligand-gated ion channel playing a role in emesis, anxiety, and intestinal smooth-muscle activation (20). GPCR is a large family of cell surface receptors that activate signal transduction pathways and cyclic AMP (cAMP) formation. Studies reported the sequence of events by which a signal molecule activates cAMP signaling. It has been shown that cAMP activates protein kinase A, which in turn phosphorylates Src and subsequently activates the Erk1/2 cascade (21). Activation of Erk1/2 has been associated with the up-regulation of COX-2 activity (22).

Previous studies showed that activation of neurotransmitter receptors has a role in colon cancer cells. Activation of  $\beta$ -adrenoceptors in colon cancer HT-29 cells increased cytosolic phospholipase A<sub>2</sub> protein expression, COX-2 mRNA and protein expression, as well as prostaglandin E<sub>2</sub> receptor (PGE<sub>2</sub>) release (23). Studies have also shown that activation of muscarinic M<sub>3</sub> receptor up-regulated COX-2 and resulted in PGE<sub>2</sub> production (24). In another study, activation of  $\alpha 7$ -nAChR ( $\alpha 7$ -nAChR) by nicotine-stimulated HT-29 cell proliferation and adrenaline production (25).

In agreement with previous findings, proteomics data described here also revealed changes in expression of AKR (26) and D-dopachrome decarboxylase. AKR is abundantly expressed in the gastrointestinal tract and catalyzes the reduction of xenobiotics



**Figure 3.** Effect of sulforaphane on AKR. The Caco-2 cells were treated with DMSO (control; *first lane*) and 5, 10, and 20  $\mu\text{mol/L}$  of sulforaphane for 48 h. Cell lysates (60  $\mu\text{g}$  of protein per lane) were analyzed by Western blot using a specific goat polyclonal antibody against AKR. The same blot was stripped and re probed with antibody against  $\beta$ -actin.



**Figure 4.** Western blot analyses of serotonin receptors 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, nAChR, and SERT in Caco-2 cells treated with sulforaphane. Blots (A–C) show a decrease of protein levels (60  $\mu\text{g}$  of protein per lane) in a dose-dependent manner after exposure to sulforaphane 5, 10, and 20  $\mu\text{mol/L}$  for 48 h. D, 20  $\mu\text{g}$  of protein per lane; dose-dependent increase of nAChR after sulforaphane treatments. Detection of  $\beta$ -actin after membrane stripping has been shown for each blot.

with broad substrate specificity. Interestingly, recent studies reported how members of AKR family efficiently reduced the antiemetic 5-HT<sub>3</sub> receptor antagonist dolasetron (27, 28).

D-dopachrome decarboxylase is involved in the detoxification of the toxic quinone product of dopamine. Chung and colleagues (29) observed a similar effect of  $\beta$ -phenylethyl isothiocyanate on D-dopachrome decarboxylase in a proteomic investigation using human hepatoma HepG2 cells. Recent studies reported that sulforaphane protects against compounds known to induce dopamine quinone production (30). Oxidation of dopamine, which is a precursor of adrenaline, to dopamine quinone causes oxidative stress.

In conclusion, this research suggests that a potential cancer preventive agent sulforaphane regulates neurotransmitter receptors in Caco-2 cells. Understanding these effects of sulforaphane could provide new insights into development of new therapies or therapeutic agents for colon cancer prevention.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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