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Involvement of Nrf2 activation in resistance to 5-fluorouracil in human colon cancer HT-29 cells

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ABSTRACT

Acquisition of drug resistance by cancer cells is attributed to various factors including alterations in apoptotic pathways, enhanced expression of multidrug resistance-associated proteins, altered drug metabolism or uptake and/or overexpression of cytoprotective genes. Thus, potential induction of defence pathways by anticancer drugs might have a marked incidence on cancer cell resistance. 5-Fluorouracil (5-FU) remains the most commonly used anticancer drug for the treatment of colorectal cancer, although objective response rates are as low as 20%. The aim of our study was to investigate the effects of 5-FU on cytoprotective systems in human colon HT-29 cells. Our results demonstrate that 5-FU induced the expression of mRNAs encoding glutathione transferases and antioxidant enzymes. To further determine the mechanisms involved in 5-FU effects, we investigated whether it activates the Nrf2/antioxidant response element pathway which is implicated in the regulation of several genes involved in cytoprotection. Translocation of Nrf2 into the nucleus after 5-FU exposure was demonstrated by immunocytochemistry and western blotting. Using an ARE-driven reporter gene assay, activation of the luciferase activity by 5-FU was also evidenced. Moreover, transfection of HT-29 cells with siRNA directed against Nrf2 inhibited induction of Nrf2 target genes and increased 5-FU cytotoxicity. In conclusion, we demonstrate for the first time that 5-FU activates the Nrf2/ARE pathway which in turn induces cytoprotective genes and modulates chemosensitivity of HT-29 colon cancer cells. Therefore, we postulate that Nrf2 might represent a potential therapeutic target in 5-FU treatment of colon cancer.

1. Introduction

The fluoropyrimidine 5-fluorouracil (5-FU) is an antitumoural agent widely used in the treatment of solid tumours, including colorectal cancers.[1] The mechanism of action of 5-fluorouracil has been associated with inhibition of thymidylate synthase (TS) and incorporation of 5-FU into RNA and DNA.[1] Furthermore, 5-FU has many side-effects and is also cytotoxic for normal cells. Understanding of the mechanism of action of 5-FU greatly increases in recent years; however, the development of drug-resistant phenotypes remains a significant limitation to its clinical use. The overall response rates to 5-FU chemotherapy in colon cancer patients remain as low as 10–20%.[2] 5-FU resistant tumours commonly express high levels of thymidylate synthase (TS) and 5-FU sensitivity is inversely related to the level of TS activity in cancer cells.[3]

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Nevertheless, the mechanisms of 5-FU resistance are multifactorial and involve expression levels of dihydropyrimidine dehydrogenase,[4] Nuclear Factor-κB[5] and DNA mismatch-repair genes,[6] the genetic status of p53[7] and cell cycle disturbances.[8]

Many anticancer drugs are responsible for the production of reactive oxygen species (ROS) in cancer cells, a phenomenon which contributes to drug-induced apoptosis. 5-FU also generates mitochondrial ROS through the p53-dependent pathway.[9,10] Therefore, antioxidant defence systems such as glutathione (GSH), γ-glutamylcysteine synthetase (γ-GCS), haem oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione reductase (GSR) and glutathione peroxidase (GPX) might play an important role in 5-FU resistance. Thus, GSH has been implicated in resistance of tumour cells to chemotherapeutic agents, including cisplatin and melphalan[11–13] and the relationship between high expression of γ-GCS, the rate-limiting enzyme in glutathione synthesis, and drug resistance has been extensively documented in human cancer cells.[14] Similarly, A549 lung cancer cells overexpressing HO-1 are resistant to epigallocatechin 3-gallate-induced apoptosis,[15] while down-regulation of this gene enhances chemosensitivity towards cisplatin in the same cells.[16] Although glutathione transferases (GST) are not involved in 5-FU metabolism, they might represent another key enzymatic system involved in 5-FU resistance. Indeed, this gene family plays a role in cellular protection against oxidative stress and in the regulation of the mitogen-activated protein kinase pathway, which participates in cellular survival and cell death signalling.[17,18]

Several genes involved in drug resistance are regulated by a cap'n'collar basic leucine zipper transcription factor, nuclear factor-erythroid 2p45 (NF-E2)-related factor 2 (Nrf2). Recently, it has been shown that constitutive activation of Nrf2 contributes to drug resistance by regulating several plasma membrane efflux pumps (MRP1 and MRP2) and phase II detoxification enzymes.[19] The role of the Nrf2-antioxidant system in cisplatin-mediated toxicity and its implication in cancer cell resistance has also been shown.[12] The down-regulation of the Nrf2-dependent response by overexpression of its negative regulator, kelch-like ECH-associated protein 1 (Keap1), or transient-transfection of Nrf2-siRNA in lung carcinoma, breast adenocarcinoma and neuroblastoma rendered cancer cells more susceptible to cisplatin, etoposide and doxorubicin.[20] All these findings support the idea that Nrf2 can influence effectiveness of chemotherapy and contribute to drug resistance.

This study was undertaken to determine the role of Nrf2 and its downstream gene targets in drug resistance of colon cancer cells. To address this question, we analysed the influence of 5-FU on Nrf2-regulated cytoprotective genes, such as GSTs and antioxidant enzymes, in the human colon adenocarcinoma HT-29 cell line.

2. Materials and methods

2.1. Chemicals, cell culture and treatment

Culture media, L-glutamine and Fetal Calf Serum (FCS) were purchased from Eurobio. 5-Fluorouracil, Dimethyl sulfoxide (DMSO), neutral red, NS-398, haemin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH and DEVD-AMC were purchased from Sigma (St Quentin Fallavier, France). HO-1 (sc-10789), Nrf-2 (sc-722) and γ-GCS (sc-22754) antibodies were purchased from Santa Cruz Biotechnologies (Tebu, France). GSTS1 antibody was a gift of Pr. John Hayes (University of Dundee, Scotland). HT-29 cells (Human colon adenocarcinoma grade II cell line) were purchased from ATCC.

2.2. HT-29 cell cultures

HT-29 cells were cultured in minimum essential medium supplemented with 10% FCS v/v and 2 mmol/L L-glutamine. For all experiments, cells were seeded at 1×10^5 per cm^2 and allowed to attach for 24 h before treatment with 5-FU.

2.3. Total RNA extraction and RT-PCR analysis

Total RNA was extracted from 5×10^6 HT-29 cells with the SV total RNA isolation system (Promega, Madison, WI), which included a DNase treatment step. RNAs were reverse-transcribed into cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed by the fluorescent dye SYBR Green methodology using the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI prism 7300 (Applied Biosystems). *Table* 1 (supplementary data) shows primer pairs for each transcript designed with primers 3 (http://fokker.wi.mit.edu/primer3/input.htm). The amplification curves were read with the 7300 SDS software using the comparative cycle threshold method. The relative quantification of the steady-state of the target mRNA levels was calculated after normalization of the total amount of cDNA using 18S RNA as reference. A dissociation curve was performed after the PCR reaction to verify the amplification specificity.

2.4. Preparation of cytosolic and microsomal fractions and nuclear extracts

Cells were homogenised with a lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol v/v, 1 mM EDTA, 1 mM EGTA, 1% NP40 v/v, 100 μM PMSF and 1 mM DTT). Nuclei and mitochondria were eliminated by centrifugation at 3000*g* for 10 min and at 8000*g* for 20 min. The supernatant containing microsomes and cytosol was subjected to centrifugation at 30,000*g* for 1 h and the resulting supernatant (cytosolic fraction) was frozen at –80 °C until use. Microsomal pellet was dissolved with a buffer containing 50 mM Tris–HCl, 20% glycerol v/v, PMSF 100 mg/l and heparin 3000 UI/l. For nuclear extracts, 10×10^6 cells were seeded into 100 mm dishes in the absence or the presence of 5-FU. Cells were then harvested, collected with phosphate buffer-saline and the nuclear proteins were prepared using the TransFactor Extraction Kit Protocol-at-a-Glance (PT3612-2) (Clontech).

2.5. Western blot analysis

Cytosolic, microsomal and nuclear proteins (20–30 μg) were subjected to electrophoresis on a polyacrylamide slab gels

and electroblotted overnight onto Hybond-enhanced chemiluminescence (ECL) nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). GSTS1 and γ-GCS were analysed in cytosolic fractions, while HO-1 and Nrf2 were detected in the microsomal and nuclear fractions, respectively. Anti-Hsc70 and anti-lamin A/C antibodies were used as loading controls for cytosolic and nuclear fractions, respectively. Equal transfer of microsomal proteins was confirmed by staining the nitrocellulose with Ponceau Red.

2.6. Immunocytochemistry

HT-29 cells were fixed with 3% paraformaldehyde for 15 min at 4 °C. After washing with PBS/0.5% saponin and 1 h incubation with blocking medium (10% donkey serum in PBS-0.5% saponin), cells were incubated for 2 h with anti-Nrf2 primary antibody, followed by 1 h incubation with biotin-conjugated secondary antibody and 30 min with streptavidin. Cells were counterstained with DAPI and observed using an Axiovert 200 M inverted microscope from Zeiss. Images were acquired with AxioVision 4.6 Software (Carl Zeiss).

2.7. Measurement of HO enzyme activity

HO activity was measured by the bilirubin generation method. Briefly, 50 μl of microsomal fraction was added to the reaction mixture (400 μl) containing rat liver cytosols (2 mg), haemin (40 μM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (0.4 U) and NADPH (1.6 mM) for 1 h at 37 °C in the dark. The formed bilirubin was measured by the change in optical density between 464 and 530 nm (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin). HO activity is expressed as micromoles of bilirubin formed per microgram of protein per hour.

2.8. Determination of PGD_2 concentration

HT-29 cells seeded in 24-well plates at a density of 500,000 cells per well were grown to confluence. PGD_2 concentrations were determined in medium culture by using a commercial Prostaglandin D_2 enzyme immunoassay kit (Cayman). The PGD_2 enzyme assay is based on the competition between PGD_2 and PGD_2-acetylcholinesterase conjugate (PGD_2 tracer) for a limited number of PGD_2 monoclonal antibody binding sites.

2.9. Plasmid constructs, transient transfection and analysis of luciferase reporter gene activity

The ARE-luciferase reporter plasmid was generated using the pGL3-promoter vector (Promega) containing an SV40 promoter upstream of the firefly luciferase gene. The 4xARE sequence, which contains 4 copies of an ARE sequence in head-to-tail orientation (5′-GTGACAAAGCACCCGTGACAAAGCACCCGTGACAAAGCACCCGTGACAAAGCA-3′), has been inserted between the *KpnI* and *SacI* restriction sites upstream of the SV40 promoter. The Dual-luciferase Reporter Assay System (Promega) was used to examine reporter gene activity in transiently transfected cells. Briefly, cells at 80% confluency were detached using trypsin–EDTA (Sigma–Aldrich, United States of America), and were washed 2-times with PBS prior transfection with a Microporator™ (LabTech, France) according to the manufacturer's instructions and using the following parameters: 0.6 μg of reporter vector (either pGL3-4xARE, pGL3 promoter or pGL3 basic) and 0.06 μg of pRL-SV40 (Promega) encoding *Renilla luciferase* (control for transfection efficiency) for 10^5 cells in suspension. Cells were electroporated at 1400 V, for 20 ms and 2 pulses. After electroporation, cells were cultured in 24-well culture plates for 24 h prior to 5-FU treatment. Nrf2-siRNA (120 nM, HP validated siRNA, Qiagen) or non-targeting siRNA (Dharmacon) were transiently transfected by Microporator™ (LabTech, France) as described above.

2.10. Measurement of intracellular reactive oxygen species by flow cytometry

Dihydroethidine (DHE) and dihydrorhodamine (DHR) fluorescent probes were used to detect intracellular superoxide anion and hydrogen peroxide production, respectively. After treatment, adherent cells (1.5×10^6) were collected and incubated in PBS containing 5 μmol/L DHE for 20 min and

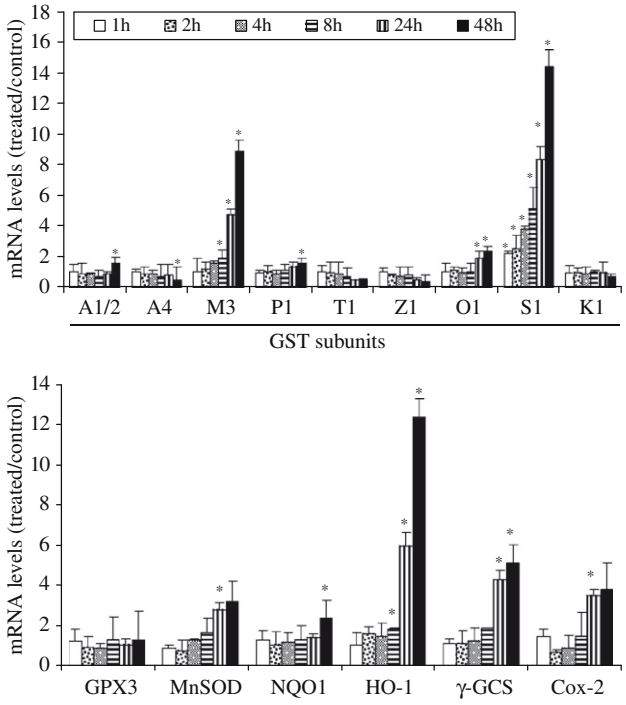

Fig. 1 – Effects of 5-FU on expression of mRNAs encoding GST and antioxidant enzymes in HT-29 colon cancer cells. The cells were treated with 5-FU (100 μg/ml) for 1, 2, 4, 8, 24 and 48 h. Expression of GST (upper graph) and antioxidant enzymes (lower graph) mRNAs were assessed by real-time PCR. The relative quantification of the steady-state mRNA levels was calculated after normalisation of the total amount of complementary DNA using, 18S RNA as reference. The results are expressed as fold change compared to untreated cells arbitrarily set at 1. The diagrams are representatives of four separate experiments. Statistical analysis was performed by comparison of untreated with 5-FU-treated cells (*, $p < 0.05$).

5 μmol/L DHR for 1 h at 37 °C. Dye oxidation (increase in FL-2 for DHE or FL-1 fluorescence for DHR) was measured using a FC500 flow cytometer (Becton–Coulter) with excitation and emission settings at 488 and 530 nm, respectively.

2.11. Measurement of cell viability

Cells were cultured in 96-well plates and were treated or not with 100 μg/ml 5-FU. After treatment exposure, the medium was discarded and replaced with FCS-free medium containing neutral red (0.05 g/l) for 3 h. Thereafter, cells were fixed with chloroform for 1 min, washed with PBS and dissolved in ethanol–acetic acid and dye uptake was determined as optical density (540 nm) using a microplate reader (iEMS Reader MF; Lab-systems, Helsinki, Finland).

2.12. Apoptosis assays

Microscopic detection of apoptosis was carried out in both floating and adherent HT-29 cells recovered after treatment using nuclear chromatin staining with 1 μg/mL Hoechst 33342 for 15 min at 37 °C. The number of cells with apoptotic nuclei (i.e. condensed or fragmented) was counted in the total cell population ($n = 200$ cells). For caspase activity, 1×10^6 HT-29 cells were seeded into 35 mm dishes. Detached cells were collected and combined with adherent cells, centrifuged, washed with PBS and resuspended in 70 μl lysis buffer (20 mM PIPES pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% KCl w/v, 10% sucrose w/v, DTT 10 mM and PMSF 100 μM). Eighty micrograms of crude cell lysate were incubated for 2 h at 37 °C with 80 μM N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (N-acetyl-DEVD-AMC). Thereafter, caspase activities were measured by spectrofluorometry (Spectra Max Gemini, Molecular Devices) at the excitation/emission wavelength pair of 380/440 nm.

2.13. Statistical analysis

Each value corresponded to the mean +/–SD of three independent experiments. The Mann–Whitney non-parametric test was used to compare all values.

3. Results

3.1. Effect of 5-fluorouracil on the expression of GST and antioxidant enzymes in HT-29 cells

In order to investigate the effects of 5-FU treatment on expression of GSTs and antioxidant enzymes, HT-29 cells were incubated with 100 μg/ml 5-FU for 1, 2, 4, 8, 24 and 48 h. While only low effects, if any, were observed for most GSTs after 5-FU treatment, GSTS1 and GSTM3 mRNA levels were increased up to 8- and 14-fold, respectively, after 48 h of incubation with the drug (Fig. 1). Interestingly, we also observed an induction of mRNAs encoding HO-1 and γ-GCS which play an important role in cytoprotective processes. By contrast, 5-FU slightly increased expression of MnSOD and NQO1 and had no effect on GPX3. Finally, mRNA coding for Cox-2, the central enzyme in prostanoid biosynthesis, was increased by 4-fold after 48 h of treatment. To determine whether changes in mRNA levels resulted in an increase in the corresponding protein levels, Cox-2, GSTS1, γ-GCS and HO-1 protein expressions were analysed by western blotting. We showed that variations observed at the mRNA levels correlated with the protein expression levels (Fig. 2).

3.2. 5-FU induces translocation of Nrf2 into the nucleus and activates the Nrf2/ARE signalling in HT-29 cells

It has been suggested that activation of Nrf2/ARE is critical for enhanced expression of anti-oxidant proteins in drug-resistant cancer cells. Activation of this pathway relies on accu-

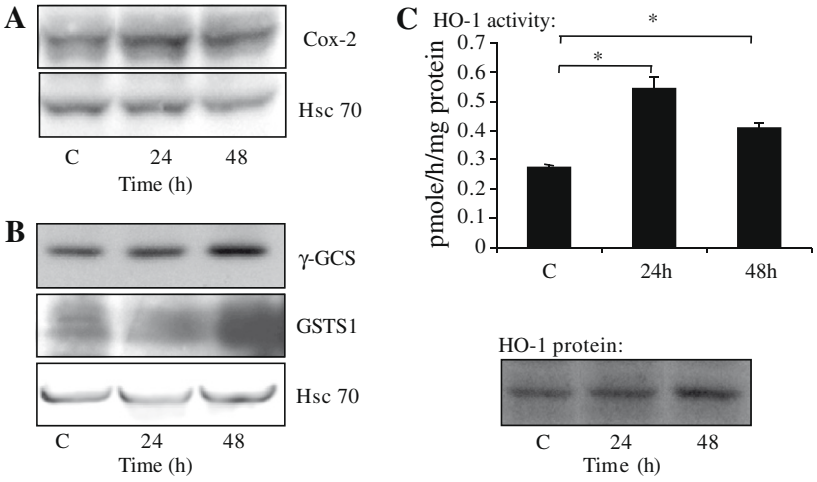

Fig. 2 – Effects of 5-FU on Cox-2, GSTS1, γ-GCS and HO-1 protein levels and HO-1 activity in HT-29 cells. Total lysates, cytosolic and microsomal proteins were prepared from HT-29 cells cultured in the absence (C) or presence of 5-FU for 24 or 48 h. Cox-2 was analysed in total lysates, GSTS1 and γ-GCS in cytosolic fractions and HO-1 in microsomal fractions. An anti-Hsc70 antibody was used as a loading control for total lysate and cytosolic fraction. HO-1 activity was measured in microsomal fractions using the bilirubin generation method. Statistical analysis was performed by comparison of untreated with 5-FU-treated cells (*, $p < 0.05$).

mulation and translocation of Nrf2 in the nucleus. In order to determine whether the Nrf2/ARE signalling pathway was activated in HT-29 cells treated with 5-FU, we determined nuclear and cytosolic levels of Nrf2 in both control and 5-FU-treated cells. As shown by immunocytochemistry, Nrf2 was localised in the cytosol of untreated cells and translocated into nucleus following treatment with 5-FU (100 μg/ml) for 24 h (Fig. 3A). This result was confirmed by western blotting which revealed a stable amount of Nrf2 in cytosolic proteins and an accumulation of Nrf2 into the nucleus in 5-FU-treated cells (Fig. 3B).

Since we demonstrated that 5-FU induces Cox-2 and GSTS1 expressions, as well as PGD_2 synthesis (Fig. 3C), we hypothesised that this increase might involve the formation of 15-deoxy-*D*12,14-prostaglandin J_2 (15d-PGJ_2), a downstream metabolite of PGD_2 which is known to activate Nrf2. Thus, we investigated the impact of Cox-2 inhibition (by using the

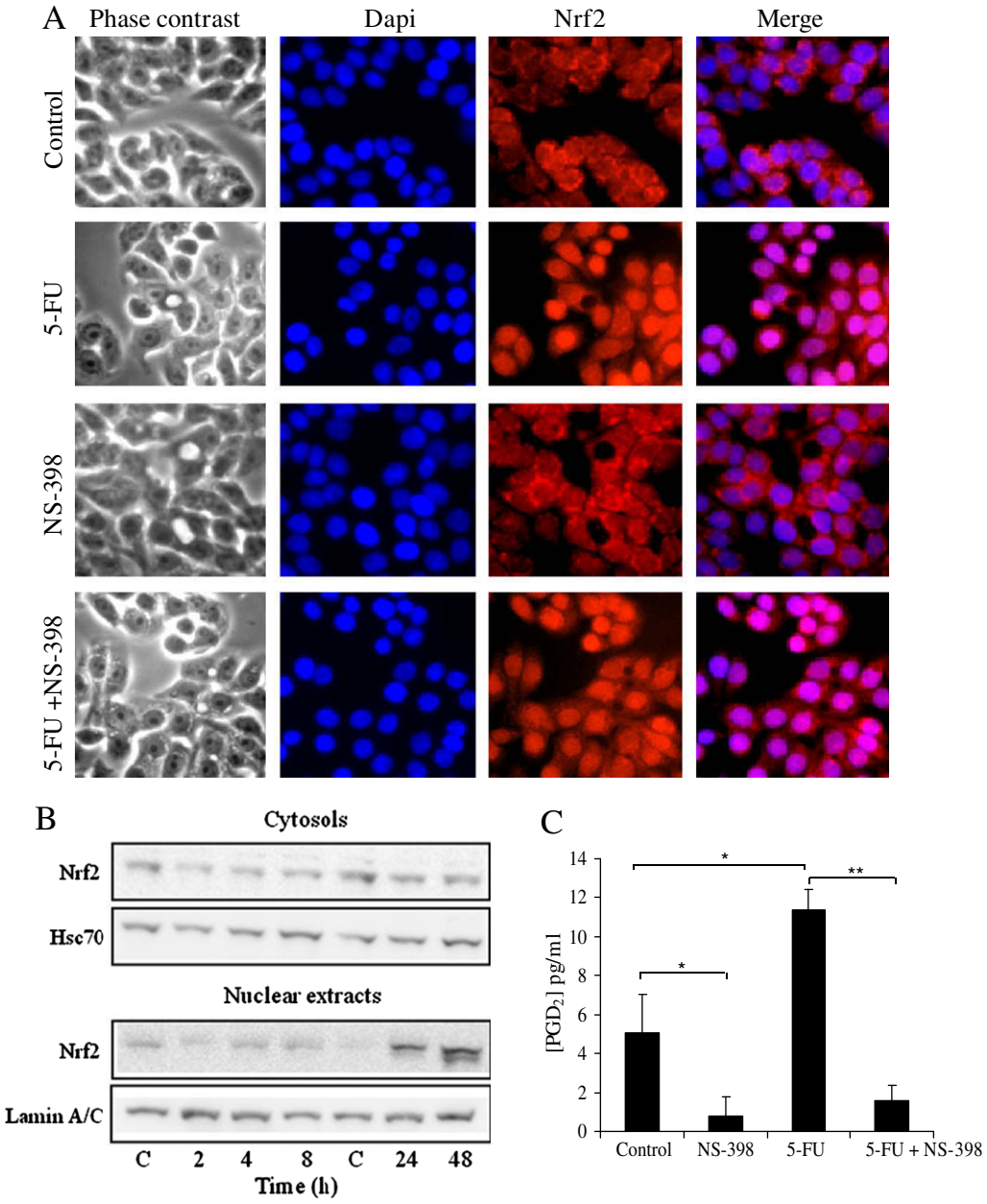

Fig. 3 – Effects of 5-FU on nuclear translocation and activation of Nrf2 in HT-29 cells. (A) HT-29 cells were treated for 24 h with 100 μg/ml 5-fluorouracil alone or in combination with 75 μM of NS-398 (a specific inhibitor of Cox-2). Immunostaining was performed with an anti-Nrf2 antibody and nucleus labelling with a DAPI solution (bar: 20 μm). (B) Nuclear and cytosolic extracts were prepared from HT-29 cells, either untreated (C) or treated with 100 μg/ml 5-fluorouracil for 2–48 h and analysed by western blotting. Anti-Hsc70 and anti-Lamin A/C antibodies were used as loading controls for cytosolic and nuclear fractions respectively. (C) Effect of 5-FU and NS-398 on PGD_2 synthesis. PGD_2 levels were measured by using a Prostaglandin D_2 enzyme immunoassay kit (*, $p < 0.05$; **, $p < 0.01$).

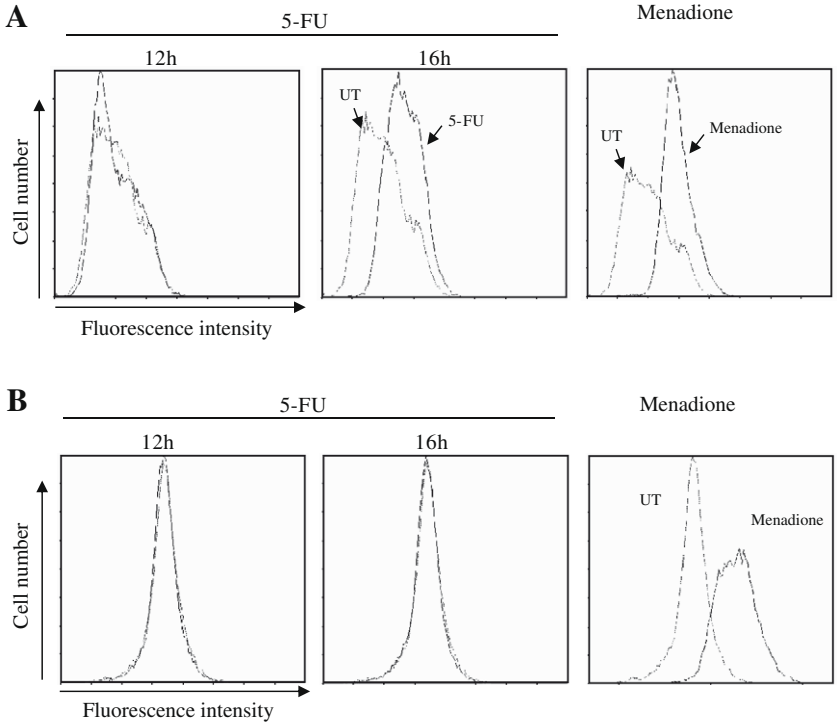

Fig. 4 – Effect of 5-FU treatment on ROS levels. The production of superoxide anion (A) and hydrogen peroxide (B) in HT-29 cells was monitored by flow cytometry by following the oxidation of dihydroethidium (DHE) and dihydrorhodamine (DHR), respectively. DCF fluorescence and DHE fluorescence were examined in cells either untreated (UT) or treated with 100 μg/ml 5-FU for 12 or 16 h. A positive control was obtained by incubating cells with 5 mM menadione for 1 h. The results are presented as an overlay between control and 5-FU or menadione histograms to visualise a shift due to production of ROS. This figure is a representative of three independent experiments.

selective inhibitor NS-398) on Nrf2 nuclear translocation in HT-29 cells treated with 5-FU. NS-398 efficiently inhibits PGD_2 synthesis (Fig. 3C). As shown by immunocytochemistry (Fig. 3A), the treatment of HT-29 cells with 5-FU for 24 h in the presence of NS-398 did not prevent translocation of Nrf2 from the cytoplasm to the nucleus. We therefore investigated whether 5-FU induced ROS levels in HT-29 cells. DHE and DHR fluorescent probes were used to detect intracellular superoxide anion and hydrogen peroxide production, respectively. Interestingly, we demonstrate an increase in superoxide anion levels after 16 h of 5-FU treatment while hydrogen peroxide levels were similar in untreated and treated cells (Fig. 4).

In order to determine whether 5-FU activated the Nrf2/ARE pathway, an ARE-luciferase reporter plasmid containing four ARE copies upstream to the luciferase gene (pGL3-4xARE) was engineered and transiently transfected in HT-29 cells. A 24 h treatment with 5-FU resulted in a 5-fold increase of luciferase activity (Fig. 5). By contrast, 5-FU had no effect on luciferase activity when cotransfection of HT-29 cells was performed with pGL3-4xARE and the Nrf2 dominant negative expression vector.

Since several genes encoding GSTs or antioxidant enzymes (HO-1, γ-GCS and NQO1) contain functional or putative ARE sequence in their 5'-flanking regions, we examined the effects of 5-FU on HO-1, γ-GCS, NQO1, MnSOD, GSTS1 and GSTM3 mRNA levels in HT-29 cells transfected with a siRNA directed against Nrf2. As shown in Fig. 6A and B, Nrf2 siRNA dramatically decreased the amount of both Nrf2 mRNA and protein. Interestingly, siRNA-mediated down-regulation of Nrf2 completely prevented the induction of HO-1 and NQO1 and partially repressed γ-GCS induction following 5-FU treat-

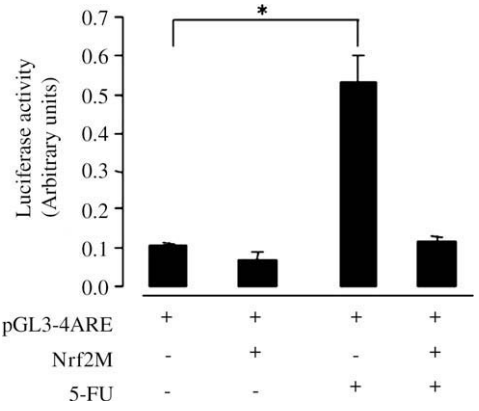

Fig. 5 – Transactivation of pGL3-4xARE reporter vector by 5-FU. Cells were transfected with the pGL3-4xARE reporter vector either in the presence or the absence of the expression vector Nrf2M (coding for a Nrf2 dominant negative) and treated with 100 μg/ml 5-FU for 24 h. Luciferase activities are presented as means ± SD of four independent experiments (*, $p < 0.05$).

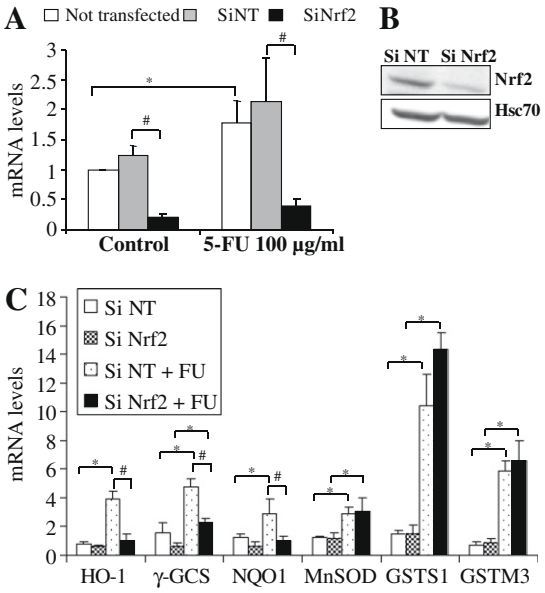

Fig. 6 – Role of Nrf2 in the induction of GST and antioxidant enzymes by 5-FU. (A) Nrf2 mRNA expression in HT-29 cells either untransfected or transfected with a non-targeting siRNA (Si NT) or a siRNA directed against Nrf2 (Si Nrf2). The cells were cultured in the absence (control) or presence of 5-FU for 24 h. (B) Western blot analysis of Nrf2 protein levels 72 h after transfection with a non-targeting siRNA (Si NT) or a siRNA directed against Nrf2 (Si Nrf2). An anti-Hsc70 antibody was used as a loading control. (C) Transcript levels of GSTs (GSTS1, GSTM3) and antioxidant enzymes (HO-1, γ-GCS, NQO1) in HT-29 cells transfected with a non-targeting siRNA (Si NT) or a siRNA directed against Nrf2 (Si Nrf2) in the absence or presence of 100 μg/ml 5-FU. Statistical analysis was performed by comparison of untreated with 5-FU-treated cells (*, $p < 0.05$) and Si NT with Si Nrf2-transfected cells (#, $p < 0.05$).

ment. By contrast, Nrf2 siRNA did not prevent 5-FU-induction of MnSOD, GSTS1 and GSTM3 (Fig. 6C).

3.3. *Administration of Nrf2 siRNA sensitises HT-29 cells to 5-fluorouracil treatment*

Since 5-FU activates the Nrf2/ARE pathway and increases expression of cell defence enzymes, we investigated the putative role of Nrf2 in HT-29 cell sensitivity to this drug. HT-29 cells were transfected with either non-targeting siRNA or Nrf2 siRNA, and, 72 h later, treated with 5-FU. A higher level of apoptosis, determined by caspase 3 activity and Hoechst 33342 staining, was observed in 5-FU-treated cells transfected with Nrf2 siRNA compared to those transfected with non-targeting siRNA ($p < 0.05$) (Fig. 7A and B). Neutral red cytotoxicity test showed that cell survival was much lower in 5-FU-treated cells transfected with Nrf2 siRNA compared to those transfected with non-targeting siRNA (Fig. 7C). These results support the hypothesis that cytotoxic effects of 5-FU could be enhanced by inhibiting the Nrf2 pathway in HT-29 cells.

4. Discussion

Although a protective role of the Nrf2-antioxidant system against carcinogenesis and chemicals has been well documented in normal cells, its prolonged activation in cancer cells might increase resistance to anticancer drugs. By using the human colon adenocarcinoma HT-29 cell line, we provide here the first evidence that 5-FU induces nuclear translocation and activation of Nrf2, which in turn leads to antioxidant enzymes up-regulation and increases resistance to toxic effects of this anticancer drug.

Multiple mechanisms appear to be involved in Nrf2 activation including its phosphorylation by MAP kinases[21,22] and conformational changes of its major upstream regulator, Keap1. Indeed, Nrf2 is normally sequestered in the cytoplasm by Keap1 and is targeted for ubiquitination and degradation by the proteasome.[23] Induction of Nrf2/Keap1 dissociation leads to Nrf2 nuclear translocation and activation of its target genes.[24] Regarding upstream factors which activate Nrf2, the involvement of ROS and 15-dPGJ_2 has been established.[25] In our study, we demonstrate that 5-FU induces the expression of Cox-2 (which converts arachidonate to PGH_2) and GSTS1 which catalyses isomerisation of PGH_2 to produce PGD_2. Induction of Cox-2 and GSTS1 is of particular interest since these enzymes contribute not only to PGD_2 production but also to the formation 15d-PGJ_2, which modulates the activities of transcription factors such as Nrf2 and nuclear factor-κB (NFκB). Therefore, we have investigated the potential role of 15d-PGJ_2 in 5-FU-dependent nuclear translocation/activation of Nrf2. Using NS-398, a selective inhibitor of Cox-2, we showed that NS-398 did not prevent translocation of Nrf2 into the nucleus. Thus, we conclude that activation of Nrf2 by 5-FU is independent of 15d-PGJ_2 synthesis. Thereafter, we have measured ROS levels in HT-29 cells in the absence or the presence of 5-FU and demonstrated that H_2O_2 levels remained unchanged, an observation in agreement with a previous study demonstrating that 5-FU did not induce H_2O_2 levels in HT-29 cells.[26] In contrast, we showed that superoxide anion levels were increased in treated-cells when compared to untreated cells. Therefore, this result suggests that Nrf2 might be activated by ROS in HT-29 cells treated with 5-FU.

Interestingly, a recent work of Shibata et al.[27] reported that *Keap1* gene deletion provoked an aberrant Nrf2 activation and is one of the molecular mechanisms explaining chemotherapeutic resistance against 5-FU in gallbladder cells. Here, we demonstrate that HT-29 cells, when transfected with siRNA directed against Nrf2, are more sensitive to 5-FU than cells transfected with a non-targeting siRNA. Thus, we confirm and extend the previous observations of Shibata et al. by showing that Nrf2 is involved in the resistance to 5-FU in a different cell type, the HT-29 colon cancer cells which express Keap1.

Our study also showed that Nrf2 is involved in antioxidant enzymes induction. Indeed, we observed either a complete or a partial inhibition of HO-1, γ-GCS and NQO1 inductions by 5-FU in HT-29 cells in which Nrf2 expression was down-regulated by transfection of specific siRNA. Conversely, no effects were observed for GSTM3, GSTS1 and MnSOD suggesting that induction of these genes by 5-FU is Nrf2-independent. In

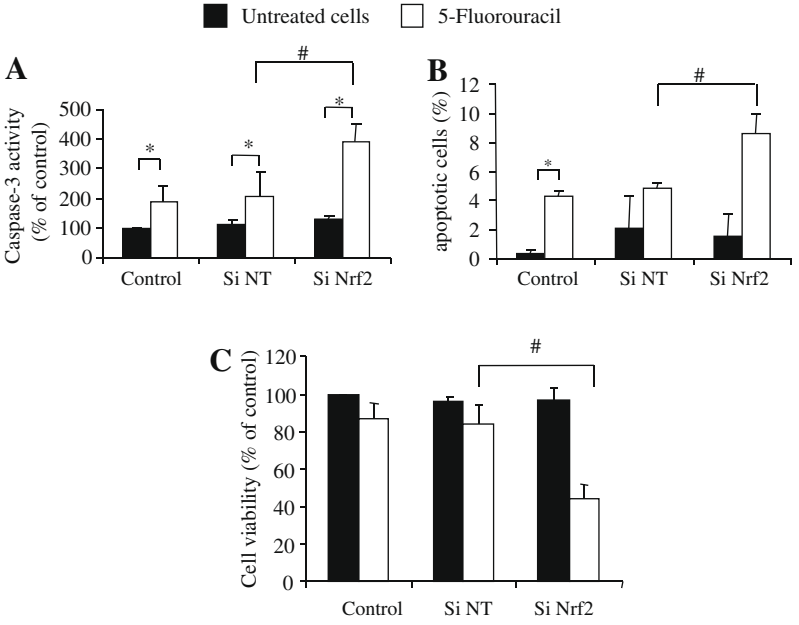

Fig. 7 – Role of Nrf2 in 5-FU sensitivity in HT-29 cells. HT-29 cells were untransfected (control) or transfected with non-targeting siRNA (Si NT) or Nrf2 siRNA (Si Nrf2) for 72 h. The cells were then cultured in the absence or presence of 100 μg/ml 5-FU for 24 h. (A) caspase 3 activity, (B) staining with Hoechst 33342 and (C) cell viability determined by the neutral red assay. The data are presented as means ± SD of three independent experiments. Statistical analysis was performed by comparison of untreated with 5-FU-treated cells (*, $p < 0.05$) and Si NT with Si Nrf2-transfected cells (#, $p < 0.05$).

order to determine whether HO-1 and γ-GCS might be involved in 5-FU resistance, we investigated the effects of 5-FU in HT-29 cells transfected with either non-targeting siRNA or siRNAs directed against either HO-1 or γ-GCS. Caspase 3 activities were not significantly different between cells transfected with HO-1 or γ-GCS and non-targeting siRNA (*Supplementary* Fig. 1). A similar result was obtained when HT-29 cells were transfected with siRNA directed against GSTM3. The substrate specificities of enzymes encoded by GSTS1 and GSTM3 genes are partially overlapping; both catalyse the isomerisation of PGH_2[28] and the reduction of organic hydroperoxides,[29,30] thereby not excluding the possibility of a compensatory effect by GSTS1.

In addition to the induction of *γ-GCS*, *HO-1* and *NQO1* genes, Nrf2 is responsible for transcriptional activation of numerous genes. In a recent study, it was estimated that more than 250 genes might be regulated by Nrf2 in human cells.[31] Thus, resistance of HT-29 cells to 5-FU cannot be attributed to only one or two target genes of Nrf2, but probably to a set of genes playing a role in cytoprotection.

Altogether, our data demonstrate for the first time that 5-FU activates the Nrf2-ARE signalling pathway in HT-29 cells. This activation leads to induction of cytoprotective systems which in turn might contribute to resistance of HT-29 cells to 5-FU treatment. Therefore, we postulate that Nrf2 might represent a potential therapeutic target in 5-FU treatments of colon cancer.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.05.017.

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