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Inhibition of GSK-3β reverses the pro-apoptotic effect of proadifen (SKF-525A) in HT-29 colon adenocarcinoma cells

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ABSTRACT

Proadifen (SKF-525A) is a well-known inhibitor of cytochrome P450 monooxygenases. Besides the prevention of drug metabolism it affects the proliferation of cancer cells, although the mechanisms of possible anti-cancer activity of proadifen have not been fully understood yet. The aim of this study therefore was to evaluate the potential anti-proliferative effect of proadifen on HT-29 colon cancer cells. Our results show that proadifen inhibited the growth of HT-29 cells by the accumulation of cells in the G1 phase of the cell cycle, reduction of metabolic activity and colony formation and by the induction of apoptosis. Analyses of Western blots and flow cytometry revealed time- and dose-dependent phosphatidylserine externalization, caspase-3 activation and PARP cleavage. Intense upregulation of NAG-1 and ATF3 and downregulation of Mcl-1 and Egr-1 were also observed. Further investigation showed that NAG-1 gene silencing by siRNA had no effect on the pro-apoptotic action of proadifen. In contrast, we found that AR-A014418, the specific inhibitor of glycogen synthase kinase-3 β (GSK-3β), significantly decreased proadifen-induced apoptosis. Inactivation of GSK-3β (phosphorylation at serine 9) resulted in changes in phosphatidylserine externalization and caspase-3 activation. These data suggest that GSK-3β is an important factor in the induction of apoptosis in HT-29 colon cancer cells treated with proadifen.

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1. Introduction

Proadifen is a well-known cytochrome P450 monooxygenase inhibitor (Hofmanova et al., 2000; Ishihara et al., 2010). Because of this ability proadifen should be included in the large class of non-steroidal anti-inflammatory drugs (NSAIDs) together with inhibitors of cyclooxygenases and lipoxygenases. Moreover, there are some studies which have documented its anti-proliferative potential in cancer cells originating from different tissues (Hoferova et al., 2004; Hofmanova et al., 2000; Kleban et al., 2007). However, the molecular mechanisms involved in proadifen action are not completely understood.

Glycogen synthase kinase-3 β (GSK-3β) is a serine/threonine kinase whose activity is regulated by site-specific phosphorylation. In general, full activity of GSK-3β requires phosphorylation at tyrosine 216 and in contrast, phosphorylation at serine 9 inhibits action of this multifunctional kinase (Hughes et al., 1993; Sutherland et al., 1993). GSK-3β has been implicated in regulation of multiple cellular processes such as metabolism, cell fate determination, proliferation and survival (Hardt and Sadoshima, 2002; Harwood et al., 1995; Krylova et al., 2000; Kunnimalaiyaan et al., 2007; Wang et al., 2002). Moreover, several studies suggest that GSK-3β also possesses pro-apoptotic properties (Bijur et al., 2000; Song et al., 2002; Watcharasit et al., 2003). Although the molecular mechanism by which GSK-3β induces apoptosis has not been fully elucidated yet, it is well known that GSK-3β exerts its activity by regulation of a broad range of substrates. Among them, non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) (Lee et al., 2010a,b; Soto-Cerrato et al., 2007) has been reported as a pro-apoptotic and anti-tumorigenic

Abbreviations: A, AR-A014418, GSK-3β inhibitor; ANOVA, analysis of variance; ATF3, activating transcription factor 3; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-XL, B-cell lymphoma-extra large; DMSO, dimethyl sulfoxide; DOC-Na, sodium deoxycholate; Egr-1, early growth response protein 1; FITC, fluorescein isothiocyanate; FL-1, -2, -3, fluorescence channel-1, -2, -3; GSK-3β, glycogen synthase kinase-3 β; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KLF4, Krueppel-like factor 4; MCC-555, 5-[[6-[(2-fluorophenyl)-methoxy]-2-napthalenyl]-methyl]-2,4-thiazolidinedione; Mcl-1, induced myeloid leukemia cell differentiation protein; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); NAG-1, non-steroidal anti-inflammatory drug-activated gene-1; NP-40, nonyl phenoxypolyethoxylethanol; NSAIDs, non-steroidal anti-inflammatory drugs; P, proadifen; p53, protein 53; PARP, poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; PI, propidium iodide; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay buffer; RT, room temperature; SD, standard deviation; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; Sp-1, Specificity protein 1; TBS, tris buffered saline; Tris, tris(hydroxymethyl)aminomethane; TWEEN 20, polysorbate surfactant.

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gene (Baek et al., 2001). Upregulation of NAG-1 expression has been shown by many different anti-tumorigenic and chemopreventive drugs, such as resveratrol (Baek et al., 2002), genistein (Wilson et al., 2003), epicatechin gallate (Baek et al., 2004), celecoxib (Pang et al., 2007), prodigiosin (Soto-Cerrato et al., 2007), xanthorrhizol (Kang et al., 2009), apigenin (Zhong et al., 2010) and peroxisome proliferator-activated receptor-γ (PPAR γ) agonist – MCC-555 (Cekanova et al., 2010).

Anti-proliferative properties of proadifen have been established mainly through its ability to influence metabolic activity, cell cycle progression and cell numbers. Based on these results, therefore, we used HT-29 colon cancer cells to investigate more detailed effects of proadifen on the parameters reflecting cell proliferation and cell death. Furthermore, we were interested in evaluating the effect of GSK-3β inhibition and NAG-1 suppression on proadifen action in colon cancer cells.

2. Materials and methods

2.1. Materials

Proadifen (SKF-525A; CAS No.: 62-68-0; Sigma–Aldrich, St. Louis, MO, USA) was prepared in distilled water immediately before adding to the cell culture. AR-A014418 (GSK-3β Inhibitor VIII, CAS No.: 487021-52-3; Merck KGaA, Darmstadt, Germany) was prepared in dimethyl sulfoxide (DMSO, Sigma–Aldrich) and added to the cells 1 h before proadifen treatment. Application of GSK-3β inhibitor was performed based on the study of Soto-Cerrato and co-authors (2007) with minor modification. Concentration of DMSO did not exceed 0.1%. The control group was treated with the carrier at the highest concentration used. The final concentration of DMSO did not influence the analyzed parameters (data not shown).

2.2. Cell culture and experimental design

Human colon adenocarcinoma cell line HT-29 was purchased from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1}, amphotericin 25 μg ml^{-1}; Gibco, Invitrogen, Grand Island, NY, USA) at 37 °C in 5% CO_2 and 95% humidity. Prior to treatment, cells were seeded and left to settle for 24 h. Later, either proadifen (P, 5–50 μM) alone (MTT assay, clonogenic assay, cell cycle analysis, Western blot analysis) or in combination with AR-A014418 (A, 10 μM) (phosphatidylserine externalization analysis, caspase-3 activation analysis, Western blot analysis) was added to the cells. Depending on the type of analysis, results were analyzed 6 h, 24 h, 48 h and/or 72 h after treatment.

2.3. MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich) assay was performed to evaluate the metabolic activity of cells as previously reported (Kleban et al., 2007). At scheduled time points, 24 h, 48 h or 72 h after proadifen and/or AR-A014418 treatment, MTT from a stock solution (5 mg ml^{-1}) was added to the cells in a 96-well plate (TPP, Trasadingen, Switzerland) (final concentration 0.5 mg ml^{-1}). The reaction was stopped after 4 h incubation at 37 °C and the insoluble formazan was dissolved by addition of sodium dodecyl sulfate (SDS) at a final concentration of 3.3%. The absorbance (λ = 584 nm) was measured using a BMG FLUOstar Optima (BMG Labtechnologies GmbH, Offenburg, Germany). Results were evaluated as percentages of the absorbance of the untreated control.

2.4. Clonogenic assay

For clonogenic assay, cells were harvested (24 h after proadifen treatment), counted and 500 viable cells were seeded in six-well plates (TPP). Cells were cultivated under standard conditions. Eight days later, cells in the plates were fixed and stained with methylene blue dye (0.008% w/v) and scanned. Colonies were counted using Clono-Counter software (Niyazi et al., 2007). Results were evaluated as percentages of the untreated control.

2.5. Cell-cycle analysis

For flow cytometric analysis of the cell cycle, adherent cells were harvested by trypsinization and collected together with floating cells (5×10^5) 48 h and 72 h after proadifen treatment, washed in cold PBS, fixed in cold 70% ethanol and kept at −20 °C overnight. Prior to analysis, cells were washed twice in PBS, resuspended in staining solution (0.1% Triton X-100, 0.137 mg ml^{-1} ribonuclease A and 0.02 mg ml^{-1} propidium iodide – PI), incubated in the dark at room temperature (RT) for 30 min and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with a 488 nm argon-ion excitation laser. Fluorescence was detected via 585/42 nm band-pass filter (FL-2). ModFit 3.0 (Verity Software House, Topsham, ME, USA) software was used to generate DNA content frequency histograms and quantify the number of cells in the individual cell cycle phases.

2.6. Phosphatidylserine externalization analysis

Analysis of phosphatidylserine externalization was performed using Annexin V-FITC/PI (BD Biosciences Pharmingen, Franklin Lanes, NJ, USA) double-staining method according to the manufacturer's instructions. AR-A014418 was added to the cells 1 h before proadifen treatment. Adherent cells were harvested by trypsinization and collected together with floating cells (1.5×10^5) 48 h and 72 h after proadifen treatment, stained with Annexin V-FITC in binding buffer (10 mM HEPES, 2.5 mM $CaCl_2$, 140 mM NaCl) for 15 min, stained with PI (2 μg ml^{-1}) for 5 min and thereafter analyzed using a BD FACSCalibur flow cytometer with a 488 nm argon-ion excitation laser. Fluorescence was detected via 530/30 nm band-pass filter (FL-1; Annexin V-FITC) and via 670 nm long-pass filter (FL-3; PI). Although no significant changes in the population of PI positive cells were detected, the results were evaluated and are presented as percentages of Annexin V positive cells in the particular experimental groups.

2.7. Caspase-3 activation analysis

Activation of caspase-3 was analyzed using a FITC Active Caspase-3 Apoptosis Kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Adherent cells were harvested by trypsinization and collected together with floating cells (4×10^5) 48 h or 72 h after proadifen treatment. AR-A014418 was added to the cells 1 h before proadifen treatment. Cells were washed in cold PBS, fixed and permeabilized with BD Cytofix/Cytoperm Fixation and Permeabilization Solution for 20 min on ice, washed again twice, incubated with antibody for 30 min at RT, washed and finally analyzed using a BD FACSCalibur flow cytometer with a 488 nm argon-ion excitation laser. Fluorescence was detected via 530/30 nm band-pass filter (FL-1). Results were evaluated as percentages of positively-stained cells.

2.8. NAG-1 siRNA transfection analysis

NAG-1 siRNA transfection was performed using ON-TARGETplus SMARTpool human GDF15 siRNAs (L-019875-00-0005, Ther-

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