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Regular paper

Inhibitory effect of selenomethionine on carcinogenesis in the model of human colorectal cancer *in vitro* and its link to the Wnt/β-catenin pathway*

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Selenium compounds have been implicated as anticancer agents; however, the mechanism of their inhibitory action against cancer development has not been extensively investigated. A constitutive activation of the Wnt/β-catenin pathway is a central event in colorectal carcinogenesis. In this pathway, excessive cell proliferation is initiated by generation of β-catenin followed by overexpression of proto-oncogenes, such as c-Myc. It is believed that under physiological conditions the level of c-Myc is efficiently controlled by accessibility of the β-catenin protein through the process of phosphorylation by glycogen synthase kinase 3β (GSK-3β). Here, we determined whether selenomethionine (SeMet) can inhibit cell growth and affect the Wnt/β-catenin pathway in the HT-29 human colorectal cancer cells in vitro. The effective cytotoxic doses of SeMet have been selected after 48 h of incubation of this compound with colorectal cancer HT-29 cell line. MTT assay was used to assess cell viability and the protein and mRNA levels of β-catenin and c-Myc were determined by Western blotting and qPCR, respectively. SeMet potently inhibited growth of HT-29 cells, significantly decreased level of the β-catenin protein and mRNA concentration, down-regulated the c-Myc gene expression and up-regulated the pro-apoptotic Bax protein level. Moreover, SeMet increased the level of GSK-3β phosphorylated at serine 9 (S9) and significantly increased the level of β-catenin phosphorylated at S33 and S37. We conclude that SeMet suppresses growth of HT-29 colorectal cancer cells by a mechanism linked to the Wnt/β-catenin pathway, however, degradation of β-catenin may occur independently of GSK-3β catalytic activity and its phosphorylation status.

Key words: colorectal cancer, GSK-3β, Wnt/β-catenin pathway, selenium, selenomethionine

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Abbreviations: APC, adenomatosis polyposis coli protein; APCmin, a mutation in APC causing MIN - multiple intestinal neoplasia; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2 protein; EC9706, oesophageal squamous cell carcinoma, line 9706; GPx, glutathione peroxidase; GSK-3β, glycogen synthase kinase 3β; HaCaT, spontaneously transformed (immortalized), patient-derived, normal human keratinocyte cell line; IC, inhibitory concentration; KYse150: squamous cell oesophageal carcinoma, the KYse series from Kyoto, line 150; LRP5/6, low density lipoprotein receptor-related protein 5/6; MSA, methylseleninic acid; NSAIDs, non-steroidal anti-inflammatory drugs; p-XSC, 1,4-phenylenebis(methylene) selenocyanate; SeMet, selenomethionine; TCF/LEF, T-cell factor/lymphoid enhancer-binding factor; β-TrCP, β-transducin repeats-containing protein; Wnt, Wingless + Integration-1 (Wg + INT-1)

INTRODUCTION

Over the last few decades convincing evidence has been accumulated indicating that selenium compounds are good candidates for cancer treatment and chemoprevention. However, despite promising results, there is still little information available on the molecular genetics background underlying anti-tumorigenic effects of these compounds. The cellular responses induced by selenium in experimental models are very diverse and may depend on different forms and doses of this microelement (Brozmanova *et al.*, 2010). Major dietary selenocompounds are the following amino acids: selenomethionine (SeMet), selenocysteine and Se-methylselenocysteine (Combs *et al.*, 1998; Rayman, 2000; Brozmanova *et al.*, 2010; Korbut *et al.*, 2012). For instance, SeMet has been shown in numerous cancer models to inhibit neoplastic cell growth (Nelson *et al.*, 2005; Goulet *et al.*, 2007). It has been also reported that SeMet is less toxic than other selenium compounds, making it the selenium derivative form of choice for human interventional trials (Redman *et al.*, 1997). Moreover, unlike selenite and selenocysteine, SeMet failed to exhibit glutathione peroxidase (GPx) activity known to protect the cells from oxidative damage. This clearly suggests that cytotoxic activity of SeMet involves a different mechanism, in part unrelated to antioxidative activity of this compound (Beutler *et al.*, 1975). This was also confirmed by meta-analysis in an animal model which revealed that selenium-enriched diet showed greater potency in increasing GPx activity than SeMet (Bermingham *et al.*, 2014).

One of the most attractive targets for anti-cancer therapy seems to be the Wnt/β-catenin pathway. An abnormal activation of this signalling pathway has already been described in a wide variety of human cancers (Kikuchi, 2003; Anastas *et al.*, 2013; Han *et al.*, 2013). Interestingly, over 90% of colorectal cancers have demonstrated abnormal regulation of the Wnt/β-catenin signalling (Fevr *et al.*, 2007; Polakis, 2012).

The level of β-catenin, the central signalling protein of the Wnt/β-catenin pathway, is regulated by the activity of a destruction complex that is composed of the Axin scaffolding protein, the adenomatosis polyposis coli protein (APC), casein kinase 1 (CK1), and the glycogen synthetase kinase 3β (GSK-3β). In the absence of Wnt signalling, GSK-3β sequentially phosphorylates β-catenin at S33 and S37, resulting in β-catenin recognition by β-TrCP, an E3 ubiquitin ligase subunit, and subsequent β-catenin ubiquitination and proteasomal degradation (He *et al.*, 2004) (Fig. 1, left panel). In turn, in the presence of Wnt signalling, β-catenin accumulates in the cytoplasm and then it can translocate into the nu-

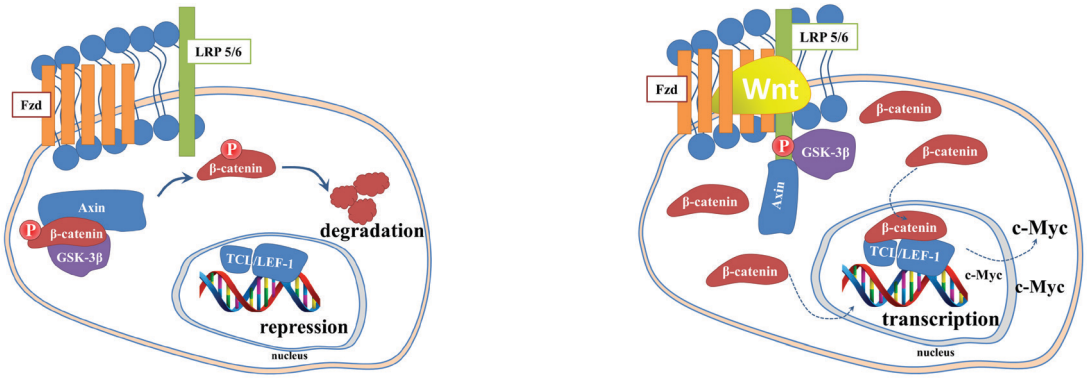

Figure 1. Schematic diagram showing a regulatory model of Wnt/β-catenin signalling.
Left panel: in the absence of Wnt, constitutive activation of GSK-3β leads to β-catenin phosphorylation and subsequent proteasomal degradation. **Right panel**: in the presence of Wnt, GSK-3β is inactive (phosphorylated), β-catenin then enters the nucleus and affects gene transcription involved in regulation of cell cycle progression, proliferation and apoptosis. Notes: Solid and dashed arrows represent causal processes in the Wnt/β-catenin pathway.

cleus. The nuclear β-catenin interacts with transcription factors – TCF/LEF, enhancing expression of c-Myc and other genes. The activity of GSK-3β is silenced by phosphorylation at S9. This phosphorylation induces a pseudo-substrate conformation in the substrate docking motifs of GSK-3β that acts as a competitive inhibitor for true substrates (Doble, 2003; McCubrey *et al*., 2014). These events play a pivotal role in the control of cell proliferation, differentiation and cell apoptosis (Clevers, 2006; Pecina-Slaus, 2010; Miller *et al*., 2012) (Fig. 1, right panel). Moreover, elevated β-catenin mRNA levels which correlate with constitutively high amounts or nuclear localization of β-catenin, were found in several tumors, including colorectal carcinomas (Bandapalli *et al*., 2009).

The link between the Wnt signalling and apoptosis has become increasingly established in the literature (Pecina-Slaus, 2010). The Bcl-2 and Bax family of proteins play an important role in the regulation of the programmed cell death known as apoptosis. While Bcl-2 promotes cell survival, the structurally similar Bax induces programmed cell death by permeabilization of the outer mitochondrial membrane. Impairment of apoptosis is generally accepted as the central event in the mechanism of cancer development and seems to be responsible for tumour resistance to cytotoxic therapeutics (Adams *et al*., 2007).

In view of the above, we designed our study to determine the effect of SeMet on colorectal cancer cell proliferation, *and* Bax-mediated apoptosis, and we examined whether Wnt/β-catenin is involved in these effects of SeMet on cancer cells *in vitro*.

MATERIALS AND METHODS

Cell lines. Human epithelial colorectal adenocarcinoma cell line (HT-29) was purchased from European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK). HT-29 cell line was maintained in RPMI 1640 medium (with L-glutamine and sodium bicarbonate) from Sigma Aldrich (St. Louis, MO, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA). Human keratinocyte cell line (HaCaT) was purchased from CLS Cell Lines Service GmbH (Heidelberg, Germany). HaCaT cell line was maintained in high glucose DMEM medium (with 2 mM L-glutamine) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) FBS (Sigma-Aldrich, St. Louis, MO, USA). Both cell lines were cultured in the presence of 1% (v/v) antibiotics/antimycotics solution (10 000 units of penicillin, 10 mg streptomycin, and 25 µg of amphotericin B per mL) from Sigma-Aldrich (St. Louis, MO, USA), in a humidified incubator with 5% CO_2 at 37°C. Subculturing was done at subconfluent densities with a solution of 0.25% Trypsin-EDTA solution (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) for 2 minutes at 37°C.

Tetrazolium-based growth assay (MTT). The growth inhibitory effect of SeMet on cellular viability was evaluated by the MTT colorimetric method using thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA). HT-29 cells were plated in 5 replicates in 96-well plates at a density of 1×10^4 cells/well in a final volume of 100 µl medium. After overnight incubation at 37°C, 5% CO_2, dilutions of SeMet were added in 5 replicates per drug concentration. Untreated cells (appropriate volumes of medium added) served as controls. After 48 h incubation, 50 µl of the MTT solution was added to each well and incubated for 4 h at 37°C. Medium was removed and the formazan product of MTT reduction was dissolved in 75 µl of DMSO per well. The optical density was measured at 550 nm. Each assay was repeated three times. Nonlinear regression graph was plotted between percentage of cell inhibition and $\log_{10}$ concentration, and IC_{50} was determined using the GraphPad Prism software (Version 5.01, GraphPad Software, Inc., San Diego, CA, USA).

Selenomethionine treatment. SeMet (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in medium to a stock concentration of 10 mM. At 48 h before the SeMet treatment, exponentially growing human colon cancer cells were seeded at a density of 1×10^5 cell/well. 24 h before the experiment, supplemented medium was replaced with a serum-free medium. SeMet concentration range and incubation time used in the study presented here were based on the results of a pilot studies. Control cells were treated with an equivalent volume of a serum free medium only.

Gene expression. Total RNA was isolated from cell samples using the SV Total Isolation Kit (Promega Fitchburg, Wisconsin, USA), according to the manufacturer's instructions. The RNA concentration of each sample was measured using the NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The purity of extracted total RNA was determined by the A260/A280 ratio. cDNA was synthesized by reverse

transcription of 500 ng of total RNA from each sample, using MMLV First-Strand Synthesis Kit (GeneDireX, Las Vegas City, NV, USA) according to the manufacturer's protocol. The reaction was performed in a T3 Thermocycler (Biometra, Göttingen, Germany). Relative gene expression was determined by qPCR according to the MIQE guidelines. The PCR primers and TaqMan probes for HPRT (a housekeeping gene, assay ID Hs99999909_m1), c-Myc (assay ID Hs00153408_m1) and β-catenin (assay ID Hs00355049_m1) were purchased from Life Technologies/Thermo Fisher Scientific (Waltham, MA, USA). The qPCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Temperature cycling and real-time fluorescence measurements were done by using Rotor-Gene RG-3000 Corbett Research thermal cycler (Qiagen, Hilden, Germany). The relative quantitation of gene expression was done by using the $2^{-\Delta\Delta CT}$ method. Each qPCR experiment was done in quadruplicate (two duplicates from two different reverse transcription reactions), and the mean C_T value was used for data analysis.

Western Blotting Analysis. Whole cell lysates were used in Western blotting analysis. Protein content was determined using the Bradford assay with an Elx808 (BioTek, Winooski, VT, USA) microtiter plate reader. Proteins were separated on NuPage Novex 10% Bis-Tris polyacrylamide electrophoresis gels (Invitrogen Ltd, Paisley, UK). After electrophoresis, proteins were transferred from gels onto nitrocellulose membranes by using an iBlot Dry Blotting System (Invitrogen Ltd, Paisley, UK). The primary antibodies used for detection were anti-β-catenin rabbit polyclonal antibody (1:4000), anti-β-catenin (phospho S33+S37) rabbit polyclonal antibody (1:1000), anti-GSK-3β (phospho S9) rabbit polyclonal antibody (1:400), and anti-Bax rabbit polyclonal antibody (1:1000). Secondary antibodies were goat polyclonal antibodies to rabbit IgG H&L (HRP). As a control for protein loading, an anti-β actin rabbit polyclonal antibody (1:5000 dilution) was used. All antibodies were obtained from Abcam (Cambridge, UK). Signals for protein bands were visualized by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol, and exposed to X-ray film (KODAK, Rochester, MN, USA). The density of each specific band was measured using a computer-assisted imaging analysis system.

Statistical Analysis. Results are presented as the mean values ± S.D. Statistical differences of data for two groups were compared by unpaired Student's *t*-test. Relative expression levels from qPCR were logarithmically transformed prior to statistical analysis for normalization. For all statistical analyses, the level of significance was set as $P<0.05$. The IC_{50} values were calculated using the GraphPad Prism® 5 software (Version 5.01, GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

The effect of SeMet administration on HT-29 and HaCaT cell viability

We investigated the effect of SeMet on the viability of HT-29 colon cancer cells and normal human keratinocytes HaCaT, and selected SeMet cytotoxic/antiproliferative concentrations for further experiments. The HaCaT cell line was used in order to get more insights as to whether SeMet can affect the cancerogenic HT-29 and non-cancerogenic (HaCaT) cell lines. The cell lines were exposed to SeMet for 48 h and MTT growth assay was performed. SeMet sensitivity values were calculated in terms of 50% inhibitory concentration (IC_{50}). We observed that SeMet causes a concentration-dependent inhibition of HT-29 growth, with an IC_{50} value of 283 μM (n=3) after 48 h of incubation with this derivative of selenium containing amino acid (Fig. 2). In contrast, IC_{50} for SeMet in HaCaT cells was 630 μM (Fig. 3).

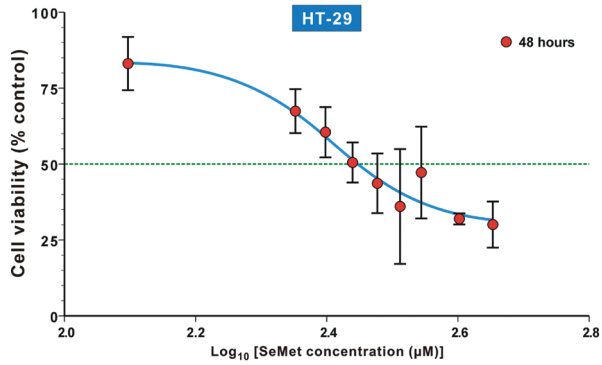

Figure 2. Determination of IC_{50} values of SeMet on HT-29 cells. HT-29 cells were treated for 48 h with SeMet at concentrations ranging from 0 to 450 μM. MTT assay was then used to determine cell viability (%). IC_{50} values were determined after plotting the absorbance values *vs.* various drug concentrations using GraphPad Prism program. The graph was then fitted with a non-linear regression and a sigmoidal dose-response curve to obtain the IC_{50} values. Data represent the means ± S.D. (n=3).

SeMet decreases expression and protein levels of β-catenin

To ascertain if β-catenin is a critical factor in the anti-carcinogenic effect of SeMet, we analysed the level of this protein by Western blotting in the HT-29 cell line after 48 h of incubation of these cells with SeMet at cytotoxic doses (IC_{25}, IC_{50}). The β-actin was used to normalize the changes in the β-catenin signal. As shown in Fig. 4, incubation with SeMet at the dose of IC_{25} tended to decrease the level of β-catenin protein but this change was not significant. However, when the IC_{50} dose of Se-

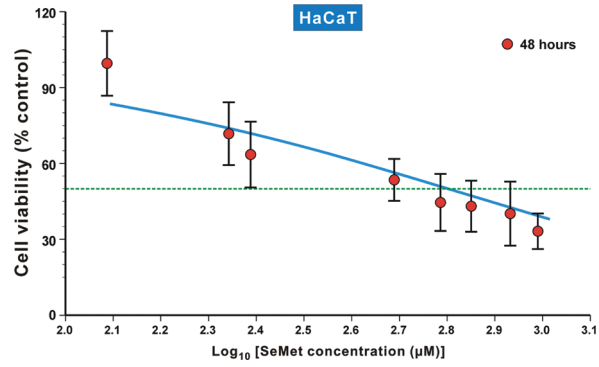

Figure 3. Determination of IC_{50} values of SeMet on HaCaT cells. HaCaT cells were treated for 48 h with SeMet at concentrations ranging from 120 to 1000 μM. MTT assay was then used to determine cell viability (%). IC_{50} values were determined after plotting the absorbance values *vs.* various drug concentrations using GraphPad Prism program. The graph was then fitted with a non-linear regression and a sigmoidal dose-response curve to obtain the IC_{50} values. Data represent the means ± S.D. (n=3).

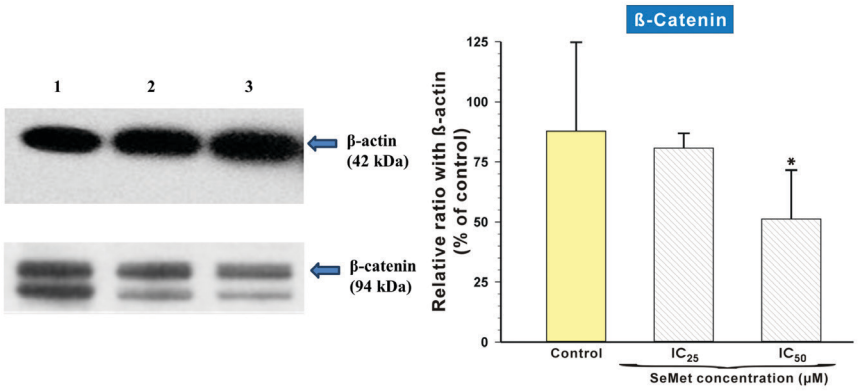

Figure 4. Effect of SeMet stimulation on β-catenin protein level.
HT-29 cells were incubated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h. **Left panel** shows a representative Western blot from SeMet treated and untreated (control) HT-29 cell line. **Right panel** shows the semi-quantitative densitometry analysis of the Western blots from HT-29 incubated with SeMet for 48 h. As shown, there is a significant decrease in β-catenin protein level following SeMet treatment with respect to the untreated control. Data are shown as the mean ± S.D. and are representative of three independent experiments. *$P < 0.05$ determined by Student's *t*-test.

Met was applied, a marked decrease (approx. 2 fold *vs* control) in β-catenin protein was observed. Similar results with SeMet were obtained at mRNA level as assessed by qPCR. As shown in Fig. 5, the relative gene expression of β-catenin significantly decreased in the SeMet-stimulated HT-29 cells when compared to untreated control cells.

SeMet ihnibits c-Myc gene expression

To determine whether SeMet can influence c-Myc gene expression, HT-29 cell line was incubated with SeMet at cytotoxic concentrations (IC_{25}, IC_{50}) and the mRNA level was investigated by qPCR. As shown in Fig. 6, the relative gene expression of c-Myc was significantly decreased (approx. 12 fold for IC_{50} *vs* control) in the SeMet stimulated HT-29 cells when compared with the untreated control. These data suggest that SeMet can inhibit the growth of HT-29 cells by inhibiting c-Myc gene expression.

Inhibition of β-catenin is due to its enhanced degradation and does not depend on GSK-3β S9 phosphorylation status

On the basis of our previous observations we found that β-catenin is down-regulated by SeMet within 48 h in colorectal cancer cell line which can result from an increased degradation of β-catenin. We observed that at cytotoxic concentrations, SeMet had increased β-catenin phosphorylation at the S33 and S37 residues (approx. 2 folds *vs.* control at IC_{50}; Fig. 7), and subsequently resulting in β-catenin proteasomal degradation. To determine the mechanism of β-catenin phosphorylation by SeMet, involvement of GSK-3β was evaluated. Treatments with SeMet significantly increased the level of phosphorylated GSK-3β in HT-29 cells (approx. by 2 folds and 6 folds *vs.* control for IC_{25} and IC50, respectively) (Fig. 8). These results indicate that SeMet caused an increase in β-catenin phosphorylation in the colorectal cancer cells and this effect appears to be independent of GSK-3β phosphorylation at S9.

SeMet increases pro-apoptotic Bax protein level

The level of Bax protein with or without incubation with SeMet was examined in order to determine the potential pro-apoptotic activity of this seleno-derivative of

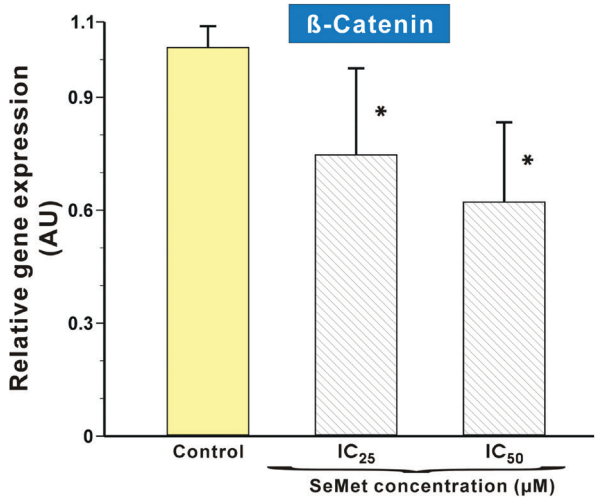

Figure 5. Effect of SeMet stimulation on β-catenin gene expression.
HT-29 cells were incubated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h and mRNA was analysed by qPCR. SeMet significantly decreased β-catenin gene expression in HT-29 cells with respect to the untreated control. Experiments were performed in quadruplicates and quantified using HPRT as a reference gene. Data are shown as the mean ± S.D. and are representative of three independent experiments. *$P < 0.05$ determined by Student's *t*-test.

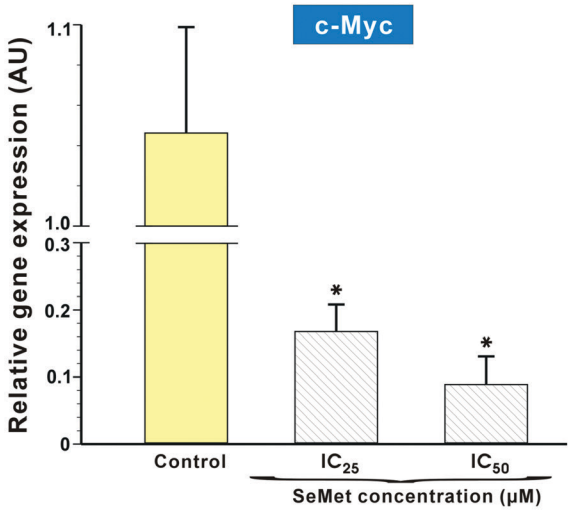

Figure 6. Effect of SeMet stimulation on c-Myc gene expression.
HT-29 cells were incubated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h and mRNA was analysed by qPCR. SeMet significantly decreased c-Myc gene expression in HT-29 cells with respect to the untreated control. Experiments were performed in quadruplicates and quantified using HPRT as a reference gene. Data are shown as the mean ± S.D. and are representative of three independent experiments. *$P < 0.05$ determined by Student's *t*-test.

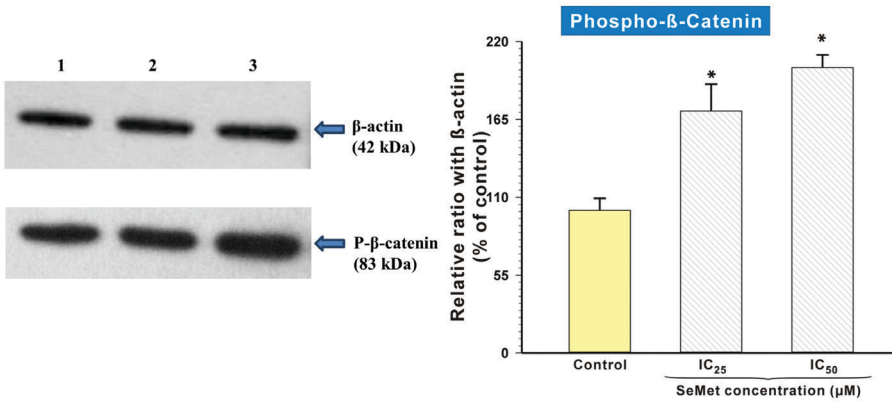

Figure 7. Effect of SeMet stimulation on phospho-β-catenin protein level.
HT-29 cells were incubated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h. **Left panel** shows a representative Western blot from SeMet treated and untreated (control) HT-29 cell line. **Right panel** shows the semi-quantitative densitometry analysis of the Western blots from HT-29 incubated with SeMet for 48 h. As shown, there is a significant increase in phopsho-β-catenin protein level following SeMet treatment with respect to the untreated controls. Data are shown as the mean ±S.D. and are representative of three independent experiments. *$P < 0.05$ determined by Student's *t*-test.

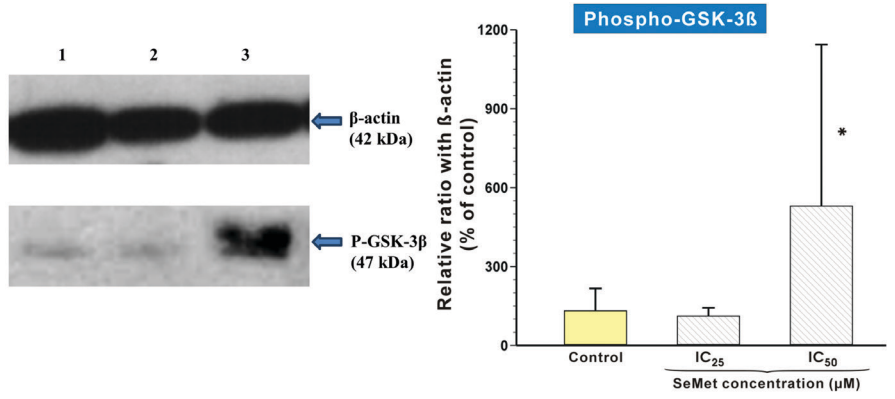

Figure 8. Effect of SeMet stimulation on phospho-GSK-3β protein level.
HT-29 cells were incubated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h. **Left panel** shows a representative Western blot from SeMet treated and untreated (control) HT-29 cell line. **Right panel** shows the semi-quantitive densitometry analysis of the Western blots from HT-29 incubated with SeMet for 48 h. As shown, there is a significant increase in phospho-GSK-3β protein level following SeMet treatment with *respect* to the untreated controls. Data are shown as the mean ±S.D. and are representative of three independent experiments. *$P < 0.05$ determined by Student's *t*-test.

methionine. As shown in Fig. 9, SeMet up-regulated pro-apoptotic Bax protein level in HT-29 cells.

DISCUSSION

Selenium is an essential trace element in mammals and is usually ingested with food or dietary supplementation either in an organic or an inorganic form (Rayman, 2000; Carroll *et al.*, 2015). Although the effect of anti-cancer action of selenium still remains unexplored, several mechanisms, such as antioxidant protection, inhibition of cancer cell growth, modulation of cell cycle and an increase in apoptosis, have been proposed to explain its anti-neoplastic activities (Brozmanova *et al.*, 2010). Recent studies suggest that at concentrations higher than nutritional requirements, selenium exerts anti-cancer potential and can be used not only for cancer prevention but also for treatment of cancer growth (Brozmanova *et al.*, 2010).

The organic selenium compounds, such as SeMet, are predominant forms of selenium which are considered as more bioavailable than its inorganic forms (Menter *et al.*, 2000).

That is why our major goal was to determine the effect of SeMet on colon cancer cells and to elucidate if Wnt/β-catenin pathway could be a molecular target for this agent. To the best of our knowledge, no data have been previously reported on the role of SeMet on Wnt/β-catenin pathway in the HT-29 colon cancer cells. According to the recent evidence, reduced β-catenin expression is involved in induction of cell death by various drugs and nutritional factors, including non-steroidal anti-inflammatory drugs (NSAIDs) and butyrate (Smith *et al.*, 2000; Bordonaro *et al.*, 2002; Emanuele *et al.*, 2004). Rao *et al.* reported that a synthetic organoselenium compound, p-XSC, significantly suppresses β-catenin expression in intestinal polyps in the APC^{min} mouse (Rao *et al.*, 2000). In another study, p-XSC also decreased protein levels of β-catenin in colon cancer cells (Narayanan *et al.*, 2004).

Here, we provide direct evidence that selenium, in the form of SeMet, exerts anti-proliferative activity against the HT-29 cell line. The mean IC_{50} value for HT-29 cells was 283 μM at exposure durations of 48 h. Similar results were reported by Redman *et al.*, who found that SeMet inhibits growth of human tumour cell lines at concentrations within the micromolar (μM) range and in a dose-dependent manner (Redman *et al.*, 1998). They also demonstrated that the sensitivities of tumour and normal cells to SeMet were highly diverse. This is consistent with our findings. We observed that the cytotoxic dose (IC_{50}) of SeMet for normal human keratinocyte (HaCaT) cell line was approx. 2 folds higher than for the tumour HT-29 cells ($IC_{50} = 630$ μM).

Furthermore, we found that administration of SeMet at effective anti-proliferative doses had significantly decreased β-catenin level in the HT-29 cell lysates. Moreover, this effect was accompanied by down-regulation of c-Myc mRNA. Similarly, Zeng *et al.* reported that submicromolar concentrations of methylselenol, generated by incubating methionase with SeMet, inhibited c-Myc expression in the HT1080 fibrosarcoma cells (Zeng *et al.*, 2009). In turn, studies conducted by Zhang *et al.* revealed that c-Myc mRNA and protein levels were significantly decreased upon methylseleninic acid (MSA) treatment in human oesophageal squamous cell carcinoma cell lines

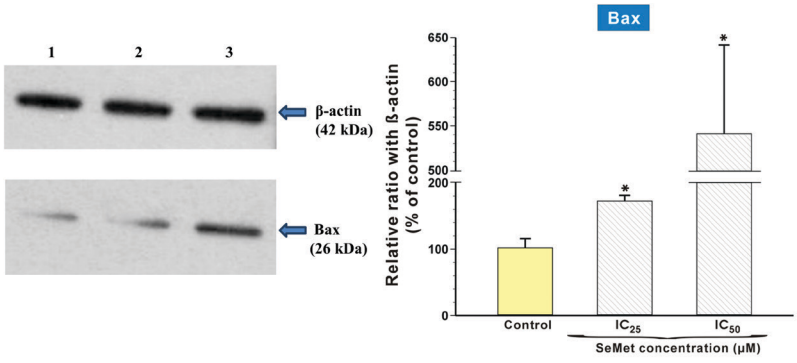

Figure 9. Effect of SeMet stimulation on Bax protein level.
HT-29 cells were treated with cytotoxic doses (IC_{25}, IC_{50}) of SeMet for 48 h. **Left panel** shows a representative Western blot from SeMet treated and untreated (control) HT-29 cell line. **Right panel** shows the semi-quantitative densitometry analysis of the Western blots from HT-29 incubated with SeMet for 48 h. As shown, there is a significant increase in Bax protein level following SeMet treatment with respect to the untreated controls. Data are shown as the mean ± S.D. and are representative of two independent experiments. *$P < 0.05$ determined by Student's *t*-test.

EC9706 and KYse150 (Zhang *et al.*, 2010). Likewise, MSA reduced β-catenin protein levels, indicating that apoptosis and growth inhibition induced by MSA could be modulated through β-catenin-TCF/LEF pathway (Zhang *et al.*, 2010). This is compatible with our observation. SeMet-induced β-catenin down-regulation in the HT-29 colon cancer cells was followed by down-regulation of c-Myc, reflecting a launch of a causal relationship between β-catenin and c-Myc, which finally leads to inhibition of cancer cell growth.

To determine whether the decrease in β-catenin protein level resulted from an increase in its degradation, the phosphorylation of β-catenin and GSK-3β was evaluated in the presence and absences of SeMet. The β-catenin protein level is normally kept low by its phosphorylation at S33 and S37, mediated by GSK-3β, which results in the β-catenin targeting for proteasomal degradation. The presence of Wnt blocks phosphorylation, thereby allowing β-catenin to accumulate and translocate into the nucleus, where it co-operates with several transcription factors (Gao *et al.*, 2014). Our data indicate that SeMet caused an increase in β-catenin phosphorylation (at S33 and S37 residues, as commonly recognized), thus, showing that cytotoxic doses of SeMet can promote β-catenin degradation. Moreover, we found a significant reduction in β-catenin mRNA after SeMet treatment. However, mechanisms underlying this effect remain unclear to us. Bandapalli *et al.* provided several lines of evidence that β-catenin can induce transcription from its own promoter, thereby up-regulating its expression. Thus, they considered β-catenin as a positive feedback activator for Wnt/β-catenin signalling (Bandapalli *et al.*, 2009). Taking this notion into consideration, it is possible that SeMet reduces β-catenin gene expression and this effect is responsible for β-catenin-protein down-regulation observed in our study. On the other hand, according to Ebert *et al.*, regulation of β-catenin transcription through β-catenin itself seems unlikely, since the β-catenin promoter does not contain a TCF-responsive element. The above, however, does not change the fact that β-catenin transcription might be regulated by other transcription factors which are triggered by the β-catenin-TCF/LEF complex (Ebert *et al.*, 2002).

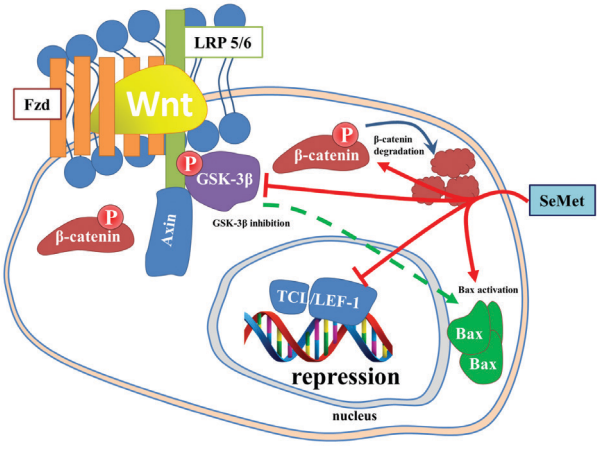

Figure 10. The effect of SeMet on the Wnt/β-catenin pathway.
We hypothesize that SeMet promotes degradation of β-catenin and suppresses its translocation into the nucleus, thereby inhibiting c-Myc gene expression. SeMet also possesses a GSK-3β-inhibitory and pro-apoptotic activity as reflected by an increased level of the Bax protein. Notes: Solid arrows: degradation of β-catenin/Bax activation; dashed arrow: possible Bax activation *via* S9 phosphorylated GSK-3β; blunt-ended arrow: inhibition of GSK-3β activity/inhibition of c-Myc gene expression.

It has been clearly established that phosphorylation at S9 correlates with inhibition of the GSK-3β activity (McCubrey *et al.*, 2014). However, involvement of GSK-3β phosphorylation in Wnt signalling remains unexplored and has not been carefully studied thus far (Gao *et al.*, 2014). Our present findings provide an alternative to the widely accepted molecular mechanism for regulation of cytoplasmic β-catenin by activation of β-catenin degradation pathway. Interestingly, we found that β-catenin protein down-regulation was accompanied by simultaneous phosphorylation of GSK-3β at S9 upon SeMet treatment. This remains at odds with the observation of Safio *et al.* who reported that administration of methyl selenic acid (MSeA) had no significant effect on the level of total GSK-3β (in HT-29 or HCT-8) and failed to alter the level of phosphorylated GSK-3β in HCT-8. In contrast, GSK-3β phosphorylated at S9 was significantly decreased in HT-29 cells (Saifo *et al.*, 2010). On the other hand, Liu *et al.* have found that the Siah-1 protein, a p53-inducible mediator of cell cycle arrest, tumour suppression, and programmed cell death, interacts with the carboxyl terminus of APC and promotes degradation of β-catenin in mammalian cells that is independent of GSK3β-mediated phosphorylation (Liu *et al.*, 2001). In turn, Dihlmann *et al.* demonstrated that NSAIDs cause an increase in the level of phosphorylated form of β-catenin (S33 + S37 + T41) in colorectal and colon cancer cell cultures, and this alteration was mediated by simultaneous increase in GSK-3β phosphorylation. However, no changes in GSK-3β phosphorylation level were observed in normal embryonic kidney cells (HEK 293) (Dihlmann *et al.*, 2003). Their results, demonstrating that aspirin can elevate GSK-3β phosphorylation at S9 in colon cancer cells, suggest that NSAID might stabilize selected S/T-phosphorylation by inhibiting a phosphatase known to revert proteins into their unphos-

phorylated forms (Dihlmann *et al.*, 2003). Thus, it cannot be excluded that in our studies SeMet triggered the Wnt/β-catenin in a similar manner as previously described, and SeMet might indirectly inactivate specific phosphatases targeting β-catenin and GSK-3β. However, the proposed mechanism remains to be elucidated in further studies.

The GSK-3β plays pivotal roles in cancer development which remain complex and are still controversial (McCubrey *et al.*, 2014). GSK-3β is overexpressed in many tumour types including: colon, liver, ovarian and pancreatic tumours (McCubrey *et al.*, 2014). Besides promoting β-catenin proteasome degradation, it can phosphorylate and stabilize Wnt-coreceptor LRP5/6 which fosters β-catenin signalling. Consecutively, inhibitors of this kinase could arrest β-catenin-mediated gene expression and may eventually be used in treatment of certain cancers (McCubrey *et al.*, 2014). Zhou *et al.* demonstrated that suppression of GSK-3β inhibited pancreatic cancer growth and angiogenesis (Zhou *et al.*, 2012). Similarly, treatment of leukaemia cell lines with a GSK-3β inhibitor resulted in GSK-3β S9 phosphorylation, β-catenin stabilization, cyclin B down-regulation, inhibition of cell growth, cell cycle arrest at G_2/M, and promotion of apoptotic cell death (Mirlashari *et al.*, 2012). Interestingly, according to Tan *et al.,* GSK-3β inhibition promotes p53-dependent apoptosis through a Bax-mediated mitochondrial pathway. They noticed that a modulation of GSK-3β by inhibitors (LY2119301 and lithium) was able to convert the p53 response from growth arrest to apoptosis. In their opinion, increased S9 phosphorylation of GSK-3β by LY2119301 or lithium could displace Bax, promoting its activation and cell death (Tan *et al.*, 2005). Therefore, in agreement with these studies, we provided evidence that SeMet was able to up-regulate pro-apoptotic Bax, suggesting that this effect can be mediated by an increased S9 phosphorylation of GSK-3β.

In summary, we hypothesize that GSK-3β phosphorylation at S9 may not be the limiting component for Wnt-signalling in colon cancer cells. Even more, GSK-3β inhibition, resulting from SeMet treatment, may function as a tumour suppressor and can promote apoptotic cell death.

CONCLUSIONS

Our studies revealed that SeMet inhibits growth of the HT-29 colon cancer cell line by targeting the Wnt/β-catenin pathway and this is due, at least in part, to an increase in the Bax-mediated apoptosis. However, the latter requires confirmation in further studies conducted under *in vitro* and *in vivo* conditions. We conclude that β-catenin and GSK-3β appear to act as key targets for SeMet in colorectal cancer cells, leading to down-regulation of c-Myc gene expression. Moreover, SeMet-induced β-catenin degradation seems to occur independently of the status of GSK-3β catalytic activity and S9 phosphorylation (Fig. 10).

Conflicts of interest

The authors declare no conflict of interest.

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