Influence of the level of γ-glutamyltranspeptidase activity on the response of poorly and moderately differentiated rhabdomyosarcoma cell lines to all-*trans*-retinoic acid

Teodoro Palomares[a], Begoña Castro[b], Maite del Olmo[b], Ainhoa Iglesias[c], Pedro Bilbao[a] and Ana Alonso-Varona[b]

Differentiation therapy with retinoic acid has been considered a potential approach for treating rhabdomyosarcoma. Analysis of retinoids as differentiating agents for rhabdomyosarcoma is, however, rendered incomplete by the fact that some rhabdomyosarcoma cell lines are retinoic acid resistant. Therefore, the aim of the present work was to study the effect of all-*trans*-retinoic acid on two rat tumour cell lines, derived from the same rhabdomyosarcoma tumour model (i.e. the moderately differentiated low metastatic F21 cell line and the poorly differentiated high metastatic S4MH cell line), to discover how degree of differentiation and glutathione metabolism influence response to this retinoic acid derivative. We observed that whereas in the S4MH cell line all-*trans*-retinoic acid induced a significant inhibition of tumorigenic potential, in F21 cells all-*trans*-retinoic acid enhanced tumour growth and only at a higher dose was there a slight antiproliferative effect. These effects were in consonance with the activity level of γ-glutamyltranspeptidase, which was significantly increased in F21 cells, but not in S4MH cells, in response to the all-*trans*-retinoic acid-induced increase in reactive oxygen species. The pro-tumour effect observed in F21 cells was reversed by adding buthionine sulphoximide, a specific cellular glutathione-depleting agent, to the all-*trans*-retinoic acid treatment. This combination produced a decrease in γ-glutamyltranspeptidase activity, and an increase in oxidative stress and apoptosis. Our findings suggest that the response to all-trans-retinoic-acid of the tumour cell lines studied is influenced by the strong relationship between intracellular glutathione content, γ-glutamyltranspeptidase activity and degree of differentiation of the rhabdomyosarcoma cell line, and that this relationship should be taken into account when identifying 'retinoid-sensitive' tumours. *Anti-Cancer Drugs* 17:1127–1139 © 2006 Lippincott Williams & Wilkins.

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Departments of [a]Surgery, Radiology and Physical Medicine, [b]Cell Biology and Histology and [c]Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country, Leioa, Vizcaya, Spain.

Correspondence to T. Palomares, Department of Surgery, Radiology and Physical Medicine, School of Medicine and Dentistry. University of the Basque Country. Leioa, 48940 Vizcaya, Spain.
Tel: +34 94 601 2813; fax: +34 94 601 2781;
e-mail: teodoro.palomares@ehu.es

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Introduction

One of the most important characteristics of normal cells is their controlled growth and differentiation. Crucial to these biological processes is the role played by cellular thiol status, which is dependent on cellular energy metabolism [1,2]. It has been shown that DNA synthesis may be regulated by glutathione (GSH, the major intracellular nonprotein thiol in mammalian cells) [3] and that GSH synthesis is essential for normal growth [4]. Moreover, it has also been described that as the cell progresses from proliferation to differentiation, cellular GSH content is decreased [1].

With respect to tumour cells, we and others have demonstrated that nondifferentiated high metastatic cells have a significantly higher GSH content than moderately differentiated cell lines [5,6]. Moreover, we have also observed that whereas elevation of intracellular GSH is associated with mitogenic stimulation, GSH depletion decreases the rate of cell proliferation and inhibits cancer growth [7].

As cell proliferation and differentiation are deregulated in tumour cells, a rational therapeutical approach used in preclinical and clinical studies triggered differentiation using chemical compounds, such as retinoic acid (RA). Retinoids play a potential role as promising chemotherapeutic agents by inhibiting uncontrolled cell growth, inducing cellular differentiation and promoting apoptosis [8]. As a result of this, they are being assayed as therapeutic agents for a variety of human cancers, such as of breast [9], lung [10], ovarian [11], head and neck [12], prostate [13] and liver [14], as well as leukaemia [15]. In the particular case of rhabdomyosarcoma (RMS), several

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studies have suggested a potential role for differentiation therapy in the treatment of these tumours [16], the most common malignant soft-tissue tumours in children, with retinoids being considered as an alternative therapeutic approach. In fact, several authors have demonstrated that the physiological RA derivative all-*trans*-retinoic acid (ATRA, the first example of a clinically useful cyto-differentiating agent) inhibits the growth of rat and human RMS cells [17–19].

The action mechanisms responsible for the effects of RA have not yet been completely elucidated. On the one hand, it is thought that these effects are mainly mediated by two subfamilies of nuclear retinoid receptors, RA receptors and retinoid X receptors [20], which exert an anticancer effect through expression of transcriptional activators such as activator protein-1 [21]. It has been, however, demonstrated that resistance to the growth-inhibitory and differentiation effects of RA expressed by several RMS cell lines is linked not only to the reduced expression and activity of RA receptors, but also to the fact that other factors may also be required for RA resistance [22]. On the other hand, retinoids can also interact directly with the GSH-dependent protein kinase C, a key regulatory enzyme in signal transduction [23]. In addition, in tumour cells, retinoids induce an increase in reactive oxygen species (ROS) [24], which has been associated with oxidative DNA damage and apoptosis [25]. To avoid redox imbalance and oxidative stress, cells have a wide array of antioxidant defences. Interestingly, one of the most important is GSH and its related enzymes [26]. In fact, there is increasing evidence supporting the idea that a decrease in GSH is often associated with the apoptotic programme through a mitochondrial-dependent apoptotic pathway, whereas recovery of GSH content may enable the cells to avoid the apoptotic signal [27].

Taken together, and in view of the fact that both intracellular GSH content and RA have important effects on cell growth, differentiation and apoptosis, it does not seem implausible to suggest that GSH levels could influence the effects of RA. As there is still little information concerning the relationship between degree of differentiation of tumour cells, their GSH metabolism and their response to the antiproliferative effects of RA, the present study examines the possible influence of intracellular GSH levels during differentiation induced by ATRA in the moderately differentiated low metastatic F21 cell line and the poorly differentiated high metastatic S4MH cell line, derived from the same rat RMS tumour model.

Materials and methods

Tumour cell lines

Two variants from the same parental RMS cell line with different metastatic potentials were used: S4MH (highly metastatic) and F21 (poorly metastatic) [28]. Cells were cultured in a humidified atmosphere (5% CO_2, 95% air) at 37°C in Dulbecco's minimum essential medium supplemented with 15 and 10% fetal calf serum, respectively, 100 units/ml penicillin and 100 µg/ml streptomycin (Sigma, St Louis, Missouri, USA).

Exponentially growing cell cultures were used in all experiments. After a brief exposure to phosphate-buffered saline (PBS)–ethylenediaminetetraacetic acid (EDTA) (2 mmol/l) and centrifuging, the pellet was re-suspended in Hank's solution and cells were enumerated with a Coulter counter (Coultronics, Margency, France). Viability, as determined by Trypan blue exclusion, ranged from 95 to 98%.

Drugs

ATRA and L-*S*,*R*-buthionine sulphoximine (BSO) were obtained from Sigma. For the in-vitro studies, BSO was dissolved in Dulbecco's minimum essential medium and administered to cells (100 µmol/l) for 6 h, after which cells were washed free of the drug. ATRA was dissolved in 100% ethanol to obtain 10^{-2} mol/l stock solutions, stored in the dark at −20°C and then diluted in the medium to obtain appropriate final concentrations (10^{-8} or 10^{-6} mol/l). Maximum concentration of ethanol in the culture (below 0.1%) did not influence cell growth, differentiation or γ-glutamyltranspeptidase (γ-GT) activity. The medium containing ATRA or the control solvent was replaced every 48 h.

For the in-vivo experiments, ATRA was diluted in 90% Clinoleic (Baxter, Erlangen, Germany) plus 10% ethanol and BSO was dissolved in sterile physiological saline solution (0.9% NaCl), pH adjusted to 7.2. Drugs were administered intraperitoneally and dosages used were adjusted for body weight (5 mg/kg of ATRA and 50 mg/kg of BSO).

Immunohistochemical staining of culture cells

Cells were seeded on microscopic chamber slides and cultured under the conditions described above. After ATRA exposure (10^{-6} mol/l), cells were fixed in 3% formaldehyde in PBS for 15 min followed by 0.3% Triton X-100 in PBS for 5 min. For immunohistochemical staining, the monoclonal antibody against rat myogenin (1:50; Santa Cruz Biotechnology, Heidelberg, Germany) was applied for 30 min at 37°C [29]. Next, slides were rinsed in PBS solution and incubated for 30 min at room temperature in a moist chamber with Image-iT signal enhancer (Invitrogen, Carlsbad, California, USA). After rinsing with PBS, slides were finally incubated with fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse IgG (Alexa Fluor SFX kit; Invitrogen). No fluorescent labelling was seen in control experiments, when cells were incubated only with secondary antibody.

Images were acquired with an Olympus Fluoview FV500 (Olympus, Barcelona, Spain) confocal microscope using AOTF controlled sequential acquisition. The excitation of FITC was performed with an Ar laser at 488 nm and specific fluorescence emission was selected in the region 458–514 nm using appropriate filter settings.

Cell proliferation

Tumour cells were seeded in 24-well microplates at a density of 10^4 cells/well in 10^3 µl of growth medium plus fetal calf serum and allowed to attach and grow for 24 h. The cells were then treated with ATRA, BSO or ATRA plus BSO. At 24, 48 and 72 h after addition of drugs, proliferation was measured by using a haemocytometer to count the cells growing in each well. Experiments were performed in quadruplicate and each assay was repeated three times.

Measurement of intracellular generation of reactive oxygen species

The intracellular content of ROS was evaluated by using the oxidation-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, Oregon, USA), which mainly measures the production of hydrogen peroxide and hydroxyl radicals [30]. Cells seeded in 24-well culture plates at a density of 10^4 cells/well were exposed to ATRA alone or in combination with BSO. After drug exposure, cells were washed twice with PBS and incubated at 37°C with 5 µmol/l H_2DCFDA for 30 min. After washing twice, the cellular fluorescence intensity was measured at 530 nm after excitation at 485 nm in a fluorimeter (Fluoroskan Ascent; Labsystems, Helsinki, Finland). Checks were performed to ensure that drugs were not interfering with dichlorofluorescein fluorescence. Experiments were performed in quadruplicate and each assay was repeated three times.

Glutathione determination

After drug exposure, the medium from the 24-well plates was removed, and cells were washed twice with PBS and frozen rapidly by immersion of plates in liquid nitrogen. Intracellular GSH content was determined by the fluorimetric method as reported by Hissin and Hilf. [31]. Briefly, 150 µl of homogenization solution (5% trichloroacetic acid in 2 mmol/l EDTA) was added to the wells and, after a short sonication, plates were centrifuged and 50 µl from each well was transferred to a 96-well plate for duplication. To each well, 175 µl of the PBS 0.1 mmol/l–EDTA 5 mmol/l (pH 8.0) solution, 15 µl of NaOH 1 mol/l and 10 µl of the fluorochrome *o*-phthalaldehyde (Sigma) solution (10 mg/ml in methanol) were added. After mixing and incubation at room temperature for 30 min in the dark, the fluorescence intensity was measured in a fluorimeter (Fluoroscan Ascent) at 450 nm emission after excitation at 360 nm. GSH measurements were expressed in nmol/mg cell protein. Protein content was determined by Lowry's method [32]. Each assay was repeated three times and all experiments were performed in quadruplicate wells.

Measurement of γ-glutamyltranspeptidase

Activity of γ-GT was assayed essentially according to Grisk *et al.* [33], using γ-glutamyl-*p*-nitroanilide (Sigma) as substrate and glycyl-glycine (Sigma) as γ-glutamyl moiety acceptor. In a typical experiment, 10^6 cells were incubated for 30 min at 37°C in 0.75 ml of substrate with or without 40 mmol/l glycyl-glycine in 5 mmol/l Tris–HCl buffer, pH 8.5. The production of free *p*-nitroanilide was measured by spectrophotometry at 405 nm. Experimental values were obtained by subtracting the value of samples without glycyl-glycine from the value of samples with glycyl-glycine. The units of enzyme activity were calculated using a molar extinction coefficient of 9.9 for *p*-nitroanilide formed, in which 1 unit of γ-GT is defined as 1 µmol/min of substrate transformed/ml/min. Enzyme activity was expressed as mU of γ-GT activity per mg of cell protein. Protein content was also determined by Lowry's method.

Detection of apoptosis

Cell viability and apoptosis were measured by Annexin V–FITC (Becton Dickinson, Plymouth, UK) and propidium iodide (PI) staining. The assay detects early-stage apoptosis on the basis of phosphatidyl serine binding with Annexin V. Cells were labelled after treatments according to the manufacturer's instructions and analysed by flow cytometry. Briefly, Annexin V conjugate (5 µl/10^4 cells) was added to cell suspension and incubated at room temperature for 15 min. Cell viability was determined by PI as dead-cell indicator (1 µg/ml). At least 10^4 cells per sample were analysed for quantitative fluorescence using a Coulter EPICS ELITE flow cytometer (Coulter, Hialeah, Florida, USA). Fluorescence emitted per cell unit was measured by flow cytometry using an Ar laser ($\lambda_{ex} = 488$ nm). Fluorescent signals emitted were collected with appropriate filters (BP 525 nm and A 610 nm for FITC and PI, respectively). All the experiments were repeated three times.

Animals and tumour model

Specific pathogen-free female Wistar (WAG) rats (Iffa Credo Laboratories, L'Arbreole, France) were used for in-vivo studies. Rats (10–12 weeks old) were given food and water *ad libitum* and kept on a 12 h day/night cycle. The animals were handled according to institutional ethical guidelines and all protocols complied with the UK Coordinating Committee on Cancer Research *Guidelines for the Welfare of Animals in Experimental Neoplasia* [34].

Each experimental group consisted of eight rats that were inoculated subcutaneously in the back with 0.1 ml of cell suspensions of syngeneic F21 or S4MH tumour cells at a

concentration of 10^7 cells/ml in Hank's balanced salt solution.

Measurement of tumours, drug activity and systemic toxicity

One set of experiments was focused on evaluating the effect on tumorigenic potential of F21 and S4MH cells previously exposed *in vitro* to ATRA. The tumours were evaluated three times per week by the same observer. Tumour weight (g) was determined by measurement of the longest axis (L) and shortest axis (W) of each tumour with a slide calliper, and calculating the assumed unit density by the following formula: 1/2 ($L \times W^2$). The effect of ATRA on F21 and S4MH cell line tumorigenicity was evaluated until day 26 post-tumour cell inoculation as the ratio of the mean weight of tumours in rats inoculated with ATRA-treated cells to the mean in control rats (T/C ratio).

In addition, we investigated the effects of in-vivo ATRA administration, alone and in combination with BSO, on tumour growth in rats bearing F21 or S4MH tumours. For this set of experiments, ATRA (5 mg/kg) and BSO (50 mg/kg) treatments were initiated when the tumour weight reached 50–150 mg (day 0), and continued daily for 14 days. Control rats received injections of equal volumes of the vehicles of the corresponding drugs. Tumour volumes were estimated by the formula 1/2 ($L \times W^2$). Relative tumour volume (RTV) was calculated using the formula RTV (%): $V_X/V_i \times 100$, where V_X represents the tumour volume on a given day of measurement and V_i is the volume of the same tumour at the start of treatment. ATRA and BSO effects on tumours were evaluated using two different methods: by calculation of the T/C values (i.e by dividing the RTV of treated rats by that of control rats) and by calculating the growth delay factor (GDF), which was defined as the mean number of tumour-doubling times gained by treatments calculated using the formula $GDF = (TD_{tr} - TD_{con})/TD_{con}$, where TD_{tr} is the tumour-doubling time of treated tumours and TD_{con} that of control tumours. Homogeneity in the distribution of the different experimental groups with respect to tumour volume before the beginning of the treatment was verified by statistical analysis (analysis of variance with one factor). The combined therapy was considered to act synergistically if T/C of BSO and ATRA was less than (T/C of BSO alone) × (T/C of ATRA alone).

Toxicity end-points included lethal toxicity, body weight loss and haematological toxicity. Rats were monitored daily for lethal toxicity and body weight, which was recorded immediately before treatment and once daily during and after the treatment. Data on body weight changes were calculated as percentages relative to the initial body weight. On the day after the end of the treatment (day 26), five rats taken randomly from each group were used for the evaluation of haematological parameters. The peripheral white blood cell count, red blood cell count, total haemoglobin concentration and haematocrit were determined using a Coulter counter. When experiments were completed, all rats were killed by cervical dislocation.

Statistical analysis

The test of significance was carried out using Student's t-test, the χ^2-test and the factorial analysis of variance, as appropriate. Values were considered to be statistically different from controls when $P < 0.05$.

Results

Effects of all-*trans*-retinoic acid on phenotypic differentiation of S4MH and F21 cell lines

Under basal conditions, undifferentiated S4MH cells are small, round and randomly distributed on the culture plate (Fig. 1a). In contrast, F21 cells have a more differentiated, fibroblastic morphology and are spindle shaped. In addition, the level of myogenin expression is higher in the F21 cell line than in S4MH cells (Fig. 1c).

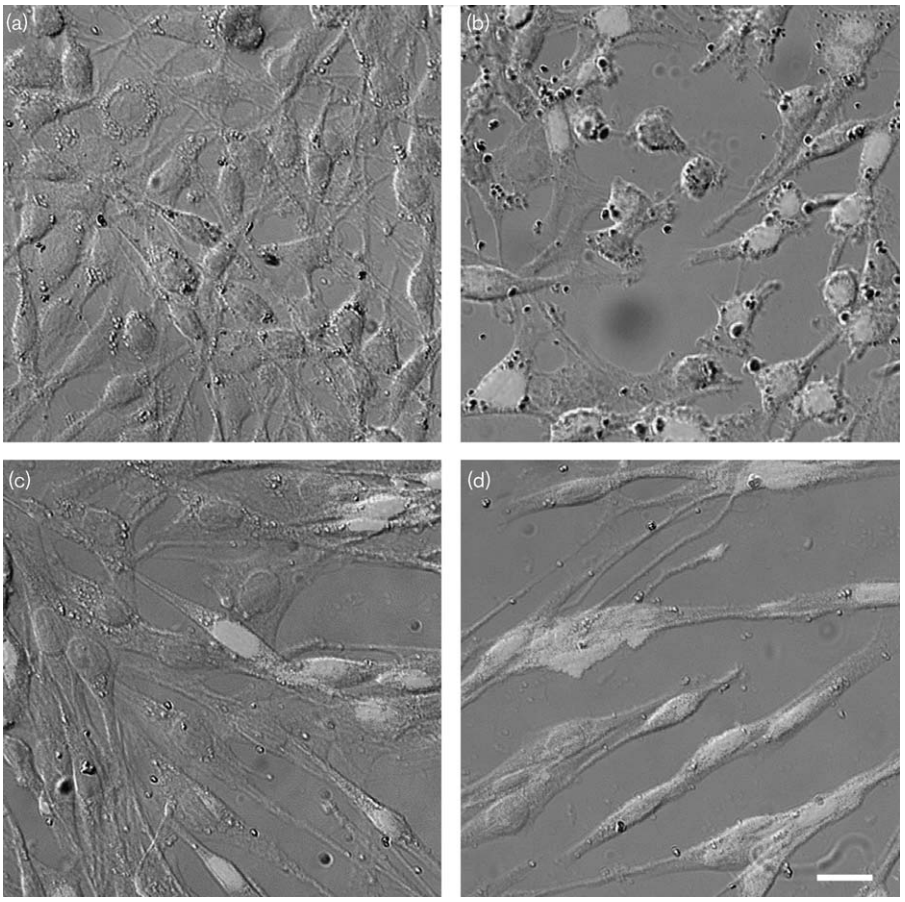
In order to determine whether ATRA treatment induces cell differentiation, we cultured both F21 and S4MH cells with 10^{-6} mol/l of ATRA. After 72 h of ATRA exposure, whereas a moderate increase in myogenin expression was observed in F21 cells (Fig. 1d), in treated S4MH cells we found significantly higher myogenin expression than in untreated cells (Fig. 1b).

Effects of all-*trans*-retinoic acid on glutathione content, proliferation and apoptosis of S4MH and F21 cells

We have previously demonstrated that under basal conditions, GSH content and proliferation rate of S4MH cells are nearly twice those of F21 cells [5]. In the present study, we initially determined the in-vitro effects of ATRA treatment on GSH content and on cell proliferation in these tumour cell lines. As shown in Fig. 2(a), ATRA produced a concentration-dependent decrease in the GSH content of the S4MH cell line. At 24 h and at doses of 10^{-8} and 10^{-6} mol/l of ATRA, the reductions were 13 and 35%, respectively, but these figures were statistically significant only at the higher concentration. In contrast, in F21 cells ATRA treatment induced a significant increase in GSH content (approaching 151% at 24 h at both doses of ATRA; Fig. 2c).

Regarding cell proliferation, ATRA significantly reduced the S4MH growth rate. After exposure to 10^{-8} and 10^{-6} mol/l of ATRA, cell proliferation was maximally reduced (2.0- and 2.1-fold, respectively) at 48 h (Fig. 2b), but the decrease in growth rate was maintained at 72 h (1.4-fold at both doses of ATRA). In the case of F21 cells, a 1.4-fold reduction in cell proliferation was also observed at a dose of 10^{-6} mol/l ATRA at 72 h. At a dose of 10^{-8} mol/l ATRA, however, cell proliferation was observed to be 1.2

Fig. 1



Confocal microscopy analysis of the morphological appearance (phase contrast) and immunofluorescence localization of myogenin expression. Left panels show untreated S4MH (a) and F21 (c) cells; right panels show 10^{-6} mol/l all-*trans*-retinoic acid-treated S4MH (b) and F21 (d) cells. Bar, 20 μm.

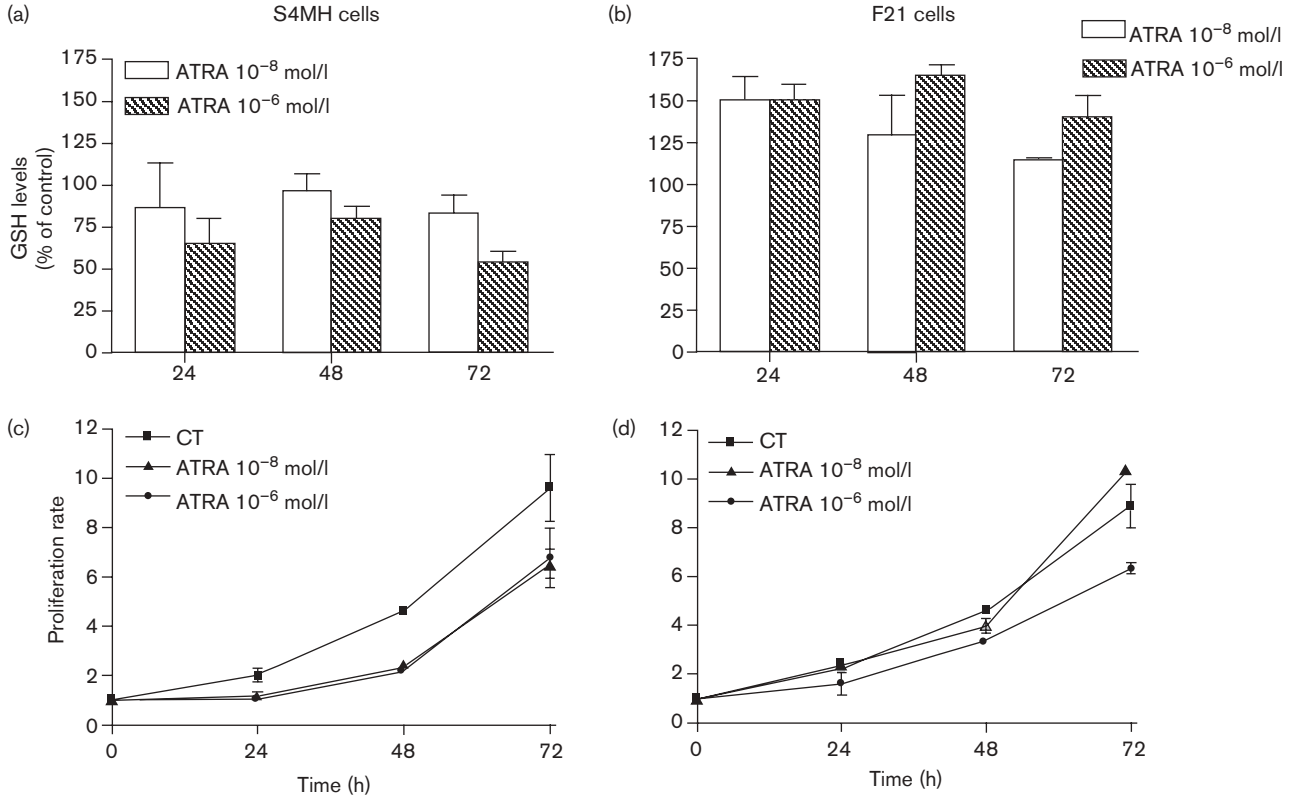
times higher ($P < 0.05$) at 72 h than for the control cells (Fig. 2d).

Finally, we analysed the effects of ATRA treatment on apoptosis induction in S4MH and F21 cells. As shown in Fig. 3, in both tumour cell lines exposed to the lower dose of ATRA, no modifications in the percentage of apoptotic cells were achieved compared with control cells at 48 h. Exposure to 10^{-6} mol/l of ATRA did, however, produce a significant increase in apoptosis at 48 h, but only in the S4MH cell line (1.6-fold increase as compared with control cells; $P < 0.05$). In the F21-treated cells, the apoptotic rate approached that of controls, and the difference was not significant.

Effects of all-*trans*-retinoic acid on γ-glutamyltranspeptidase activity of S4MH and F21 cells

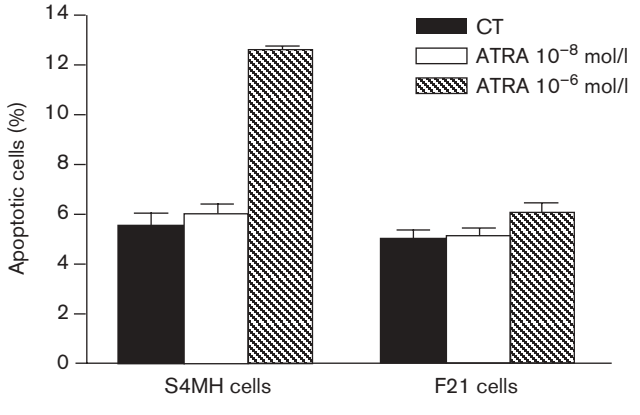
As a result of the differences observed between S4MH and F21 cells regarding GSH levels, proliferation and apoptosis after treatment with ATRA, taken together with the facts that an increase in the level of γ-GT activity has been associated with differentiation, and that this enzyme plays an important role in providing a substrate for GSH synthesis and in preventing apoptosis, we decided to investigate the possible involvement of γ-GT in these different responses. Under basal conditions, both tumour cell lines showed measurable enzymatic activity, but the level of γ-GT activity was found to be 2.3 times higher in the F21 cells than in the S4MH cells (87.8 ± 5.8 versus 37.5 ± 4.3 mU/mg protein, $P < 0.05$). When tumour cells were exposed to 10^{-8} or 10^{-6} mol/l of ATRA, a significant decrease in γ-GT activity took place in S4MH-treated cells (nearly 2.0- and 3.0-fold, respectively, compared with control cells; $P < 0.05$). In contrast, in F21 cells, γ-GT activity was significantly increased by ATRA treatment (2.3 and 1.6 times that of control cells at doses of 10^{-8} or 10^{-6} mol/l, respectively), with significant differences between doses ($P < 0.01$; Fig. 4).

Fig. 2



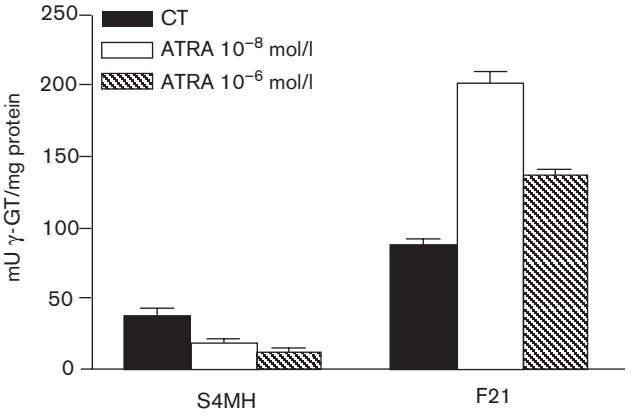
Effect of all-*trans*-retinoic acid (ATRA) (10^{-6} and 10^{-8} mol/l) on the glutathione (GSH) levels (a and c) and on cell proliferation (b and d) of S4MH and F21 tumour cell lines. GSH levels are expressed as a percentage of control values. Cell proliferation is expressed as relative values (mean ± SD) compared with mean values of the control at 0 h. All the experiments were repeated three times. CT, control.

Fig. 3



Apoptosis of S4MH and F21 cells exposed to all-*trans*-retinoic acid (ATRA) (10^{-6} and 10^{-8} mol/l). Cells were treated for 48 h, and then labelled with Annexin V–fluorescein isothiocyanate and propidium iodide for the determination of apoptosis. Flow cytometry data are expressed as the percentage of apoptotic cells. All the experiments were repeated three times. CT, control.

Fig. 4



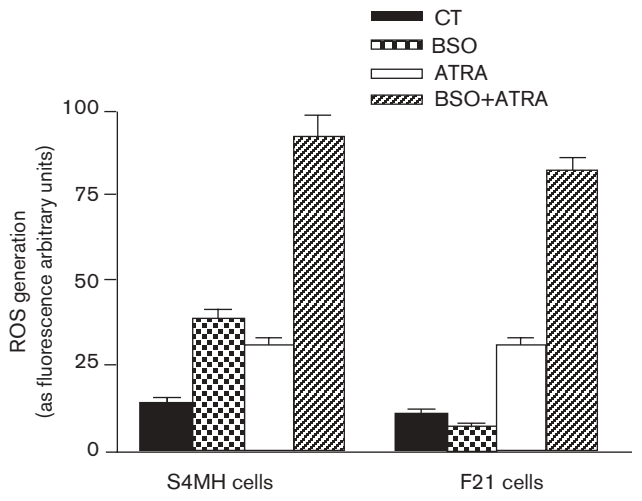
γ-Glutamyltranspeptidase activity of S4MH and F21 cells under basal conditions (control) and after exposure to 10^{-6} and 10^{-8} mol/l of all-*trans*-retinoic acid (ATRA). Enzyme activity is expressed as mU/mg of cell protein. Protein content was determined by Lowry's method. Data represent the mean ± SD of three experiments. CT, control.

Effects of buthionine sulphoximide addition to all-*trans*-retinoic acid treatment on reactive oxygen species content of S4MH and F21 cells

In a previous work, we demonstrated that exposure to 100 μmol/l of BSO (a specific blocking agent of GSH biosynthesis) induced significant GSH depletion in S4MH cells, but not in F21 cells [5]. Thus, in order to determine the influence of GSH metabolism present in each tumour cell line on the response of the tumour cells to ATRA, S4MH and F21 cells were first exposed to BSO. We then compared the changes induced by BSO (100 μmol/l) and ATRA (10^{-8} mol/l), alone and in combination, in the ROS content of these tumour cells.

Under basal conditions, a significant increase (1.3-fold) in ROS content was observed in S4MH cells compared with F21 cells (Fig. 5). Compared with controls, ATRA treatment produced a significant increase in ROS content in both tumour cell lines (2.2 and 2.8-fold in S4MH and F21 cells, respectively) at 10 h of drug exposure. In contrast, at this time, there was an increase in ROS (2.7-fold compared with control cells; $P < 0.01$) only in the case of S4MH as a result of BSO treatment, whereas a significant reduction (1.6-fold compared with controls; $P < 0.01$) was observed in F21 cells. When tumour cells were, however, exposed to the combination of BSO and ATRA, significantly higher redox stress was produced in both cell lines and, compared with untreated cells, a 6.5- and 7.4-fold increase in ROS was observed in S4MH and F21 cells, respectively.

Fig. 5



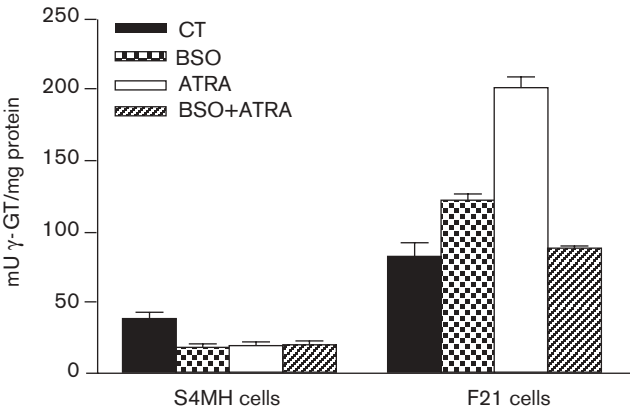
Analysis of intracellular reactive oxygen species (ROS) generation in S4MH and F21 cells under basal conditions (Control) and in response to buthionine-sulphoximide (BSO) (100 μmol/l) or all-*trans*-retinoic acid (ATRA) (10^{-8} mol/l), and BSO (100 μmol/l) plus ATRA (10^{-8} mol/l). ROS content was measured at 10 h of drug exposure using the fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate. Data are expressed in arbitrary fluorescence units. Experiments were performed in quadruplicate, and the results are expressed as the mean ± SD of three experiments. CT, control.

Effects of buthionine sulphoximide addition to all-*trans*-retinoic acid treatment on γ-glutamyltranspeptidase activity and glutathione content of S4MH and F21 cells

In our analysis of the effects of BSO and ATRA on the γ-GT activity of S4MH and F21 tumour cells, we found that at 10 h of exposure to 100 μmol/l of BSO, the effect was similar to that produced by treatment with 10^{-8} mol/l of ATRA described above. Whereas a 2.2-fold decrease in γ-GT activity was achieved in S4MH cells, in F21 cells this enzymatic activity was increased 1.4-fold compared with control cells ($P < 0.05$). As can be seen in Fig. 6, when S4MH cells were treated with BSO + ATRA, γ-GT activity was also reduced (2.0-fold compared with controls), with no significant differences compared with treatment with ATRA or BSO alone. In the case of F21 cells, however, a significant reduction in γ-GT activity was produced by this combined treatment compared with administering each drug alone; however, compared with untreated cells, no significant differences were registered.

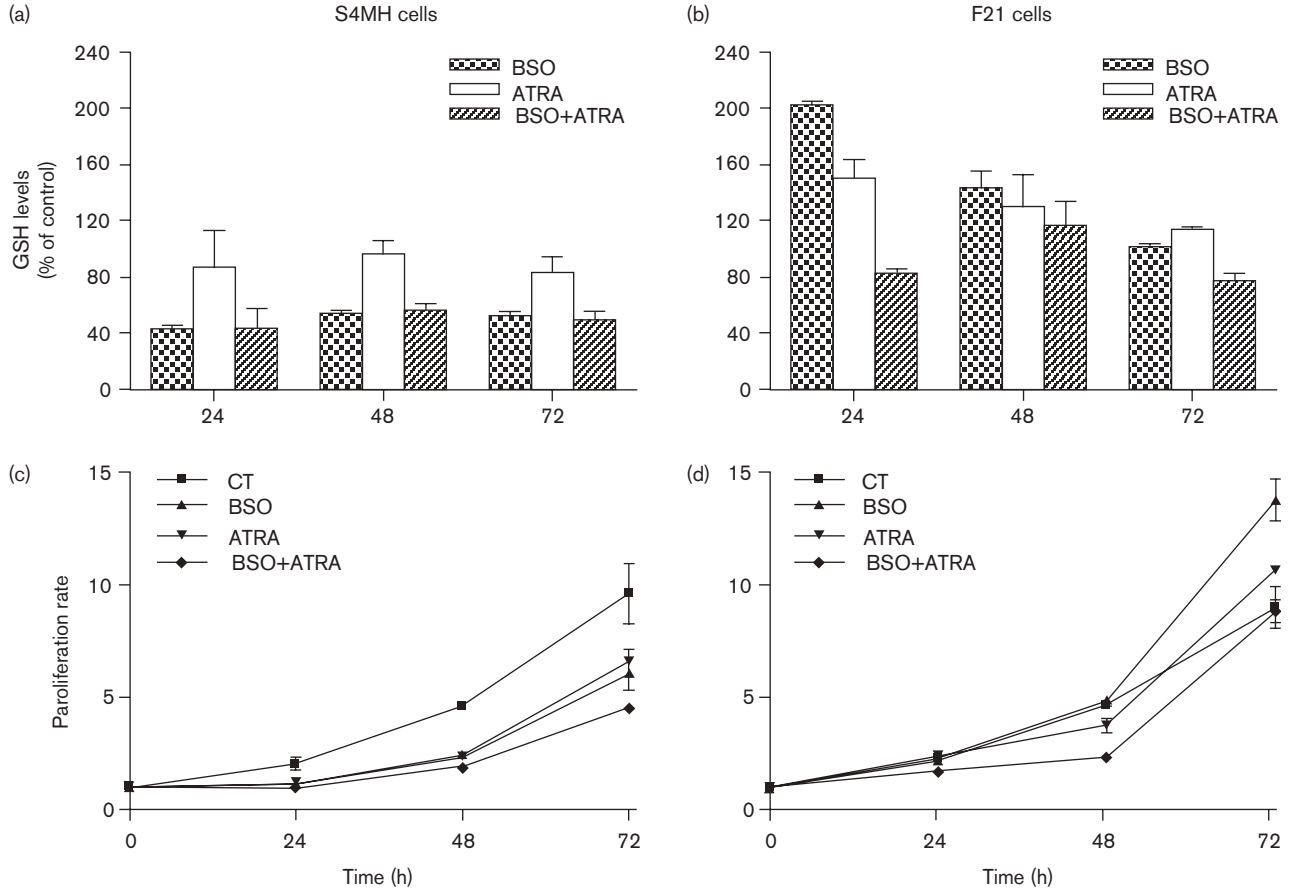
These results coincide with the effects produced by both agents on intracellular GSH levels at 24 h. In F21 cells, whereas exposure to BSO or ATRA alone induced a significant increase in GSH content (a 200 and 151% increase, respectively, compared with untreated cells), with the combined treatment, the BSO-induced and ATRA-induced GSH increase was annulled and no significant differences in GSH levels were observed compared with control cells (Fig. 7b). In contrast, in the case of S4MH cells, only BSO treatment induced significant differences in GSH content. Thus, whereas

Fig. 6



Effect of buthionine sulphoximide (BSO) (100 μmol/l) addition to all-*trans*-retinoic acid (ATRA) (10^{-8} mol/l) treatment on γ-glutamyltranspeptidase (γ-GT) activity of S4MH and F21 cells. γ-GT activity was measured at 10 h of drug exposure. Values shown are means ± SD of three separate experiments. CT, control.

Fig. 7



Effect of buthionine sulphoximide (BSO) (100 μmol/l) and all-*trans*-retinoic acid (ATRA) (10^{-8} mol/l), alone and in combination on intracellular glutathione (GSH) levels (a and c) and on cell proliferation (b and d) of S4MH and F21 cells. GSH is expressed as a percentage of control values. Cell proliferation is expressed as relative values (mean ± SD) compared with mean values of the control at 0 h. Experiments were performed in quadruplicate and each assay was repeated three times. CT, control.

ATRA resulted in no significant modification of GSH levels, BSO alone and in combination with ATRA produced a similar decrease (56%) in GSH content compared with untreated cells (Fig. 7a).

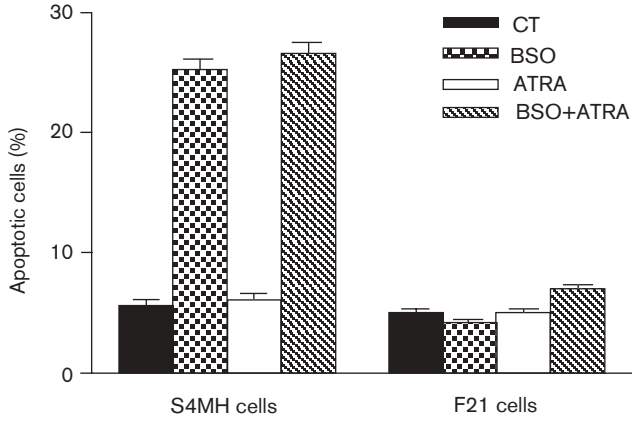
Effects of buthionine sulphoximide addition to all-*trans*-retinoic acid treatment on cell proliferation and apoptosis of S4MH and F21 cells

Regarding the effects of BSO and ATRA on proliferation rate, we observed that at 72 h, whereas both agents administered alone had a similar effect on the growth rate (nearly a 1.5-fold decrease compared with controls) in the S4MH cell line, in the F21 cells, proliferation was 1.5 and 1.2 times higher in cells treated with BSO and ATRA, respectively ($P < 0.05$). The combined treatment of BSO and ATRA, however, produced an additive antitumour effect in both tumour cell lines. In the S4MH cells, there was a greater reduction of proliferation (2.1-fold compared with controls), whereas in the F21 cells, the combined treatment reversed the increase in proliferation induced by BSO or ATRA observed at 72 h and, compared with controls, no significant differences were found at this time in the growth rate (Fig. 7c and d). These results are in accordance with the effects of BSO and ATRA on apoptosis. While the lower dose of ATRA produced no modifications in the percentage of apoptotic cells, as has been described above, BSO treatment increased the percentage of apoptotic cells (11.7-fold increase compared with controls) only in the case of the S4MH cells. The combined treatment, however, produced significant increases in apoptosis in both cell lines, although the effect was 3.8 times higher in S4MH cells than in F21 cells (a 12.4- and a 1.4-fold increase in percentage of apoptotic cells, respectively, compared with controls; Fig. 8).

Effects of in-vitro all-*trans*-retinoic acid pretreatment on tumorigenic potential of S4MH and F21 cells

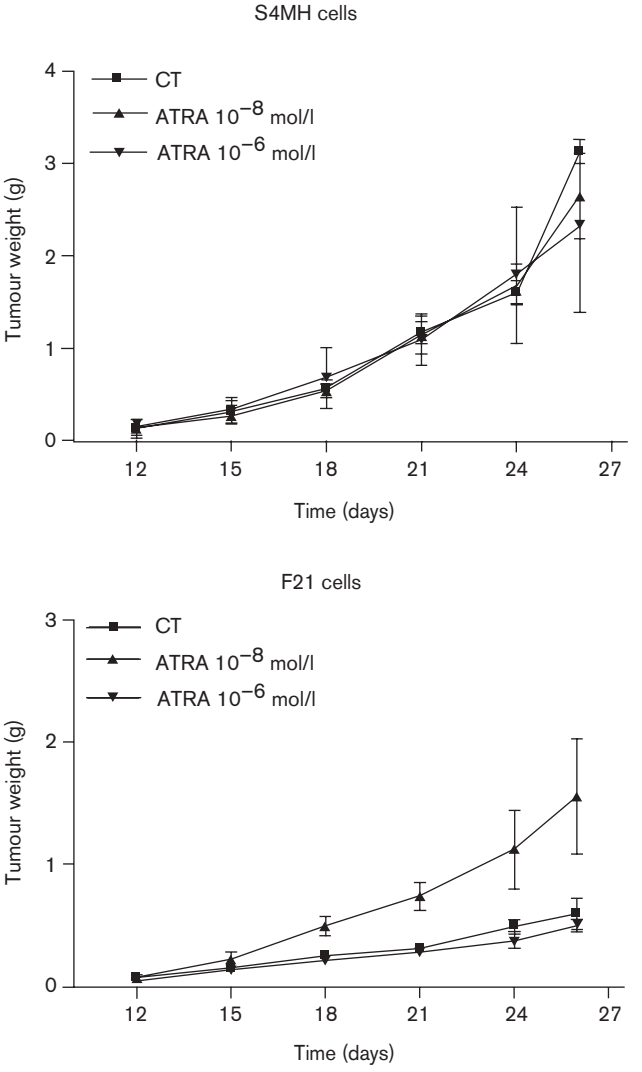
Under basal conditions, the S4MH cell line showed a significantly higher tumorigenic potential than the F21

Fig. 8



Apoptosis of S4MH and F21 cells exposed to buthionine sulphoximide (BSO) (100 μmol/l), all-*trans*-retinoic acid (ATRA) (10^{-8} mol/l) and BSO (100 μmol/l) plus ATRA (10^{-8} mol/l). Percentage of apoptotic cells was evaluated at 48 h of drug exposure. Data represent the mean ± SD of three experiments. CT, control.

Fig. 9



Tumorigenic potential of S4MH and F21 cells previously exposed *in vitro* to all-*trans*-retinoic acid (ATRA). Culture cells were treated with 10^{-6} or 10^{-8} mol/l of ATRA for 72 h and injected into rats after removing ATRA from the culture medium. Tumours were evaluated three times per week for 4 weeks. Tumour weight was calculated by the following formula: 1/2 ($L \times W^2$) where L is the longest axis and W is the shortest axis. Data are expressed as tumour weight (g), and represent the mean ± SD of three experiments. CT, control.

cell line. On day 26 after tumour cell inoculation, S4MH tumour weight was 5.5 times higher ($P < 0.01$) than F21 tumour weight (3.3 ± 0.8 and 0.6 ± 0.1 g, respectively).

To evaluate the effect of ATRA on the tumorigenic potential of S4MH and F21 cell lines, the tumour cells were cultured in the presence of 10^{-8} or 10^{-6} mol/l of ATRA for 72 h. Afterwards, ATRA was removed from the culture medium, and the cells were injected into rats. As shown in Fig. 9, no significant differences in tumour weights were found between rats inoculated with ATRA-treated S4MH cells and rats bearing untreated S4MH cells. Likewise, in the case of rats inoculated with F21 cells previously exposed to 10^{-6} mol/l of ATRA, no significant differences in tumour growth were observed compared with controls. In contrast, the tumorigenic potential of F21 cells was significantly increased by previous in-vitro exposure to 10^{-8} of ATRA, resulting in a mean tumour weight that was 260% higher than in rats bearing untreated F21 cells.

In-vivo effects of all-*trans*-retinoic acid treatment, alone and in combination with buthionine sulphoximide, on S4MH and F21 tumour growth

On the basis of similar in-vitro effects induced by ATRA and BSO on the γ-GT activity and proliferation of S4MH and F21 cells, and in an attempt to establish whether a correlation also exists with the in-vivo effects of ATRA and BSO on the tumorigenic potential of these tumour cells, in a second series of in-vivo experiments S4MH and F21 tumour-bearing rats were treated daily with 5 mg/kg of ATRA, alone and in combination with 50 mg/kg BSO.

No significant weight loss or lethal toxicity was induced by these treatments. As can be seen in Table 1, ATRA and BSO produced similar effects on tumorigenic potential. In the case of S4MH tumour-bearing rats, both drugs significantly reduced tumour growth. On the day following treatment, the *T*/*C* values registered were 46 and 44% with ATRA and BSO, respectively ($P < 0.05$), whereas the GDF values were 1.3 and 1.2, respectively. In contrast, in F21 tumour-bearing rats, treatment with ATRA or BSO produced a significant increase in tumour growth. In fact, in both cases the tumour volume of

Table 1 Effect of in-vivo ATRA treatment, alone and in combination with BSO, on F21 and S4MH tumour growth

Tumour	Group[a]	RTV[b]	*T/C* (%)[c]	TD (days)[d]	GDF[e]
S4MH	control	27.4 ± 4.7	–	1.1	–
	BSO	11.9 ± 1.2	43.6	2.5	1.3
	ATRA	12.6 ± 1.3	46.1	2.4	1.2
	BSO + ATRA	10.1 ± 1.2	37.0	2.9	1.7
F21	control	6.2 ± 1.1	–	4.3	–
	BSO	12.9 ± 1.3	207.6	2.3	–
	ATRA	12.4 ± 1.3	200.0	2.4	–
	BSO + ATRA	4.9 ± 1.0	79.3	6.1	0.3

GDF, growth delay factor; BSO, buthionine sulphoximine; ATRA, all-*trans*-retinoic acid.
[a]Rats were treated daily with BSO (50 mg/kg) or ATRA (5 mg/kg), alone and in combination, for 14 days.
[b]Relative tumour volume expressed as V_X/V_i index, where V_X represents the tumour volume on the day following the end of treatment (day 26) and V_i is the volume of the same tumour at the start of treatment.
[c]% Tumour: control ratio = RTV_{tr}/RTV_{con}, where RTV_{tr} represents the relative tumour volume of treated tumours and RTV_{con} that of untreated tumours.
[d]Tumour-doubling time.
[e]Growth delay factor = $(TD_{tr} - TD_{con})/TD_{con}$, where TD_{tr} is the tumour-doubling time of treated tumours and TD_{con} is that of untreated tumours.

treated rats was twice that of the controls (*T/C* of 200 and 207% with ATRA and BSO, respectively).

On the other hand, we observed that when ATRA and BSO were administered in combination, an additive antitumour effect was achieved in both rats bearing S4MH tumours and rats bearing F21 tumours. The significant reduction in S4MH and F21 tumour weights induced by the combined treatment was achieved not only compared with controls, but also ATRA or BSO administered alone ($P < 0.05$). The tumour volume decrease, however, was significantly higher in rats inoculated with S4MH cells (*T/C* of 37%) than in rats bearing F21 tumours (*T/C* of 79%), and the corresponding GDF values were 1.7 and 0.3, respectively.

Discussion

It has been shown that RMS, a tumour characterized by its poor response to chemotherapy, may respond to differentiation therapy with ATRA [18]. It has also been demonstrated that the therapeutic potential of differentiation induction may be impaired by tumour heterogeneity [35]. Moreover, some authors have reported that the degree of differentiation is crucial for RA sensitivity and that in several RMS cell lines, ATRA treatment fails to inhibit growth or induce myogenic differentiation [22]. To our knowledge, however, there is no description of the possible differences in tumour response to ATRA depending on the degree of differentiation and GSH metabolism of the tumour cell line.

In the present study, we demonstrate that ATRA treatment may produce different effects in tumour cell lines derived from the same RMS tumour model, but having different degrees of differentiation, and the effects are strongly associated with the GSH metabolism present in each of these cell lines. *In vitro*, ATRA was shown to have a significant antiproliferative effect on the undifferentiated S4MH cells, decreasing GSH levels and cell proliferation, and inducing myogenin expression. In the moderately differentiated F21 cell line, however, the antiproliferative effects of this RA derivative were observed only at higher doses. Moreover, at lower doses, a significant increase in the tumorigenic capacity of this tumour cell line was observed, which correlated with the GSH increase secondarily induced by ATRA exposure. *In vivo*, while ATRA treatment reduced the growth of S4MH tumours, it significantly increased the development of F21 tumours.

The effect of retinoids on GSH metabolism has also been documented. While some authors have found no modifications in GSH levels induced by these drugs by themselves [26], other investigators have described a transient decrease in GSH content after ATRA exposure [36]. A consensus, however, exists regarding the notion that intracellular reduced GSH may modulate the response to retinoids and even that a reduction of GSH levels may be required for cells to become sensitive to RA. As mitochondrial GSH is the primary defence against ROS generated from the electron transport chain [37], the reduction in GSH levels could be a consequence of the peroxide detoxification and maintenance of mitochondrial function that occurs under the stressful conditions of mitochondrial ROS overproduction, as has been suggested in the case of retinoids such as fenretidine (by targeting between complex I and II of the mitochondrial respiratory chain) [38]. Other authors have also shown the role of mitochondria in ATRA-induced antitumour effects [39]. In addition, it has been demonstrated that in cells preincubated with thiol compounds, no growth inhibition by RA is produced [40]. It has also been suggested that the GSH redox system may be a possible mechanism whereby ATRA synergistically potentiates the effect of other antiproliferative agents [41]. Finally, the efficacious ability of ATRA to inhibit glutathione-*S*-tranferase activity [42] has also been suggested as a possible mechanism for the potentiation of cytostatic drugs by this RA [36].

Therefore, GSH and its related enzymes would appear to play a key role in ATRA-related effects. Curiously, the ATRA-induced effects found in the present study are very similar to our previous observations obtained with the use of BSO [5], a potent inhibitor of GSH synthesis. Other authors have also speculated that the mechanism of ATRA could resemble that of BSO in other effects induced by these agents, such as skin depigmentation [43]. The similar responses observed in our study with the two drugs may be due, at least in part, to a modification of redox status induced by the two

antitumour agents. In S4MH cells, the BSO-derived GSH level reduction is produced by selective inhibition of the γ-glutamylcysteine synthetase enzyme [44]. In the case of ATRA, the decrease in GSH could be secondary to consumption of this important scavenger after reduction of ROS, which is significantly increased by the ATRA treatment. As occurs with BSO, in the undifferentiated S4MH tumour cell line, once a decrease in GSH level has been induced, it cannot be restored effectively by γ-GT (a key glycosylated plasma membrane enzyme involved in GSH metabolism and one of the numerous enzymes supposed to be regulated during the differentiation process) [45], whose activity was reduced by ATRA, with the consequent reduction in cell growth. In F21 cells, intracellular ROS levels are increased at percentages similar to those of S4MH cells, but F21 cells respond through a significant increase in the activity of γ-GT. We found, moreover, that with exposure to the lower dose of ATRA, this increased γ-GT activity and the consequent GSH increase correlated with an increased rate of cell proliferation, as also occurs with BSO treatment [5]. Other authors have also suggested a typical regulation of γ-GT after ATRA treatment, but in contrast to our results, on the effects of this retinoid on S4MH cells, they report that increased γ-GT activity is associated with functional maturation and cell differentiation [46]. Several lines of evidence, however, support the claim that γ-GT could play an important role in the utilization of GSH by tumours, such as rat sarcomas [47], to protect tumour cells in conditions of oxidative stress [48].

All these results suggest that a possible mechanism of resistance to ATRA is mediated by high γ-GT expression. Under this hypothesis, the ATRA-induced oxidative stress produced by increased ROS and the consequent reduction in thiols could be reversed through a rapid restoration of GSH levels in tumour cell lines with high γ-GT activity. In addition, when the induced oxidative stress is sufficiently low, the increase in γ-GT activity could function as a survival signal with a corresponding increase in cell proliferation, as suggested here. A number of studies have provided convincing evidence corroborating this hypothesis. First, it has been reported that depending on the level of oxidative stress, ROS can function as a pro-life signal in one setting (mild increase in ROS such as that occurs in pro-oxidant reactions mediated by γ-GT activity) [49] and pro-death in another (high concentration of ROS) [50]. In fact, in our study the increased oxidative stress in S4MH cells produced by the higher dose of ATRA raised the percentage of apoptotic cells. In these tumour cells, it appears that the action of ATRA at low doses (which only produced a moderate increase in ROS) was mainly related to a cell cycle arrest rather than an apoptotic mechanism, which has also been suggested previously in other tumour models [51,52]. Furthermore, the significant ROS increase induced by the addition of BSO to ATRA significantly reduced tumour growth in S4MH, but no additive effect in the percentage of apoptosis was observed in these tumour cells, which may possibly be due to the low dose of ATRA used, as has been described above. Other authors have previously shown that ATRA-induced apoptosis is a slow and time and concentration-dependent event [39]. In the case of F21 cells, the BSO and ATRA combination not only reversed the pro-tumour effect produced by ATRA or BSO alone, but also induced a significant increase in apoptosis. It should be recalled that in response to oxidative stimuli caused by ROS, GSH and its related enzymes play a key role in protecting cells against this oxidative stress [27]. When the process becomes prolonged (augmentation of GSH demand), cellular systems might no longer be able to maintain a normal GSH/oxidized glutathione ratio. The amount of GSH is decreased, which is often associated with the apoptotic programme [25], as we have also observed in our study. This point coincides with the reversion of increased γ-GT activity induced by ATRA or BSO alone observed in F21 tumour cells. Secondly, we and others have linked the activation of this enzyme with enhanced tumorigenic potential of tumour cells, suggesting as a novel therapeutic window the possibility of modifying cellular γ-GT activity [5,49]. Thirdly, it has been demonstrated that high γ-GT activity can trigger signalling pathways and activate transcription factors such as nuclear factor-κB [53]. This factor is thought to be a pivotal regulator involved in oxidative stress responses, controlling inducible expression of a variety of genes including inhibition of apoptosis [54]. This could be one of the mechanisms accounting for the lack of apoptosis increase in ATRA-treated F21 cells observed in our study.

Conclusions

In our view, there are two main conclusions to be drawn from this GSH status-dependent antiproliferative effect of RA: (1) the particular GSH metabolism of each tumour could modulate the effects of RA on tumour cell lines with different degrees of differentiation and (2) the ability of a tumour cell line to restore rapidly its GSH level is more important than the intracellular GSH level present at the moment of RA administration. In fact, although S4MH cells have higher basal levels than F21 cells, they are more sensitive to ATRA effects, which correlated with γ-GT expression.

Taken together, these results suggest that the response of tumour cell lines to RA treatment is highly dependent on the strong relationship between intracellular GSH levels, γ-GT activity and degree of differentiation of the cells in question. This possibility should, therefore, be taken into account in identifying 'retinoid-sensitive' tumours to be included in therapeutic strategies involving ATRA.

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