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ES936 stimulates DNA synthesis in HeLa cells independently on NAD(P)H:quinone oxidoreductase 1 inhibition, through a mechanism involving p38 MAPK

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ABSTRACT

The indolequinone ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione) is a potent mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1). Here, we report that ES936 significantly stimulated thymidine incorporation in sparse cultures of human adenocarcinoma HeLa cells, but was without effect in dense cultures. Stimulation of DNA synthesis was not related with a DNA repair response because an increase in thymidine incorporation was not observed in cells treated with 2,5 bis-[1-aziridyl]-1,4 benzoquinone, a well-established antitumor quinone that causes DNA damage. Conversely, it was related with an increase of cell growth. NQO1 inhibition was not involved in ES936 stimulation of DNA synthesis, because the same response was observed in cells where NQO1 expression had been knocked down by small interfering RNA. Stimulation of DNA synthesis was reverted by treatment with ambroxol, a SOD mimetic, and by pyruvate, an efficient peroxide scavenger, supporting the involvement of alterations in cellular redox state. Pharmacological inhibition of p38 with either SB203580 or PD169316 completely abolished ES936-stimulated DNA synthesis, indicating the requirement of p38 activity. This is the first report that demonstrates the existence of an ES936-sensitive system which is separate from NQO1, modulating the redox state and cell growth in HeLa cells through a p38-dependent mechanism. Our results show that the effect ES936 exerts on DNA synthesis may be either positive or negative depending on the cellular context and growth conditions.

1. Introduction

ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione) is a potent mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1) in the nanomolar range [1]. It was previously proposed that ES936 could be useful to study NQO1 functions in cellular systems as an alternative to dicoumarol, the classical NQO1 competitive inhibitor, which however has the drawback of a low specificity when used on cellular systems [1–3]. The initial observation that ES936 strongly inhibits the growth of human pancreatic cancer cells (which overexpress NQO1) was thus interpreted as the result of NQO1 inhibition [4]. However, recent investigations have revealed that ES936 may exert its effects on cells through mechanisms other than NQO1 inhibition. In this way, a series of indolequinones based on ES936 was used to evaluate NQO1 inhibition and growth inhibitory activity in the human pancreatic BxPC-3 and MIA PaCa-2 tumor cell lines. It was found that potent inhibitors of NQO1 activity were relatively poor inhibitors of cell proliferation, and conversely, compounds which were not inhibitors of NQO1 enzymatic activity demonstrated potent growth inhibition [4,5]. Thus, NQO1 inhibitory activity can be clearly dissociated from growth inhibitory activity [5]. Moreover, these compounds have been recognized recently as thioredoxin reductase inhibitors, which could explain growth inhibition of human pancreatic cancer cells by indolequinones [6].

According to these previous observations, it is expected that the effects of ES936 exerted on cell growth are dependent on the cellu-

Abbreviations: DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DZQ, 2,5 bis-[1-aziridyl]-1,4 benzoquinone; ES936, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione; FCS, fetal calf serum; Het, hydroethidine; NQO1, NAD(P)H, quinone oxidoreductase 1; ROS, reactive oxygen species; siRNA, small interfering RNA.

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lar context. Therefore, we wanted to study how ES936 affects cell growth in a different cellular model. For our studies we have chosen human adenocarcinome HeLa cells cultured either at low or high cellular density, conditions where the cells express different levels of antioxidant enzymes [7–9].

Despite the well-known effect of ES936 as a growth inhibitor of human pancreatic cancer cells, we demonstrate here for the first time that ES936 stimulated DNA synthesis in HeLa cells cultured in the absence of serum. This effect, observed in sparse but not in dense cultures, was not related to a DNA repair response but to growth stimulation under serum-free conditions. The analysis of ES936 effects on HeLa cells in which NQO1 expression had been knocked down by siRNA techniques allowed us to conclude that NQO1 inhibition is not a major factor involved in ES936-stimulated DNA synthesis. A pharmacological approach using well-established inhibitors of MAPK signalling pathways indicated that ES936-stimulated DNA synthesis required the activity of the MAPK p38 in HeLa cells. Taken together, our results reinforce the notion that ES936 has different targets other than NQO1 in the cells. Furthermore, a major novelty of our results is that the effects of ES936 on cell growth of human cancer cells may be more complex than initially supposed because, not only inhibitory, but also stimulatory responses can be observed in cells, due to its action on signalling transduction pathways which are strongly dependent on the cellular context and growth conditions.

2. Materials and methods

Unless otherwise indicated, all chemicals were purchased from Sigma (Spain).

2.1. Cell cultures and treatments

HeLa adenocarcinoma cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were cultured in Dulbecco's modified essential medium (DMEM) and supplemented with 10% fetal calf serum (FCS, Linus-Cultek, Spain), 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml amphotericin B and 4 mM L-glutamine. Cells were cultured in 6-well plates (9.6 cm^2/well) and the culture medium was changed every two days until cells reached the densities required for each experiment: about 8000 viable cells/cm^2 (sparse) or 90,000 cells/cm^2 (dense, about 80% confluent, see Fig. 1). Cells were photographed unstained by Nomarski interference contrast microscopy at a 200× magnification using a Leica DM 5000 B microscope equipped with a Leica DC 500 digital camera. The percentage of surface occupied by cells was analyzed using ImageJ software.

Once cells had reached the required densities, they were preincubated in the corresponding serum-free medium for 24 h and then treated with ES936 in medium without serum for another 24 h period. Viability of cells was estimated by the Trypan blue-exclusion assay after separation of cells from culture dishes using a Trypsin-EDTA detaching solution (Sigma, Spain).

The indolequinone ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione), synthesized as described by Beall et al. [10], was kindly provided by Dr. Christopher J. Moody (University of Nottingham, United Kingdom). Stock solutions of ES936 were prepared at 10 mM in DMSO, and added to cells to reach a final concentration ranging from 30 to 1000 nM. Concentrations of ES936 stock solutions were verified by spectrophotometric reading at 292 nm using an extinction coefficient of 22.16 mM^{-1} cm^{-1} (C.J. Moody, personal communication).

The involvement of EGF receptor (EGFR)-dependent signalling pathways and MAPK cascades in the effects of ES936 was tested by simultaneous treatment of cells with ES936 and several well-known inhibitors of these routes. The EGFR inhibitor AG1478 was prepared at 15 mM in DMSO and then given to the cells at final concentrations ranging from 0.1 to 10 μM. SB203580 and PD169316 (Calbiochem, Nottingham, UK), two inhibitors of p38 MAPK cascade, were prepared at 10 mM in DMSO and diluted with the culture medium to concentrations ranging from 0.1 to 20 μM. PD98059, an inhibitor of the ERK1/2 pathway was prepared at 20 mM in DMSO. Cells were treated with PD98059 at concentrations ranging from 1 to 50 μM. The Jun kinase inhibitor SP600125 (Calbiochem, Calbiochem, Nottingham, UK) was prepared at 15 mM in DMSO. Final concentrations of SP600125 in cell treatments ranged from 1 to 10 μM. Putative involvement of changes in cellular redox state were studied by treatment of HeLa cells with ES936 in the presence of 0.1 mM ambroxol, a SOD mimetic, or 10 mM pyruvate, a well-established H_2O_2 scavenger [7,11,12]. The same amount of the corresponding vehicle was added to the controls in all experiments, with no significant effect being observed. Concentrations for all compounds were chosen that did not affect cell viability, estimated by the Trypan blue-exclusion method.

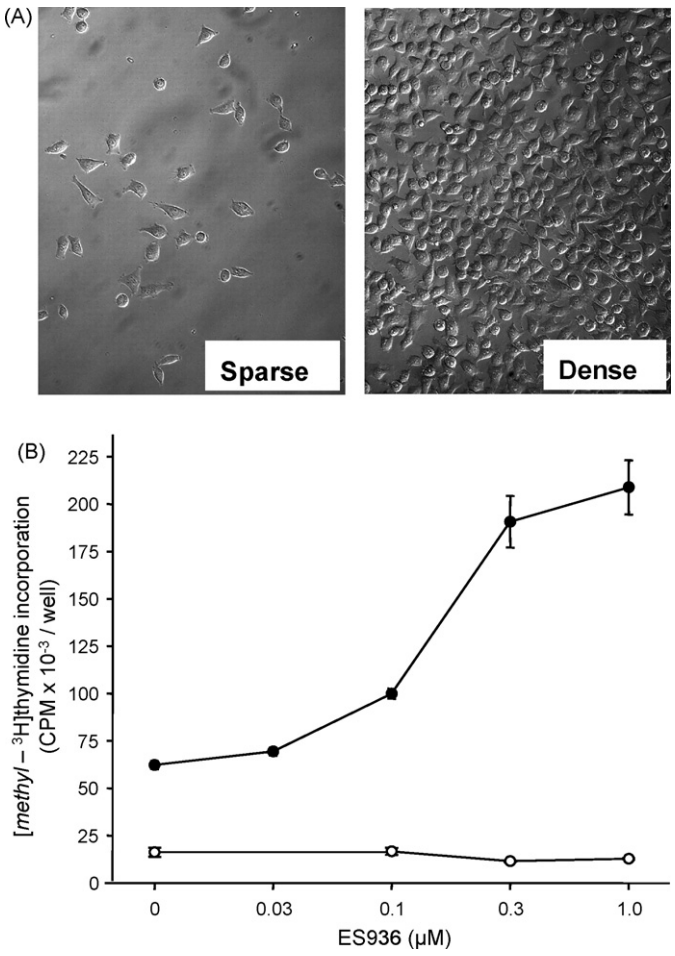

Fig. 1. (A) Representative images of sparse and dense cultures of HeLa cells used in this study. Micrographs were taken at an original magnification of 200×. (B) Effect of ES936 on DNA synthesis in HeLa cells. ES936 had a minor inhibitory effect on dense cultures ($p < 0.001$), but strongly stimulated thymidine incorporation in sparse cultures of HeLa cells ($p < 0.001$). Stimulation of DNA synthesis was already observed at 100 nM ES936, and this effect reached a plateau at 300 nM and 1 μM. Data are means ± SD ($n = 3$).

2.2. Determination of DNA synthesis

DNA synthesis was measured from the ability of cells to incorporate radiolabelled thymidine. Briefly, cells grown to either low

or high density were precultured for 24 h in the absence of serum and then incubated with 0.25 μCi/ml [methyl-^{3}H]thymidine for another 24 h. The latter period corresponded to the treatment with ES936. As a control for maximal DNA synthesis, cells were also stimulated with 10% FCS after the 24 h preculture in serum-free medium. After incubation with ES936 plus [methyl-^{3}H]thymidine, culture plates were put on ice and cells were washed twice with cold 0.9% NaCl. Cold trichloroacetic acid at 5% was added, the supernantants were discarded, and cells were then lysed with 0.1 N NaOH. Lysates were used for measuring incorporation of insoluble-TCA radioactivity using a liquid scintillation counter (Beckman, Palo Alto, USA).

2.3. Preparation of cytosolic fractions

All procedures were carried out at 4 °C. Cells were separated from culture dishes as described above, concentrated by centrifugation at 1000 × *g* for 5 min and washed with cold 130 mM Tris–HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT and 1 mM PMSF. Cells were centrifuged again and resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris–HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and 20 μg/μl each of chymostatin, leupeptin, antipain and pepstatin A (CLAP)). Cells were disrupted for 5 min with the aid of a glass–glass Dounce homogenizer and then for 30 s with a mechanical cell homogenizer (Ultraturrax T-25, IKA Labortechnik). After disruption of cells, the concentration of the lysis buffer was rised to 100 mM Tris by adding enough volume of 250 mM Tris buffer, pH 7.6 containing 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and CLAP). Unbroken cells and debris were separated by centrifugation at 800 × *g* for 5 min and the supernatant was saved. Cytosolic fractions were separated from membranous material by ultracentrifugation at 100,000 × *g* for 30 min.

2.4. Determination of NQO1 activity

NQO1 activity in cytosolic fractions was measured from the dicoumarol-inhibitable, NADH and menadione-dependent reduction of cytochome *c* [13]. Assays were carried at 37 °C with constant gentle stirring. The assay mixture (1 ml) contained 70 μg of cytosolic protein in 50 mM Tris–HCl (pH 7.5), 0.08% Triton X-100, 0.5 mM NADH, 10 μM menadione and 77 μM cytochrome *c*. Assays were carried out either in the absence or in the presence of 10 μM dicoumarol and absorbance was recorded at 550 nm in a Beckman DU-640 UV–vis spectrophotometer. NQO1 activity was calculated from the difference in reaction rates obtained with and without dicoumarol. An extinction coefficient of 18.5 mM^{-1} cm^{-1} was used in calculations of specific activities [13]. Protein determinations were carried out with the Bradford method.

2.5. NQO1 immunodetection

Prior to electrophoresis, cytosolic fractions (about 70 μg protein) were mixed with concentrated SDS-dithiothreitol loading buffer final concentration, 10% sucrose, 2 mM EDTA, 1.5% (w/v) SDS, 20 mM dithiothreitol, 0.01% (w/v) bromophenol blue and 60 mM Tris–HCl, pH 6.8. After boiling for 5 min, samples were separated by SDS-PAGE (12% acrylamide) and blotted onto nitrocellulose sheets. Blots were stained with Ponceau S for visualization of protein lanes and verification of equal protein loading, and then stained with a polyclonal goat antibody raised against human NQO1 (Santa Cruz Biotechnology, CA, USA) diluted to 1:100 and a secondary anti-goat antibody coupled to horseradish peroxidase diluted to 1:5000. Developing of images was performed by enhanced chemiluminescence (ECL Plus, Amersham Bioscience, Uppsala, Sweden). Photographic films were scanned to obtain digital images for quantification of intensity reaction using Quantiscan software (Biosoft, USA). Data obtained from quantification of stained bands (in arbitrary units) were normalized to that of the corresponding lane stained with Ponceau S to correct for minor differences in protein loading between samples.

2.6. Measurement of p38 phosphorylation

About 10^7 cells were centrifuged at 500 × *g* for 5 min, washed with PBS, and centrifuged again under the same conditions. Cell pellets were resuspended in 250 μl of extraction buffer (50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM sodium ortovanadate, 1 mM PMSF and (CLAP). After gentle agitation, extracts were centrifuged at 10,000 × *g* for 15 min at 4 °C to remove undisrupted cells and debris. Supernatants were saved for determinations of total protein and polyacrylamide gel electrophoresis followed by western blotting. Blots were then assayed for p38 activation using a monoclonal antibody raised against the phosphorylated form of p38 (1:500 dilution). Equal loading of each lane was assessed by stripping of bound antibodies from membranes as described above, and staining with a polyclonal antibody (1:1000 dilution) which recognizes total p38 (Santa Cruz Biotechnology). Developing of images was also performed by enhanced chemiluminescence.

2.7. Measurement of intracellular reactive oxygen species

Reactive oxygen species levels in cells were quantified using the probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (Het) for peroxides and superoxide respectively [11]. Briefly, cells were incubated in the dark with the corresponding probe (final concentrations: 10 μg/ml DCFH-DA and 4 μM Het) for 30 min at 37 °C. After washing, fluorescence was determined by flow cytometry at 525 nm (FL1) for fluorescein, and 620 nm (FL3) for ethidium.

2.8. Knockdown of NQO1 gene expression by siRNA silencing

We followed a gene expression knockdown procedure, in which transfection with siRNA is carried out while cells attach to the substrate. For these experiments HeLa cells were seeded at low density (4000 cells/cm^2) in 6-well culture plates. The knockdown of NQO1 gene expression was achieved with a NQO1-targeted siRNA (ID #9543, Ambion, USA). Negative controls were run with unspecific scrambled siRNA (Ambion). Stock solutions of siRNA were prepared in DNAase-free distilled water (Ambion) at final concentration of 4 μM for NQO1-targeted siRNA, and 50 μM for the negative control.

Prior to cell treatments, for each well we first mixed 2 μl of siPORT NeoFX transfection reagent (Ambion, USA) with 98 μl of transfection optimized medium OPTIMEM I (Gibco, Paisley, UK), and the mixture was incubated for 10 min at room temperature. Simultaneously, we prepared the required amount of siRNA in OPTIMEM in a final volume of 100 μl/well. Transfection reagent and siRNA were then mixed and the resulting solution was further incubated for 10 min at room temperature. The number of cells to be used for each experiment (36,000 cells/well) were suspended in 1.3 ml of DMEM containing 10% FCS and then added to the transfection cocktail to reach a final volume of 1.5 ml/well. Final concentration of siRNA in each well was 20 nM. The final mixture was added to each well and cells were allowed to attach to the substrate and cultured for a minimum of 24 h. Efficiency of gene silencing was tested by immunodetection of NQO1 polypeptide in western blots as described above. Cells transfected with either NQO1-targeted or unspecific scrambled sequence siRNA were treated with 300 nM ES936 for 24 h in serum-free medium at the time of optimal silencing. DNA synthesis was

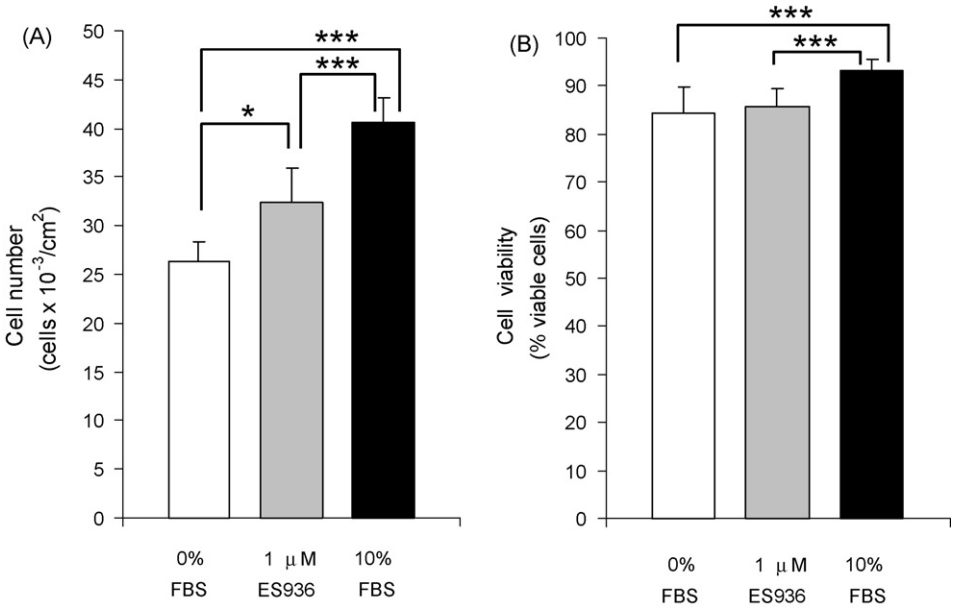

Fig. 2. Effect of ES936 on cell number and viability in sparse cultures of HeLa cells. (A) Cell counts. ES936 treatment produced a significant increase in the number of cells. (B) Cell viability. ES936 did not affect the viability of the cells. Significant differences are depicted with asterisks (*$p < 0.05$; ***$p < 0.001$). Data are means ± SD ($n = 6$).

then measured from the incorporation of radiolabelled thymidine (see above).

2.9. Statistical analysis

Data shown in this article are means ± standard deviations (SD) from at least three different determinations. Significant differences between means were assessed using Student's *t* test. The effect of the tested concentrations of ES936 and the different pharmacological inhibitors was analyzed by one-way analysis of variance. Differences were considered significant with $p < 0.05$. Data were analyzed using Graphpad Prism 4.00 (Graphpad Software Inc, San Diego, CA, USA) or Microcal™ Origin™ 4.10 (Microcal software Inc., Northampton MA, USA).

3. Results

3.1. Effects of ES936 on DNA synthesis and growth of HeLa cells

First, we tested how treatment with ES936 affected the ability of cells to incorporate radiolabelled thymidine in sparse and in dense cultures of HeLa cells (Fig. 1A and B). As depicted in Fig. 1B, DNA synthesis was not altered by ES936 up to 100 nM in confluent HeLa cultures, where NQO1 levels were upregulated by 10–15 times [7,8]. A decrease in thymidine incorporation was then observed at 300 nM and 1 μM, which fits with the reported effect of ES936 inhibiting the growth of pancreatic cancer cells, which also overexpress NQO1 (see Introduction). Strikingly, in the case of sparse cultures, treatment with ES936 at concentrations of 300 nM–1 μM increased DNA synthesis about 3-times.

ES936 has been reported to provoke DNA damage [1]. Since the stimulation of DNA synthesis we show here could be related with the repair of DNA strand breaks, we tested how thymidine incorporation was affected by a well-characterized antitumor quinone that damages DNA, such as the 2,5 bis-[1-aziridyl]-1,4 benzoquinone (DZQ) [14]. Unlike the results observed for ES936, the synthesis of DNA was absolutely unaffected by the presence of DZQ in the culture media at concentrations of up to 500 nM (data not shown). These concentrations are well above those enough to provoke a significant inhibition of cell growth in the same cell type [15]. These results support that DNA reparation mechanisms are unlikely to be related with the stimulation of DNA synthesis induced by ES936 treatment in HeLa cells.

We then hypothesized that stimulation of thymidine incorporation could be related to an effect of ES936 on cell growth. In accordance with this interpretation, treatment with 1 μM ES936 resulted in a significant increase in the number of cells compared with the controls, reaching one half of the number obtained after stimulation with 10% FCS (Fig. 2A). Viability of cultures, measured from the Trypan blue-exclusion assay, was maintained at values of about 85% after 24 h treatments in serum-free medium, regardless ES933 was added or not, whereas the viability was higher than 90% in cells stimulated with 10% FCS (Fig. 2B). Taken together, our results support that ES936 can stimulate the growth of HeLa cells in sparse cultures.

3.2. ES936 stimulation of DNA synthesis does not involve NQO1 inhibition

ES936 was claimed to be a very specific NQO1 inhibitor and no effect was found on the activity of two other major reductases of the cell, such as NADPH:cytochrome P450 reductase and NADH:cytochrome b_5 reductase, even at concentrations of ES936 up to 10 μM [1]. However, it has been demonstrated more recently that inhibition of cell growth by ES936 in human pancreatic cancer cells is not related with NQO1 inhibition [4,5]. Moreover, growth inhibition by indolequinones can be explained on the basis of their effect as thioredoxin reductase inhibitors [6]. Thus, it is of importance to test whether or not NQO1 inhibition was related with the stimulation of cell growth that we show here. For that, we first studied how ES936 affected NQO1 activity in sparse HeLa cells and found that, under our experimental conditions, a significant inhibition of NQO1 activity was only achieved at the maximal ES936 concentration (1000 nM). However, a concentration of 300 nM which produced almost maximal stimulation of DNA synthesis, had a negligible effect on NQO1 activity under our treatment conditions (Fig. 3).

To fully ascertain whether or not stimulation of DNA synthesis by ES936 was mediated by NQO1 inhibition we performed a genetic approach by testing the effect of ES936 in HeLa cells in

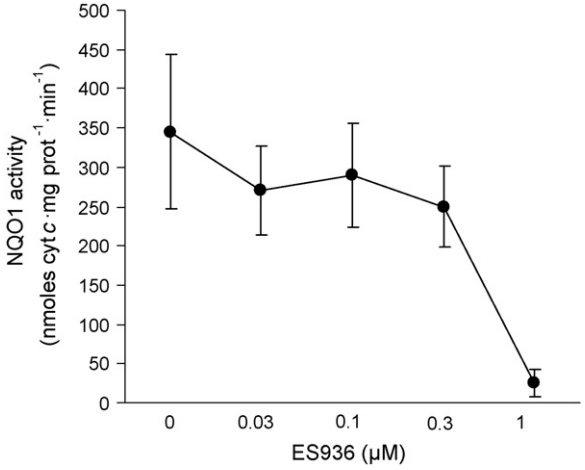

Fig. 3. Inhibition of NQO1 activity by ES936 in sparse HeLa cells. Cells grown at low density were incubated with ES936 at the indicated concentrations for 2 h in culture medium. Cells were then lysed for preparation of cytosolic fractions and NQO1 activity was then assayed in these fractions. Data represent means ± SD ($n = 3$), $p < 0.01$).

which a knockdown of NQO1 gene expression was carried out by siRNA techniques. Control cells were subjected to the same transfection procedure but using an unspecific scrambled siRNA sequence. Transfection of HeLa cells with a NQO1-targeted siRNA produced a dramatic decrease of NQO1 polypeptide at 2 days post-transfection compared with cells transfected with the scrambled sequence (Fig. 4A). This effect was transient, and a recovery of NQO1 gene expression was observed at 7 days, and normal levels of NQO1 polypeptide were observed at 9 days post-transfection with NQO1-targeted siRNA (Fig. 4A). Thus, we decided to start experiments at 2 days post-transfection. At this time cells were precultured in serum-free medium for 24 h (zero time for these experiments), and then treated with 300 nM ES936 in medium without serum containing radiolabelled thymidine for another 24 h (final time). Samples were taken at this time for measuring thymidine incorporation and for monitoring NQO1 levels. As depicted in Fig. 4B, levels of NQO1 polypeptide even decreased after the second 24 h incubation in serum-free medium containing 300 nM ES936. However, ES936 was equally effective in stimulating DNA synthesis independently of the NQO1 status of the cells (Fig. 4C). Thus, our results clearly demonstrate that inhibition of NQO1 is not a major factor involved in stimulation of DNA synthesis by ES936 in sparse cultures of HeLa cells.

3.3. ROS scavengers abolish ES936 stimulation of DNA synthesis

A plausible mechanism to explain the DNA stimulation by ES936 involves a moderate increment in the levels of superoxide or, after its spontaneous dismutation, of hydrogen peroxide, because it has long recognized that at low levels these ROS can promote cell growth in HeLa cells [16,17]. Addition of ambroxol or pyruvate, two well-known scavangers of superoxide and hydrogen peroxide respectively [7,11,12], abolished the stimulation of DNA synthesis by ES936 (Fig. 5). Ambroxol produced a general inhibition of growth, decreasing the incorporation of thymidine in cultures maintained in the absence of serum without ES963, and in cultures stimulated with 10% FCS, but its effect was more pronounced in cultures treated with ES936. Interestingly, pyruvate did not affect the DNA synthesis in cells cultured in serum-free medium without ES936 and in cells stimulated with 10% FCS, but almost abolished the increase of DNA synthesis induced by ES936. However, using DCFH-DA and Het as probes for detection of peroxides and superoxide respectively we were unable to detect by flow cytometry any

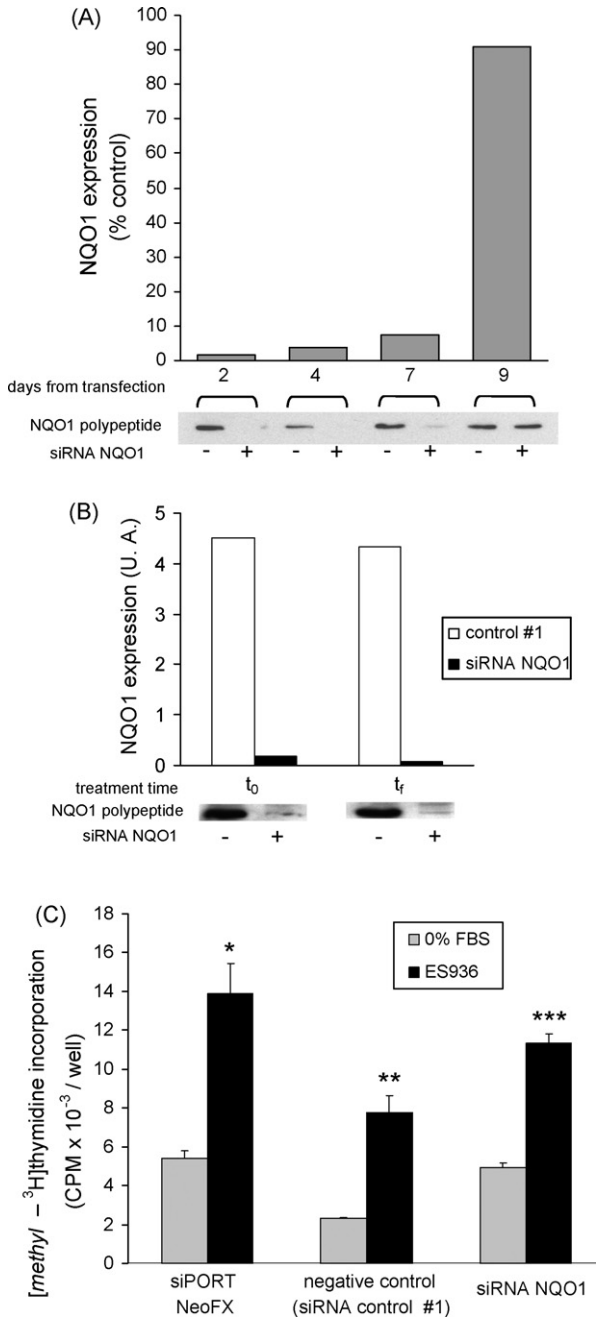

Fig. 4. NQO1 knockdown by siRNA and the effect of ES936 on DNA synthesis in silenced cells. (A) Optimization of the protocol for NQO1 silencing. Cells in sparse cultures were transfected with either a NQO1-targeted or an unspecific scrambled siRNA and then cultured by 2–9 days. Compared to mock-trasfected cells, levels of NQO1 dramatically decreased in cells transfected with the NQO1-targeted sequence at 2 and 4 days, but the polypeptide returned to normal levels at 9 days. (B) Quantification of NQO1 polypeptide in cells treated with ES936. After 2 days of transfection with either a NQO1-targeted or an unspecific scrambled siRNA cells were treated with ES936 as described in Section 2. NQO1 levels were assessed before (t_0) and after (t_f) ES936 treatment. As shown in the figure, NQO1 levels remained extremely low under our conditions for ES936 treatments. (C) The effect of ES936 on DNA synthesis was tested in HeLa cells subjected to incubation with the transfection agent alone (siPORT NeoFX), in cells transfected with the unspecific scrambled sequence (negative control), and in cells transfected with the NQO1-targeted siRNA (siRNA NQO1). As shown in this figure, ES936 was effective in stimulating DNA synthesis in all cases (note an unspecific toxic effect of transfection with the scrambled siRNA, which results in a decrease of radioactive counts for both control and ES936-treated cells). Data in (A and B) depict representative results of three independent determinations. Data in (C) are means ± SD ($n = 3$).

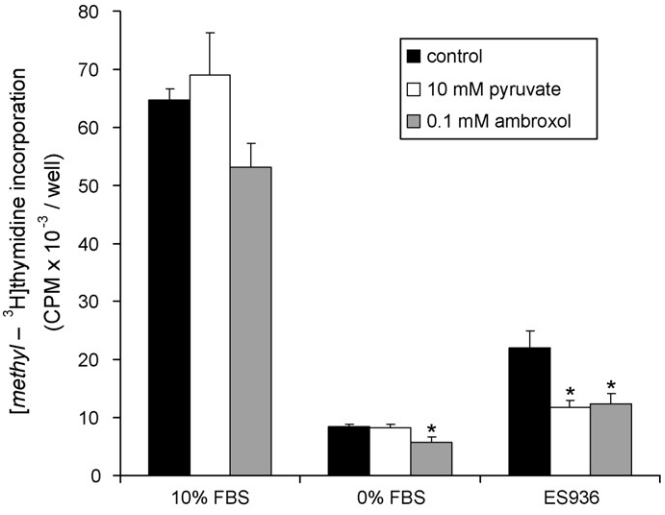

Fig. 5. Effect of ROS scavenging on ES936-stimulated synthesis of DNA. Cells were treated with 300 nM ES936 in the absence of serum, either alone or in the presence of 10 mM pyruvate, a scavenger of hydrogen peroxide, or 0.1 mM ambroxol, a SOD mimetic. Cells stimulated with 10% serum and cells cultured in serum-free medium without ES936 were also treated with the ROS scavengers. Amboxol produced a decrease of thymidine incorporation in all culture conditions. Pyruvate had no effect on cells stimulated with 10% serum and on cells cultured in serum-free medium without ES936, but almost completely abolished the increase of DNA synthesis stimulated by ES936. Experiments were carried out in triplicate. Significant differences with respect to each control without ROS scavenger (closed bars) are depicted with asterisks ($p < 0.05$). Data are means ± SD ($n = 3$).

significant increase of these ROS following ES936 treatment in HeLa cells (data not shown).

3.4. DNA synthesis stimulated by ES936 involves an EGFR- and p38-dependent signalling pathway

We used AG1478, a specific inhibitor of the EGFR, to investigate if the stimulation of DNA synthesis was mediated by activation of EGFR in a ligand-independent manner. As depicted in Fig. 6, AG1478 completely suppressed DNA synthesis stimulated by ES936 in a

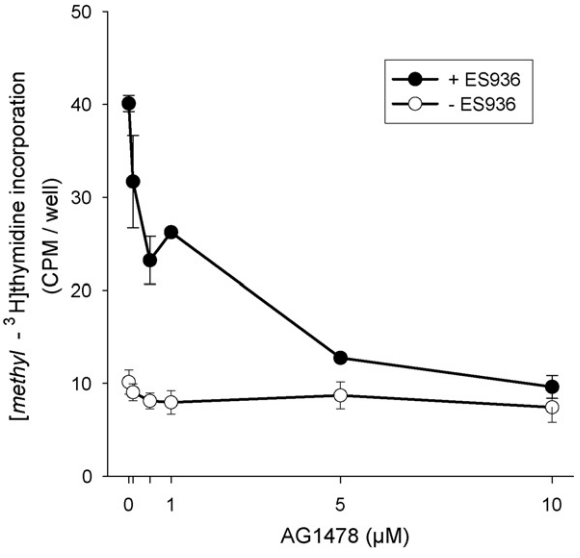

Fig. 6. Effect of AG1418, an inhibitor of EGFR-dependent signalling, on ES936-stimulated DNA synthesis. Cells were treated with ES936 in the presence of AG1418 at various concentrations. The EGFR inhibitor produced a dramatic decrease of thymidine incorporation in cells treated with ES936. Data are means ± SD ($n = 3$), $p < 0.001$.

range of concentrations that were effective to inhibit both EGFR phosphorylation and cell proliferation in other cell lines [18–20].

To determine if MAPK activity was essential for the stimulatory action of ES936 on DNA synthesis of HeLa cells, we used well-established inhibitors specific for each kinase (Fig. 7A–D). ES936-induced DNA synthesis was not affected significantly in cells treated in the presence of the ERK pathway inhibitor PD98059 at concentrations of up to 15 μM, and about a 50% inhibition was obtained at higher (50 μM), but likely unspecific concentrations (Fig. 7A). A partial inhibition of ES936-stimulated DNA synthesis was observed in cells treated with ES963 in the presence of the JNK inhibitor SP600125 at concentrations between 5 and 10 μM (Fig. 7B). Interestingly, DNA synthesis was totally abolished in cells treated with the classic p38 kinase inhibitor SB203580 (Fig. 7C) Significant inhibition of ES936-stimulated DNA synthesis was already achieved even at very low concentrations of SB203580 (100–500 nM). DNA synthesis was further inhibited at SB203580 concentrations between 10 and 20 μM. Since it has been reported that SB203580 can also act on JNK with an IC_{50} of 3–10 μM [21], we also tested the effect of PD169316, a very selective inhibitor acting on p38 MAPK in the nanomolar range [22]. Similarly to SB203580, a strong inhibition of ES936-stimulated DNA synthesis was observed with PD169316, even at concentrations between 100 and 500 nM. Concentrations of PD169316 above 10 μM completely abolished DNA synthesis in cells treated with ES936 (Fig. 7D). These results indicate that disruption of the p38 pathway suppresses DNA synthesis stimulated by ES936.

Given the observed effects of SB203580 and PD169316 on ES936-activated DNA synthesis, we wanted then to study if ES936 was able to activate p38 phosphorylation in sparse HeLa cells. As depicted in Fig. 8 (which represents the results obtained at 5, 10 and 30 min following stimulation with 300 nM ES936), levels of p38 phosphorylation were already very high in control cells cultured in serum-free medium and no increase was observed due to ES936 treatment. The same was observed for longer incubation times with ES936 (up to 6 h, results not shown). Thus, although our pharmacological approach indicates that p38 MAPK activity is clearly essential for ES936 to activate DNA synthesis in sparse HeLa cells, this compound does not activate directly p38 MAPK phosphorylation.

3.5. The effect of ES936 on DNA synthesis is dependent on the cellular context and growth phase

Previous evidence has established that ES936 can inhibit the growth of human pancreatic cancer cells through a mechanism that is separate from NQO1 inhibition, but more likely involving the inhibition of thioredoxin reductase [4–6]. Ours are the first results reporting that ES936 can also activate DNA synthesis and growth of sparse HeLa cells. Thus, we wanted to study how ES936 affected DNA synthesis in other cell lines cultured in the absence of serum, as a function of cell density. For this study we used mouse fibroblastic 3T3 cells, human hepatoblastoma HepG2 cells, and human colon carcinoma Caco-2 cells. In the case of 3T3 cells (Fig. 9A), a non-malignant cell line that arrests growth in G_0 after 24 h of serum deprivation, ES936 was able to stimulate DNA synthesis both in sparse and in dense cultures, although the profile of the cellular response and concentrations required to achieve this effect were different in the two growing conditions. In sparse cells, thymidine incorporation was doubled compared to controls without serum at concentrations of ES936 between 100 nM and 1 μM. In dense cells, a strong stimulation was observed at ES936 concentrations above 500 nM. Contrary to what we had observed previously in HeLa cells, in the case of HepG2 cells (Fig. 9B) ES936 stimulated DNA synthesis in dense cultures, although this effect was only observed at concentrations higher than 1 μM. When ES936 was tested in sparse

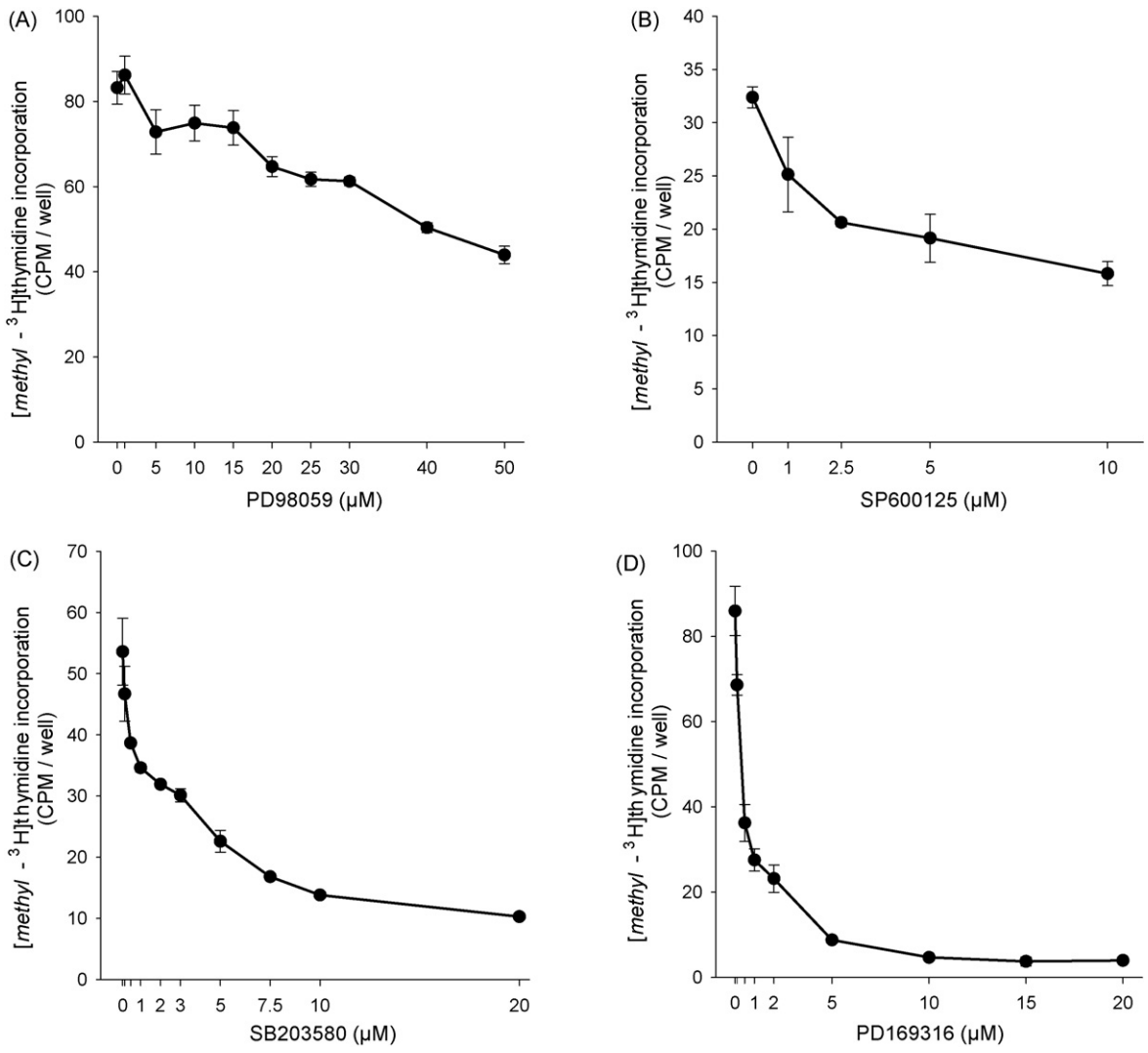

Fig. 7. Effect of MAPK inhibition on ES936-stimulated DNA synthesis. Cells were treated with ES936 in the presence of several inhibitors of MAPK signalling. (A) PD98059, a MEK inhibitor which abolishes activation of ERK1/2, $p = 0.42$; (B) SP600125, a JNK inhibitor, $p < 0.001$; (C) SB203580, a p38 MAPK inhibitor, $p < 0.001$; (D) PD16169316, a p38 MAPK inhibitor, $p < 0.001$. Significant inhibition of ES936-activated synthesis of DNA was observed for the two p38 MAPK inhibitors tested, which supports the involvement of this route in the mitogenic cell response to ES936 of sparse HeLa cells. Data are means ± SD ($n = 3$).

cultures, a strong inhibition of DNA synthesis was observed above 300 nM. In the case of Caco-2 cells, sparse cultures were not stimulated by ES936 but, on the contrary, DNA synthesis was inhibited at concentrations above 300 nM (Fig. 9C). However, when present in dense cultures these cells were not responsive to ES936 and neither activation nor inhibition were observed for all the range of ES936 concentrations tested (Fig. 9C).

Taken together, our results demonstrate that the effect ES936 exerts on DNA synthesis may be either positive or negative depending on the cellular context and growth conditions.

4. Discussion

Because of their potential use in antitumor therapy, there is strong interest in studying the effects of indolequinones on the growth of cancer cells. The indolequinone ES936, a mechanism-based irreversible inhibitor of NQO1 [1], has proven to be very effective in suppressing growth of highly malignant pancreatic cancer cells, through a mechanism that does not involve NQO1 inhibition, but more likely previously unrecognized side effects such as the inhibition of thioredoxin reductase [4–6].

In this paper, we demonstrate for the first time that the effects of ES936 in the growth of cancer cells may be more complex than initially supposed, because not only inhibition, but also no effect or even a significant activation of DNA synthesis can be obtained upon treatment with ES936, depending on the cellular context and growth conditions. In this way, DNA synthesis was activated by ES936 in HeLa cells cultured at low density, but dense cultures were not affected significantly. Fibroblastic 3T3 cells were stimulated by ES936 in sparse and in dense cultures, although concentrations required to achieving this effect were different. Hepatoblastoma HepG2 cells were stimulated in dense cultures,

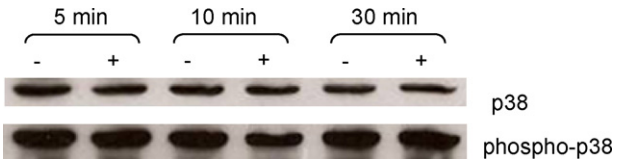

Fig. 8. Measurement of p38 MAPK phosphorylation. Cells were treated with ES936 for the indicated times and then processed as described in Section 2. Western blots were probed for phosphorylated and total p38 MAPK. Experiments were repeated three times. A representative result is depicted in the figure.

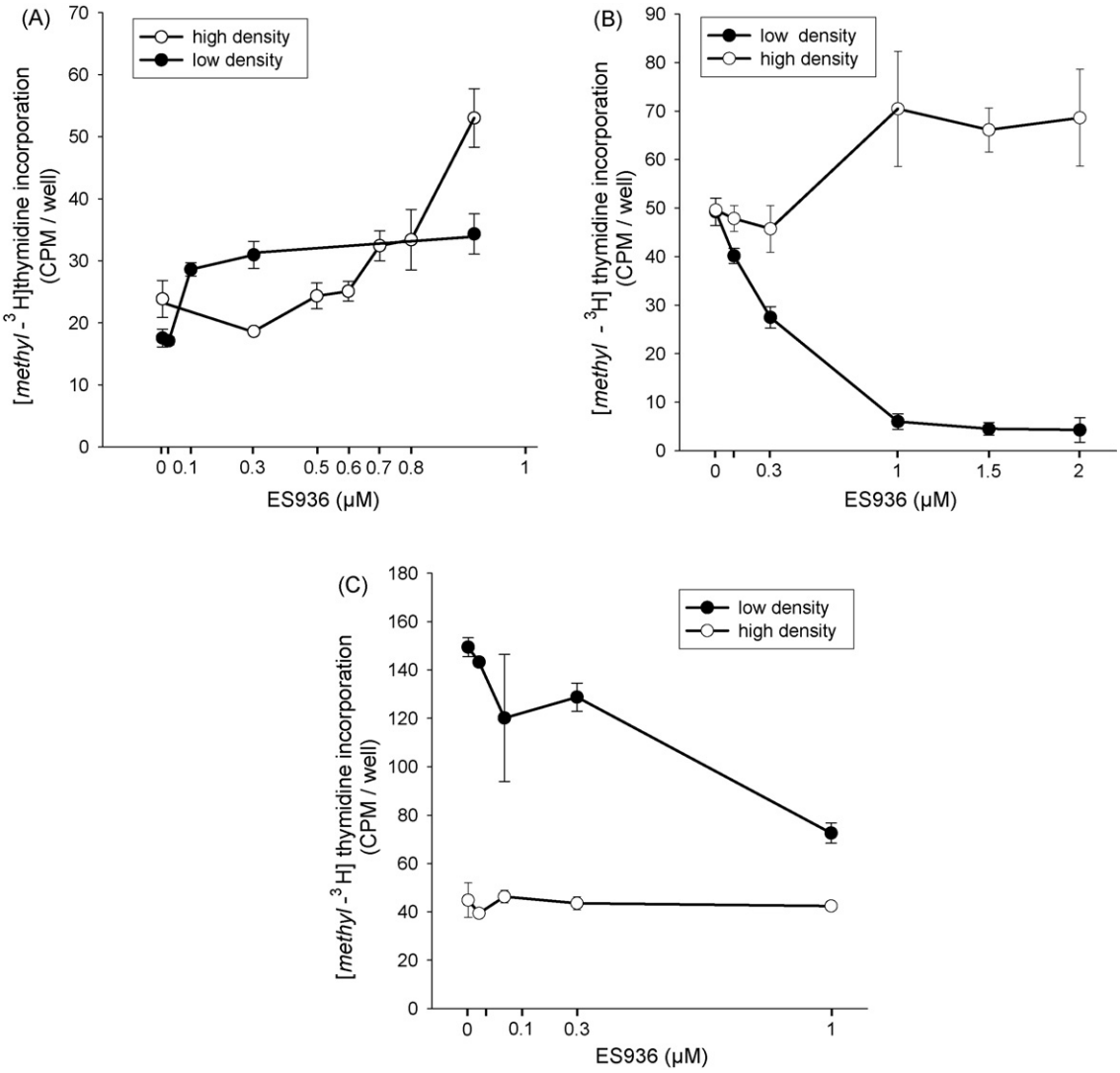

Fig. 9. Effect of ES936 on DNA synthesis in different cell lines. (A) Mouse 3T3 fibroblasts, sparse cells: $p < 0.001$, dense cells: $p < 0.001$; (B) human hepatoblastoma HepG2 cells, sparse cells: $p < 0.001$, dense cells: $p < 0.01$; and (C) human colon carcinoma Caco-2 cells, sparse cells: $p < 0.001$. The effect of ES936 on thymidine incorporation was fully dependent on the cellular context (type of cell and growth conditions). See Section 3.5 for a complete explanation. Data are means ± SD ($n = 3$).

but a strong inhibition of DNA synthesis was observed in sparse cultures. Finally, carcinoma Caco-2 cells were unaffected by ES936 at all the range of concentrations tested when cultured under dense conditions, but sparse cells were strongly inhibited at ES936 concentrations above 300 nM. Caco-2 cells are null for NQO1 activity due to the expression of the NQO1*2 polymorphism encoding a protein that undergoes a rapid proteasomal degradation [23]. Thus, the strong inhibitory action of ES936 on sparse cultures of Caco-2 cells reinforce the notion that inhibition of tumor cells growth by indolequinones such as ES936, previously observed for pancreatic cancer cells, does not rely on NQO1 inhibition [4,5].

The diversity of cellular responses to ES936 may be explained on the basis of the generation of ROS and alterations of redox balance, because responses elicited by ROS in cells are diverse, and include growth stimulation, transient or permanent growth arrest, senescence, apoptosis or necrosis, depending on the intensity and duration of the stimulus, the specific site where ROS are generated, and the cell type [24]. In addition, dependence on density of culture is consistent with the well-known regulation of the expression of several antioxidant enzymes as a function of cell density and growth phase in many cell types [7–9,25–27]. Moreover, cell density-dependent regulation of antioxidant enzymes expression may be even the opposite in different cells lines. For instance, NQO1 levels are upregulated at high density in HeLa cells, but downregulated under the same conditions in HepG2 cells [15]. Interestingly, catalase was also upregulated at high density in HeLa cells, but no change was observed in HepG2 cells [15]. In another way, the small stress protein HSP27 accumulates in confluent Caco-2 cells and blocks cell death by decreasing ROS levels [28]. This could explain why ES936 is toxic in sparse Caco-2 cells but it has no effect on the viability of confluent Caco-2 cultures, as found for several antitumor drugs [28]. A similar density-dependent behaviour of the ES936 target, still to be identified, could explain the diversity of responses to ES936 in the different cell types.

It remains to be determined if cell density per se is the important factor that determines the different responses to ES936, or if confluency itself is the determining factor. In this way, our previous work has documented that a significant increase of NQO1 was already observed well before HeLa cells reached confluence, and NQO1 expression was partially dependent on endogenous H_2O_2 [7]. Thus, as described for the cell density-dependent expression of antioxidant enzymes, different factors in addition to cell-to-cell contacts, which might include some diffusible agent(s), could determine the contrasting response to ES936.

We observed that ambroxol and pyruvate, effective scavengers of superoxide and peroxide respectively, abolished the stimulation of DNA synthesis by ES936. However, no significant increase in these ROS could be evidenced by flow cytometry using Het and DCFH-DA as probes. The inability to demonstrate ROS increases upon ES936 treatment agrees with a previous report where no significant increase in the intracellular superoxide production was detected following incubation of pancreatic cancer cells with ES936, which was explained on the basis on the low sensitivity of this technique [4]. These results suggest that the DNA synthesis stimulated by ES936 may be mediated by subtle changes in intracellular ROS concentrations.

Remarkably, the use of a genetic approach (knockdown of NQO1 gene expression by siRNA) has allowed us to demonstrate unequivocally that NQO1 inhibition is not involved in the stimulatory effect of ES936 on DNA synthesis in sparse HeLa cells. In preliminary experiments, we tested the effects of the thioredoxin reductase inhibitor 1-chloro-2,4-dinitrobenzene under the same conditions that we previously followed for ES936, but the compound was toxic to the cells at concentrations over 10 μM, and no stimulation of DNA synthesis could be observed for any concentration (data not shown). Although we can not discard that inhibition of thioredoxin reductase could participate in the stimulation of DNA synthesis by ES936 in HeLa cells, separate enzyme systems – still to be identified – whose expression is regulated in a cell density-dependent manner might be involved.

The phosphorylation of a wide range of transmembrane receptor tyrosine kinase families, including epidermal growth factor receptor (EGFR), in the absence of ligand binding, and the activation of MAPK family can be mediated by oxidative molecules, likely contributing to the initiation of specific signalling cascades and stress-related responses within cells [29,30]. Using a pharmacological approach with well-established inhibitors of EGFR-dependent signalling (AG1478), MEK-ERK1/2 (PD98059), JNK (SP600125), and p38 (SB203580 and PD169316) we concluded that stimulation of DNA synthesis by ES936 involves ligand-independent activation of EGFR and signalling through p38. PD98059 only produced a modest inhibition of ES936-stimulated DNA synthesis, and this was observed mainly at high concentrations of the MEK inhibitor, suggesting that ERK1/2 activation does not play a major role in mediating ES936 effects. SP600125, a JNK inhibitor, showed a partial inhibition in ES936-induced DNA synthesis, indicating that JNK could also participate, at least partially, in this pathway. However, it has to be noted that concentrations of SP600125 required to inhibit the effect of ES936 are also sufficient to affect other signalling kinases, such as p38 [31]. Thus, it is also possible that partial inhibition of ES936-induced DNA synthesis by SP600125 could be the result of a side effect on p38 MAPK-dependent signalling, which strongly regulates the response of HeLa cells to ES936 (see below).

The two p38 MAPK inhibitors tested, SB203580 and PD169316, strongly abolished ES936-stimulated DNA synthesis, and a significant effect of both compounds was observed in the nanomolar range. This profile of DNA synthesis inhibition is in accordance with reported IC_{50} values for inhibition of p38 MAPK activity of about 70 and 90 nM for SB203580 and PD169316 respectively in the absence of serum [21,22]. Strong phosphorylation of p38 was observed both in control and in ES936-treated cells, indicating that although preexistent p38 activity is essential for mediating ES936 effects in HeLa cells, this compound does not activate p38 phosphorylation.

Activation of p38 MAPK has been classically related with apoptotic stimuli, and p38 MAPK-dependent signalling pathways may suppress tumor formation in vivo by inducing apoptosis acting as a sensor of ROS [32]. However, p38 MAPKs can have either a positive or a negative role in the regulation of cell cycle progression depending on the cell type and stimulus [33–35]. For instance, p38 MAPK is involved in the regulation of cell cycle progression in FRTL-5 thyroid cells by increasing nuclear Cdk2 activity [34], and in the proliferation of Swiss 3T3 cells or airway smooth muscle cells stimulated by fibroblast growth factor-2 [36,37], of hematopoietic cells stimulated by cytokines [38,39], and of vascular smooth muscle and pancreatic stellate cells stimulated by platelet-derived growth factor [40,41]. p38 MAPKs are also required for CD40-induced proliferation of B-lymphocytes [42]. In cancer cells p38 MAPK activity is required for cell cycle progression of breast cancer [43], melanoma [44] and chondrosarcoma cell lines [45]. Very recently, it has been shown that p38 MAPK is active during the log phase growth in two cell lines derived from different tumor stages, and inhibition of these pathways could reduce proliferation and growth [46].

Mitogenic and antimitogenic functions of p38 are dependent on the cell type and possibly other factors [33,45]. However, the signalling pathways and identities of signalling key intermediates, which mediate p38 MAPK involvement in cell cycle progression, are poorly characterized. Interestingly, the apoptotic-inducing activity of p38 is often uncoupled from ROS sensing, and the apoptotic effects of p38α in response to ROS are specifically suppressed without affecting other cellular processes mediated by p38α that might be important for promoting the viability and growth of cancer cells containing high levels of ROS [32]. In accordance, a PPARδ agonist enhanced proliferation through ROS-mediated p38 MAPK [47], and H_2O_2 induced an increase in the phosphorylation of EGFR in mouse embrionyc stem cells, which was blocked by the inhibition of p38 MAPKs [48]. This is in agreement with our findings that EGFR-dependent signalling and p38 MAPK activity are absolutely required for ES936 to stimulate DNA synthesis in HeLa cells through a mechanism involving alterations in cellular redox balance.

In conclusion, we have demonstrated by the first time that ES936 not only induces growth inhibition in some cancer cells, but a stimulatory response can be also observed depending on the cellular context and growth conditions. We are currently investigating which cellular factors are modulated by ES936 in pathway dependent on p38 MAPK to promote cell growth.

Conflict of interest

None.

Acknowledgements

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