Guggulsterone Suppresses Bile Acid-induced and Constitutive Caudal-related Homeobox 2 Expression in Gut-derived Adenocarcinoma Cells

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Abstract. *Background: Guggulsterone, a plant polyphenol guggulipid, has several antitumour effects and acts as an antagonist for the farnesoid X receptor. Although bile acids induce caudal-related homeobox 2 (CdX2), a transcription factor essential for intestinal development and gut tumourigenesis, the effects of guggulsterone on regulation of CdX2 in the gut are unknown. Materials and Methods: Regulation of CdX2 expression by treatment with bile acids and/or guggulsterone was analysed by immunoblot analysis in human gut-derived adenocarcinoma, Bic-1 cells. Nuclear factor-κB (NF-κB) activity and the cell cycle distribution were also examined. Results: Chenodeoxycholic acid and deoxycholic acid increased CdX2 expression in Bic-1 cells. Guggulsterone reduced bile acid-induced and constitutive CdX2 expression at 5 μM. Guggulsterone (up to 5 μM) did not affect cell viability or the cell cycle and did not attenuate bile acid-induced or constitutive NF-κB activation. Conclusion: Guggulsterone may be used as a novel drug to target CdX2 expression in certain gut adenocarcinomas.*

Guggulsterone is a plant polyphenol traditionally used to treat obesity, diabetes, hyperlipidemia, atherosclerosis and osteoarthritis (1-3). Recent studies have also shown that it is an antagonist of the bile acid receptor, farnesoid X receptor (FXR; nuclear receptor subfamily 1, group H, member 4) (4), and modulates expression of proteins by inducing apoptosis (5-7), inhibition of cell proliferation (8) and angiogenesis (9) in various tumour cells. Guggulsterone also mediates gene expression through regulation of various transcription factors, including nuclear factor-κB (NF-κB) (10, 11), signal transducer and activator of transcription-3, and CCAAT/enhancer-binding protein alpha. Thus, guggulsterone may be a candidate chemotherapeutic agent for suppressing tumour initiation, promotion and metastasis (1). However, few reports have examined the effects of this attractive agent on gut carcinogenesis.

Caudal-related homeobox 2 (CdX2) is a transcription factor essential for intestinal development (12, 13) and has been suggested to play a crucial role in the pathogenesis of gut metaplasia (*e.g.* in oesophagus, stomach and bile duct) and the development of cancer (*e.g.* colon cancer and leukaemia) (14-18). Previous studies have revealed that CdX2 is induced by bile acids and several inflammatory conditions of the gut (19). Furthermore, it has been reported that bile acids activate CdX2 expression through NF-κB (20, 21). Recent reports suggested that CdX2 may be a chemopreventive target in cancer development (22, 23). Since guggulsterone has been shown to bind to FXR (4) and/or modulate NF-κB activation (10), its effect on the regulation of CdX2 may demonstrate its potential as an anticancer agent and a chemopreventive agent for gut carcinogenesis.

The purpose of this study was to investigate whether guggulsterone may be used as a novel drug to target CdX2 expression in the gut. The aims of the study were to examine whether guggulsterone had any effect in regulating CdX2 expression with or without bile acids in gut-derived adenocarcinoma cells and to compare any such effect with its effects on apoptosis, cell survival, and NF-κB activation.

Materials and Methods

Materials. Cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), guggulsterone, and anti-β-actin antibody were obtained from Sigma (St.

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Louis, MO, USA). Foetal bovine serum (FBS) was obtained from Invitrogen (Grand Island, NY, USA). Enhanced chemiluminescence (ECL) reagent for immunoblots was obtained from GE Healthcare (Slough, UK). CdX2 monoclonal antibody was obtained from BioGenex Laboratories (San Ramon, CA, USA) and anti-FXR antibody was obtained from Aviva Systems Biology (San Diego, CA, USA).

Cell line and culture. The human gut-derived adenocarcinoma cell line Bic-1 was kindly provided by Dr. David Beer (University of Michigan, Ann Arbor, MI, USA). The cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin at 37˚C in a humidified incubator containing 5% carbon dioxide.

Immunoblot analysis. Cells were harvested by scraping and washed twice with phosphate-buffered saline (PBS) before incubation on ice for 60 min in lysis buffer [0.3% Nonidet P-40, 25 mM NaF, 150 mM NaCl, 2 mM ethylene glycol *bis* (β-aminoethlether)-*N, N, N', N'*-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetate (EDTA), 0.2% Triton X-100, 50 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 100 μM leupeptin, 2 mM 4(-2-aminoethyl)benzenesulfonyl fluoride hydrochloride]. The cells were then centrifuged at 13,000 rpm for 15 min at 4˚C. The supernatant was evaluated for protein concentration using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein was boiled with sodium dodecyl sulfate (SDS) sample buffer (Tris-glycine SDS sample buffer, 2X; Invitrogen). Equal concentrations of protein were subjected to electrophoresis on a 4-20% Tris-glycine precast gel (Invitrogen). The separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membrane was blocked with TBST [10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.1% Tween 20] containing 5% fat-free milk for 60 min before incubation with anti-CdX2 antibody (1/100 dilution) or anti-FXR antibody (1/1000 dilution) overnight. The blots were washed in TBST and then incubated with horseradish peroxidase-conjugated anti-mouse antibody (1/1000 dilution) and visualised by ECL. The relative intensities of unsaturated signals were quantified by densitometry, using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The intensities of the CdX2 protein bands were standardised to those of the corresponding β-actin protein bands.

3-(4,5-Dimethylthiozol-2-yl)2,5-diphenylethylchloromethyl ketone (MTT) assay. Cells were seeded in 96-well microplates at 1000 cells/100 μl per well and incubated for 24 h. They were treated with DMEM supplemented with 1% FBS and 0, 0.05, 0.5, 5, 25, or 50 μM guggulsterone for 48 or 72 h. After incubation, MTT solution was added to each well to a final concentration of 1.0 mg/ml MTT. Four hours later, 100 μl dimethyl sulfoxide were added to each well. The optical density of each well was measured with a microplate reader at 570 nm.

Flow cytometric analysis. Cell cycle analysis was performed on harvested cell pellets treated with 0.2% Triton X-100 in PBS and povidone iodine solution (20 μg/ml) containing RNase A (100 μg/ml). The mixture was immediately analysed by flow cytometry, and cell cycle distribution was measured using an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Electromobility shift analysis (EMSA). NF-κB double-stranded oligonucleotide probes, corresponding to the respective sites in the CdX2 promoter (21, 24), were labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (Toyobo, Tokyo, Japan). Nuclear extracts (10 μg) were incubated for 20 min at room temperature with 50,000 cpm of ^{32}P-labelled probes and 2 μg of poly(dIdC)-poly(dIdC) (Sigma) in a buffer containing 10% glycerol, 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol. The DNA-protein complexes were resolved on 5% polyacrylamide gel at 100 V for 3 h at 4˚C. The gel was dried and visualised using the BAS-1000 autoradiography system (Fujifilm, Tokyo, Japan). Competition experiments were performed by adding a 100-fold molar excess of unlabelled oligonucleotide before adding the radioactive probe. In some experiments, movement of protein–DNA complexes into the gel was blocked by incubating the complexes with 2 μg antibody against the p50 subunit of NF-κB (sc-7178X; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis. All data were expressed as mean±standard deviation (SD). The significance of differences between groups was estimated using the two-tailed Student's *t*-test using Microsoft Excel. Results were considered statistically significant if the *p*-value was less than 0.05.

Results

Bic-1 cells express FXR and CdX2 and bile acids induce CdX2 protein. Bic-1 cells expressed basal FXR protein which was confirmed by an electrophoresis band at the molecular marker with 54kDa. Bic-1 cells also expressed CdX2 at 40 kDa (Figure 1A). CdX2 protein expression increased when Bic-1 cells were treated with the bile acids CDCA and DCA for 24 h (Figure 1B). To verify the effects of CDCA and DCA on CdX2 protein expression, Bic-1 cells were treated with different concentrations of each bile acid for 24 h. Both CDCA and DCA increased CdX2 protein expression in a concentration-dependent manner, with maximum effects observed at 100 μM [346% (p=0.003) and 196% (p=0.02) increases compared to the control, respectively; Figure 1C-F].

Guggulsterone inhibits CdX2 expression induced by bile acids in Bic-1 cells. To investigate whether guggulsterone, an FXR antagonist, inhibits CdX2 expression induced by bile acids, Bic-1 cells were treated with different concentrations of guggulsterone with or without bile acids (100 μM CDCA and DCA) for 24 h. Guggulsterone significantly reduced CDCA-induced CdX2 protein expression to 32% (p=0.01) compared to 100 μM CDCA alone, and 59% (p=0.16) compared to the control (Figure 2A, C). Guggulsterone also significantly reduced DCA-induced CdX2 protein expression to 44% (p=0.02) compared to 100 μM DCA alone, and 83% (p=0.50) compared to the control (Figure 2B, D).

Guggulsterone reduces constitutive CdX2 expression in Bic-1 cells. To investigate whether guggulsterone inhibited CdX2

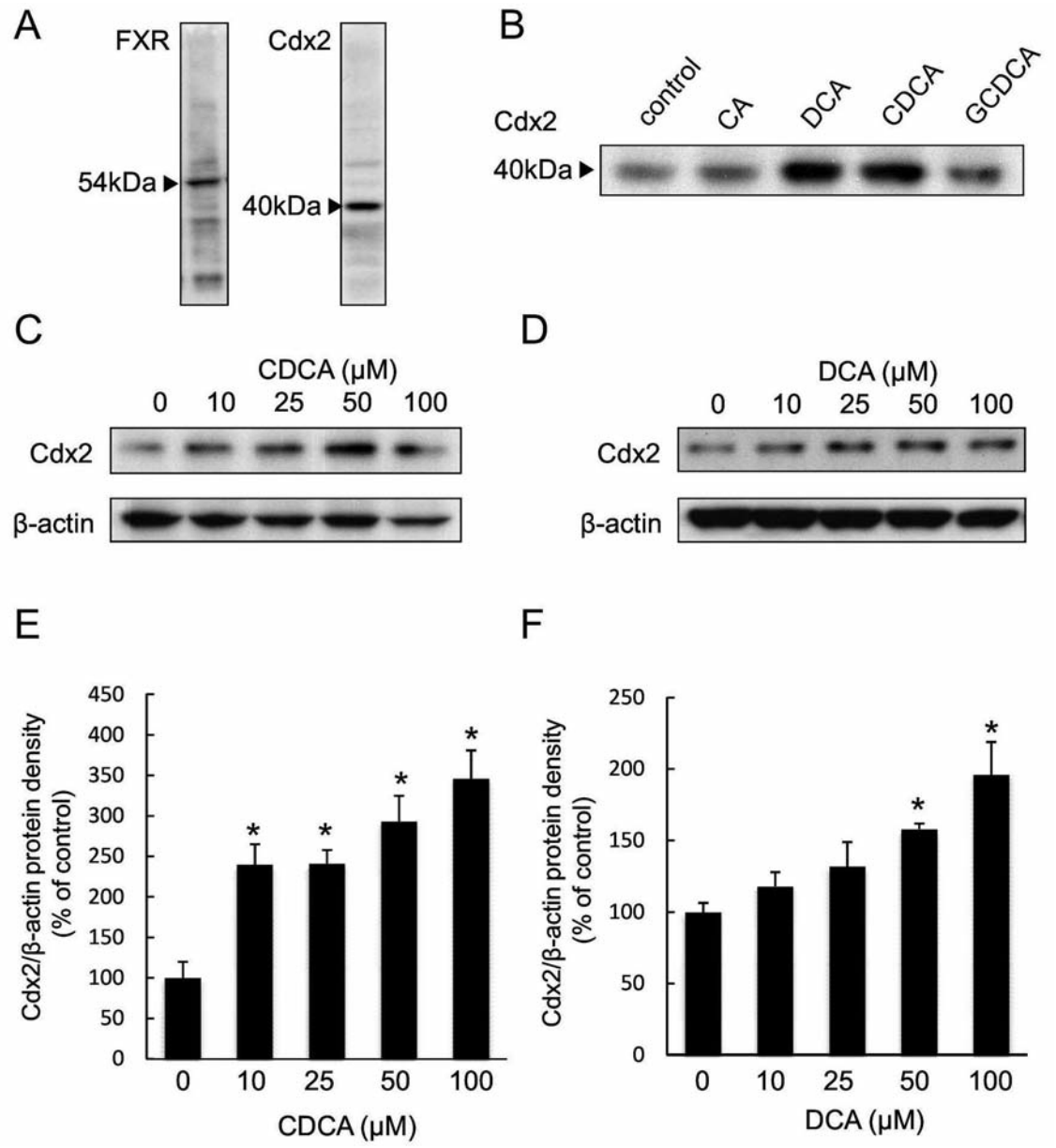

Figure 1. *FXR and CdX2 are expressed in Bic-1 cells, and bile acids induce CdX2 protein expression in Bic-1 cells. Immunoblot analysis of FXR and CdX2 expression. CdX2 and FXR were detected by mouse monoclonal antibody and rabbit polyclonal antibody, respectively. A: Thirty micrograms of protein extracted from Bic-1 cells were subjected to immunoblot analysis. B: After treating Bic-1 cells with the bile acids CA, DCA, CDCA, and GCDCA for 24 h, 50 μg of protein extract were subjected to immunoblot analysis. C, D: After treating Bic-1 cells with different concentrations of CDCA (C) or DCA (D) for 24 h, 50 μg of protein extract were subjected to immunoblot analysis. Blots are representative of three separate experiments. E, F: CdX2 protein expression was quantified by densitometry and standardised to β-actin with different concentrations of CDCA (E) or DCA (F) for 24 h. Values are mean±SD of three separate experiments. *p<0.05 compared to the Control.*

protein expression in the absence of bile acids, Bic-1 cells were treated with different concentrations of guggulsterone for 24 h. CdX2 protein expression decreased significantly after treatment with guggulsterone for 24 h, with maximum effect observed at 5 μM (54%, p=0.03 compared to the control; Figure 3A, B).

Guggulsterone has no effect on cell viability, cell cycle, or apoptosis at a low concentration in Bic-1 cells. Since guggulsterone has been reported to reduce cell viability by inducing cell cycle arrest and apoptosis (8, 10), these functions of guggulsterone were evaluated in the present experimental system. To investigate the effect of

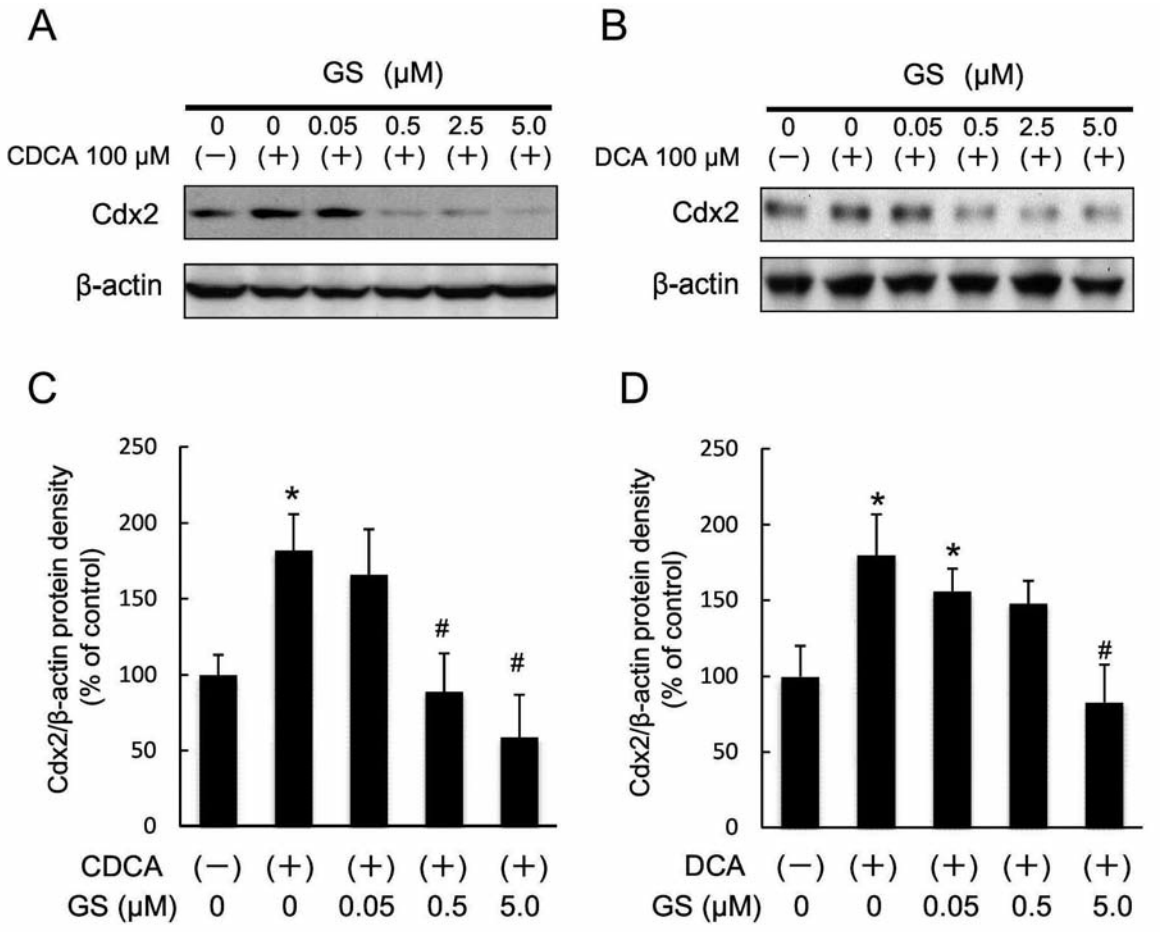

Figure 2. *Guggulsterone, an FXR antagonist, inhibits bile acid-induced CdX2 expression in Bic-1 cells. After treating Bic-1 cells with different concentrations of guggulsterone and 100 μM CDCA or DCA for 24 h, 50 μg of protein extract were subjected to immunoblot analysis. A, B: Blots are representative of three separate experiments. C, D: CdX2 protein expression was quantified by densitometry and standardized to β-actin. Values are mean±SD of three separate experiments. *p<0.05 compared to the control; #p<0.05 compared to treatment with bile acid only; GS: guggulsterone.*

guggulsterone on cell viability, Bic-1 cells were treated with guggulsterone for 48 or 72 h and their viability was assessed using the MTT assay. Treatment with 50 μM guggulsterone for 72 h significantly reduced Bic-1 cell viability compared to that of the control, whereas concentrations up to 5 μM did not do so (Figure 4A). Furthermore, treatment with up to 50 μM guggulsterone for 48 h did not reduce cell viability (data not shown). To investigate the effects on the cell cycle, Bic-1 cells were treated with 5 μM guggulsterone for 48 h, and the cell cycle was analysed by flow cytometry. The results revealed that 5 μM guggulsterone had no effect on the cell cycle in Bic-1 cells (Figure 4B). These data indicate that concentrations of guggulsterone lower than 5 μM are appropriate for assessing the cell cycle-independent antagonistic effects of guggulsterone on FXR in Bic-1 cells.

Guggulsterone does not affect CDCA-induced or constitutive NF-κB activation at a low concentration in Bic-1 cells. Previous studies showed that bile acids directly induce CdX2 expression through NF-κB in oesophageal epithelium (20, 21). EMSA was performed to examine the effects of CDCA and guggulsterone on the binding activity of NF-κB. Nuclear protein extracted from Bic-1 cells was bound to labelled oligonucleotides containing NF-κB binding site 1 or 2 from the *CdX2* promoter, and this interaction was eliminated by competition with an unlabeled oligonucleotide containing an NF-κB-binding site or by an anti-p50 antibody (Figure 5A). Although treatment of Bic-1 cells with 100 μM CDCA increased NF-κB binding to binding sites in the *CdX2* promoter, treatment with 5 μM guggulsterone did not greatly reduce the constitutive or CDCA-induced binding activity of NF-κB to the *CdX2* promoter (Figure 5B). Taken together, these findings indicate that 5 μM guggulsterone inhibits CdX2 expression independent of NF-κB inactivation in Bic-1 cells.

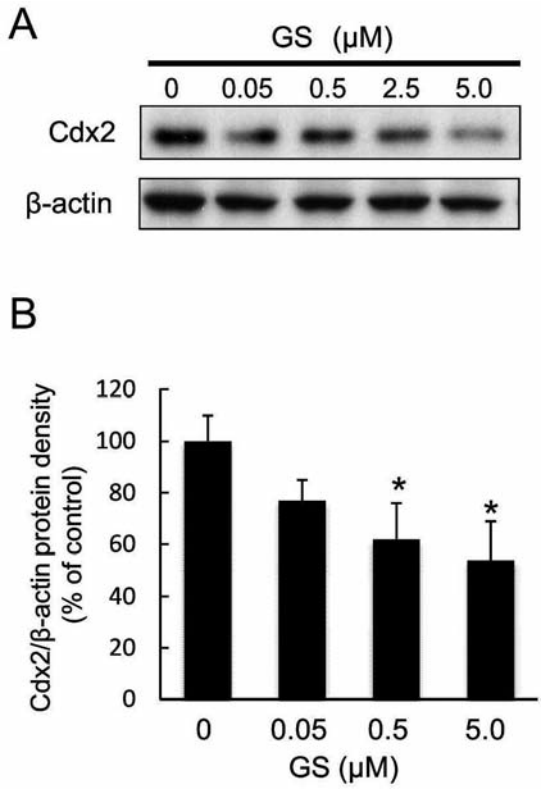

Figure 3. *Guggulsterone reduces constitutive CdX2 expression in Bic-1 cells. After treating Bic-1 cells with different concentrations of guggulsterone for 24 h, 50 μg of protein extract were subjected to immunoblot analysis. A: Blots are representative of three separate experiments. B: CdX2 protein expression was quantified by densitometry and standardised to β-actin. Values are mean±SD of three separate experiments. *p<0.05 Compared to the control; GS: guggulsterone.*

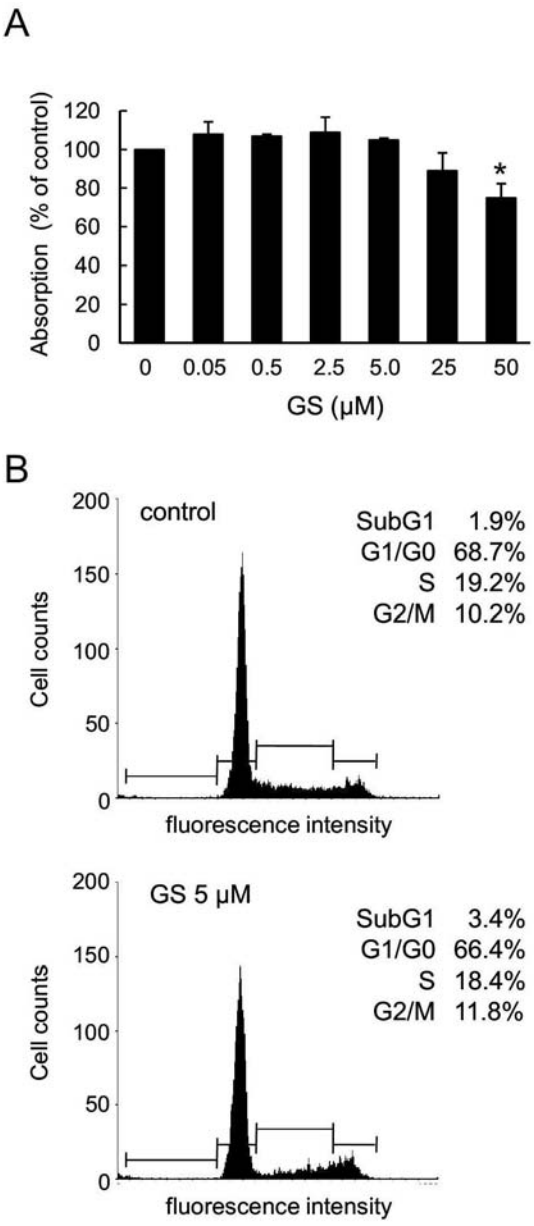

Figure 4. *Guggulsterone (5 μM) has no effect on cell viability, cell cycle, or apoptosis in Bic-1 cells. A: Bic-1 cells were treated with different concentrations of guggulsterone for 72 h, and their viability was assessed using the MTT assay. Values are mean±SD of three separate experiments. *p<0.05 Compared to the control. B: Bic-1 cells were incubated in the absence or presence of 5 μM guggulsterone for 24 h. Thereafter, the cells were washed, fixed, stained with propidium iodide and analysed for DNA content by flow cytometry. GS: guggulsterone.*

Discussion

This study investigated the effect of guggulsterone, an FXR antagonist, on the pathogenesis of gut-derived adenocarcinoma, with emphasis on the regulation of CdX2 expression. The main findings were: (a) CDCA and DCA, which are natural FXR agonists, induced CdX2 expression in Bic-1 cells; (b) guggulsterone reduced CdX2 expression in the presence and absence of bile acids; and (c) this suppressive effect of guggulsterone on CdX2 was observed at guggulsterone concentrations that did not affect cell viability or the cell cycle, nor did they attenuate NF-κB activation.

Guggulsterone has been reported to have antitumour activity in several types of cancer cells, including prostate cancer (5, 6), lung cancer, acute leukaemia and breast cancer (7, 8). In addition, recent studies revealed that guggulsterone inhibited angiogenesis *in vitro* and *in vivo* in human prostate cancer cells (9) and suppressed NF-κB activation in leukaemia, breast cancer and multiple myeloma cells (10, 11). However, it is not known whether guggulsterone modulates CdX2 expression in gut-derived cells. FXR is reported to be significantly expressed throughout the intestine, from the duodenum to the colon, in mice (25). It is also reported to be directly activated by bile acids and

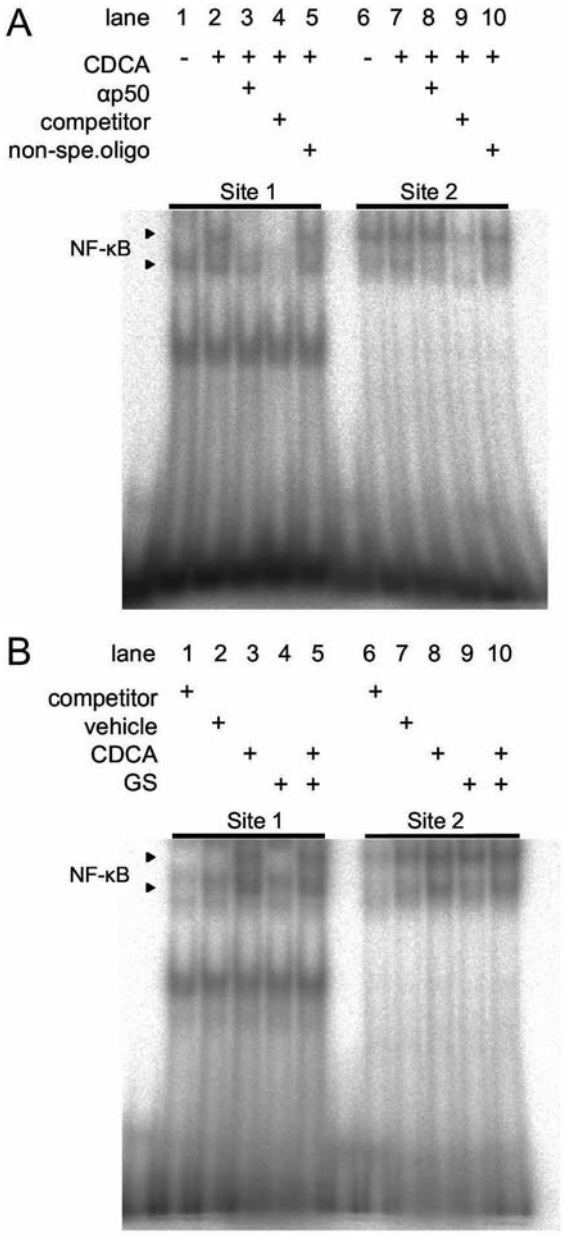

Figure 5. *Guggulsterone (5 μM) does not affect CDCA-induced or constitutive NF-κB activation in Bic-1 cells. A: Nuclear proteins were extracted from Bic-1 cells and 10 μg was incubated with labelled oligonucleotides corresponding to NF-κB-binding sites 1 and 2 in the CdX2 promoter (lanes 1 and 6). Treatment with 100 μM CDCA increased the intensity of the DNA–protein complex (lanes 2 and 7). Presence of NF-κB in this complex was assessed by supershift analysis using a polyclonal antibody specific for p50 (lanes 3 and 8). The specificity of the p50-containing DNA–protein complex was assessed using a 100-fold molar excess of unlabeled NF-κB-binding nucleotide and nonspecific oligonucleotide (lanes 4, 5, 9, and 10). B: The specificity of NF-κB was assessed using a 100-fold molar excess of unlabeled NF-κB-binding nucleotide (lanes 1 and 2). Treatment with 100 μM CDCA increased the intensity of the DNA–protein complex (lanes 3 and 8). Guggulsterone did not affect the intensity of the NF-κB-nucleotide complex in the absence of CDCA (lanes 4 and 9) or the presence of 100 μM CDCA (lanes 5 and 10). GS: Guggulsterone.*

regulate the synthesis, absorption and excretion of bile acids (26-28). It was therefore hypothesised that guggulsterone also regulates CdX2 expression and has chemopreventive potential for gut tumourigenesis.

Firstly, the FXR expression in gut-derived adenocarcinoma cells was confirmed and which bile acids enhance CdX2 expression was examined. It was found that CDCA and DCA enhanced CdX2 expression more effectively than did the other bile acids. Since CDCA and DCA are high-affinity agonists of FXR (27, 29), it is suggested that these effects were caused by FXR activation. In the second part of this study, it was found that the FXR antagonist guggulsterone reduced CdX2 expression induced by CDCA and DCA in Bic-1 cells in a concentration-dependent manner. These results support the idea that CdX2 expression is associated with FXR activation. In this study, the suppressive effect of guggulsterone was also observed in the absence of bile acids, suggesting that FXR may have constitutive activity without its agonist and that guggulsterone may have a strong regulating effect on CdX2 in Bic-1 cells.

Since guggulsterone has been reported to induce cell cycle arrest and apoptosis and inhibit NF-κB activation (1, 8, 10), it was also investigated whether guggulsterone affected viability, the cell cycle, and NF-κB activity in Bic-1 cells. Interestingly, guggulsterone at concentrations up to 5 μM induced neither cell cycle arrest nor apoptosis, and did not inhibit NF-κB activation in Bic-1 cells. In previous studies, higher concentrations of guggulsterone (20-50 μM) than those used in this study induced cell cycle arrest and apoptosis and inhibited NF-κB activation (8, 10). These facts indicate that the mechanisms whereby guggulsterone reduced CdX2 expression in the present study did not involve cell cycle arrest, apoptosis, or NF-κB inactivation, which were previously reported as antitumour activities.

This study had several limitations. Firstly, although it was hypothesised that guggulsterone suppressed CdX2 expression through FXR antagonism in Bic-1 cells, no definite evidence for this could be found using FXR knockdown models with the siRNA and shRNA techniques because cell viability could not be maintained for further experiments to clarify this issue with regard to FXR knockdown. Therefore, it was not possible to conclude that the suppressive effect of guggulsterone on CdX2 expression is induced by inactivation of FXR signaling. Secondly, it was found that CdX2 expression was regulated by guggulsterone in an adenocarcinoma cell line *in vitro*. To determine the chemopreventive effect of this drug, it is necessary to examine whether guggulsterone also induces suppression of CdX2 expression in normal gut epithelial cells *in vivo*. Thirdly, although recent reports indicate that CdX2 may play an important role in carcinogenesis in the colon, the oesophagus, the stomach and other non-intestinal tissues (15), it is controversial whether increasing or decreasing CdX2 expression may have a chemopreventive effect on the

development of these types of cancer. Therefore, it should be remembered that guggulsterone can act as either a chemopreventive or chemoprogressive drug in different types of cancer.

Nevertheless, guggulsterone is considered a relatively safe agent because guggulipid, the ethyl acetate of gum guggul, had no severe adverse side-effects associated with it in previous clinical studies of its hypolipidemic effect (30). This study showed for the first time that guggulsterone, which at a low concentration does not induce apoptosis, may be useful for regulating CdX2 expression in certain types of gut adenocarcinoma. Although further *in vivo* studies are required, these results suggest that guggulsterone could be an attractive drug for future anticancer therapies in gut.

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