Glutathione Modulation Reverses the Growth-promoting Effect of Growth Factors, Improving the 5-Fluorouracil Antitumour Response in WiDr Colon Cancer Cells

TEODORO PALOMARES[1], MARTA CARAMÉS[1], IGNACIO GARCÍA-ALONSO[1] and ANA ALONSO-VARONA[2]

Departments of [1]Surgery, Radiology and Physical Medicine and [2]Cell Biology and Morphological Sciences, Faculty of Medicine and Dentistry, University of the Basque Country, Leioa, Vizcaya, Spain

Abstract. *Background: A common cause of treatment failure in colorectal cancer is chemoresistance, which may be related to the redox state of cancer cells and the tumour microenvironment, where growth factors (GFs) play an important role. Glutathione (GSH), a key regulator of the redox balance, is involved in GF signalling systems and may also protect against drug-induced cellular injury. Materials and Methods: The effect of GSH modulation on 5-fluorouracil (5-FU) activity on the WiDr colon cancer cell line was studied. Cell proliferation and GSH content were assessed. Cells were exposed to the GSH modulators, L-buthionine-SR-sulfoximine (BSO) or L 2 oxothiazolidine-4-carboxylate (OTZ), before treatment with 5-FU in the presence of hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF). Results: Exposure to GFs significantly increased GSH levels and induced a pro-tumour effect. During the first 48 h of incubation, VEGF and EGF induced a near 30% reduction in 5-FU antitumour activity, while exposure to HGF abrogated the drug-induced growth inhibition. Treatment with OTZ and BSO abrogated the growth-promoting effects of GFs. Moreover, the addition of either of the GSH modulators to 5-FU produced an increase of nearly 40% in the 5-FU activity in the case of HGF or VEGF, and a 25% increase in the case of EGF. Conclusion: GSH manipulation could yield a therapeutic gain for chemotherapy with 5-FU in the presence of GFs.*

Correspondence to: Dr. Teodoro Palomares, Department of Surgery, Radiology and Physical Medicine, School of Medicine and Dentistry, University of the Basque Country, Leioa, E-48940 Vizcaya, Spain. Tel: +34 946012813, Fax: +34 946012781, e-mail: teodoro.palomares@ehu.es

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Colorectal cancer (CRC) constitutes the second leading cause of death from cancer in the Western world representing one million new cases and half a million deaths annually worldwide. This elevated death rate is related to the fact that more than 20% of all patients with CRC have metastatic disease at diagnosis, and a great number of early–stage patients will eventually go on to develop metastatic or advanced disease (1).

In the treatment of CRC, 5-fluorouracil (5-FU) still maintains its preponderant role. However, various new pharmacological strategies have been applied to enhance its effectiveness. In particular, many different schedules of administration have been developed (2) and this agent has been modulated by the addition of leucovorin (LV) (3). In the last decade, the addition of chemotherapeutic compounds (irinotecan and oxaliplatin) and new targeted therapies (bevacizumab and cetuximab) to standard 5-FU-based chemotherapy regimens have improved overall survival (4). Despite these advances in the management of CRC, there is a strong medical need for more effective and well-tolerated therapies. In fact, resistance to current cytotoxic therapies limits the treatment of CRC and results in treatment failure in the majority of advanced cancer patients. Indeed, the main obstacle to efficient cancer treatment with 5-FU is drug resistance (5).

Chemoresistance must be considered in the context of tumour cells as well as the tumour microenvironment. In relation to this, it has been reported that several growth factors (GFs), such as hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are involved in the paracrine growth mechanisms of colon cancer cells and have therefore been identified for their potential to modulate the sensitivity of tumour cells to chemotherapeutic agents (6). Indeed, GFs play a pivotal role in the regulation of CRC progression and metastasis, stimulating downstream signalling cascades involved in cell proliferation, survival and angiogenesis, and they appear to be viable molecular markers of invasive and

metastatic disease (7). Moreover, we (8) and others (9) have supported the hypothesis that the release of hepatotrophic GFs after partial liver resection, could stimulate not only the proliferation of hepatocytes but also the silent tumour cells that go on to form the metastatic microfoci in the remaining liver, producing liver tumour recurrence, which remains a problem with high incidence in clinical settings.

An increasing amount of evidence has indicated that the intracellular redox state plays an essential role in the mechanisms underlying the actions of GFs. Specifically, GFs have been reported to generate reactive oxygen species (ROS), which can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death (10). The intracellular redox homeostasis capacity is substantiated primarily by glutathione (GSH), the most prevalent intracellular non-protein thiol (11). On the other hand, GSH has long been known to be a chemoresistance factor in cancer (12). It plays an important role in the protection of cellular constituents against oxidative damage and in detoxification and repair processes following cellular injury caused by diverse anticancer agents. Therefore, GSH is able to modulate cellular susceptibility to chemotherapy. Moreover, it has been shown that colorectal carcinogenesis is associated with serious oxidative stress (13) and that colon cancer cells contain high GSH levels (14). There has also been evidence to indicate that the GSH status of colon cancer cells is a critical determinant of cell injury by various agents (15).

Based on these data, it appears plausible that modulation of GSH content could improve the cytotoxic activity of 5-FU and reverse the pro-tumour effects of GFs. Indeed, we have previously shown that the induction of GSH depletion produces a chemosensitizing effect in other tumour models (16-19).

Among GSH modulators, one of the most potent that has been investigated is buthionine-(SR)-sulfoximine (BSO), an irreversible inhibitor of the enzyme γ glutamylcysteine synthetase (the rate-limiting enzyme in GSH synthesis) (20). Another GSH modulator is L-2-oxothizaolidine-4-carboxylate (OTZ) (21), a prodrug that is converted into *S*-carboxy-L-cysteine by the intracellular enzyme 5 oxo L prolinase, which spontaneously decarboxylates to L-cysteine, the rate-limiting precursor for GSH synthesis.

To test our hypothesis, we investigated the effect of GFs on the response of WiDr cells to 5-FU and the influence of GSH modulators (OTZ or BSO) on the growth-promoting action of these GFs. We also evaluated the effects of adding these modulators to treatment with 5-FU, in the presence or absence of GFs.

Materials and Methods

Tumour cell culture. A metastatic human colon cancer WiDr cell line was selected. The line was originally obtained from the American Type Culture Collection (Rockville, MD, USA). The cell line was maintained in Minimum Essential Medium (MEM) with Earl's salts (GIBCO BRL, Rockville, MD, USA) adjusted to contain 2 mM L glutamine, 1% non-essential amino acids, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co, St. Louis, MO, USA) in a humidified atmosphere (5% CO_2, 95% air) at 37˚C.

Exponentially growing cell cultures were used in all experiments. After brief exposure to phosphate-buffered saline (PBS)/EDTA (2 mM) and centrifuging, the pellet was re-suspended in complete medium plus FCS and a cell count obtained with a Coulter counter (Coultronics, Margency, France). Viability, as determined by trypan blue exclusion, ranged from 95% to 98%.

Chemicals. 5-FU was purchased from Acofarma S.C.L. (Barcelona, Spain). Growth factors were obtained from Sigma Chemical Co. and reconstituted in agreement with their specification sheets. BSO and OTZ were also obtained from Sigma Chemical Co. For the *in vitro* experiments, drugs were dissolved in MEM at an appropriate concentration.

Determination of cell proliferation. WiDr human colon cancer cells were seeded in 24-well microplates at a density of 10^4 cells/well in 10^3 μl of growth medium plus 10% FCS, and allowed to attach and grow for 24 h. The cells were then exposed to BSO 100 μM for 24 h. Subsequently, the BSO was removed and the cells were treated with 5-FU for 24 h. The IC_{50} at 48 h was used (0.5 μg/ml of 5-FU), as determined in previous studies. In the experiments with OTZ 5 mM, the cells were exposed to the cysteine prodrug 5 mM for 4 h before the addition of 5-FU (for 24 h). After treatment, the cells were washed free of drug and allowed to grow for an additional 48 h in growth medium alone (schedule A of OTZ) or with OTZ 5 mM (schedule B of OTZ). These experiments were also carried out in the presence of HGF 7.5 ng/ml, VEGF 10 ng/ml or EGF 25 ng/ml. They were added at the same time as the 5-FU and maintained until the end of the experimental period. The concentration of each GF was chosen from preliminary studies to determine the maximal increase in growth (data not shown). At 24, 48 and 72 h after the addition of drugs, proliferation was measured using a haemocytometer to count the cells growing in each well. Each assay was repeated three times and all experiments were performed in sextuplicate wells.

Cell growth with 5-FU alone or in combination with BSO or OTZ, in the presence or absence of GFs, was calculated as a percentage with respect to the growth of cells incubated in culture medium alone (the control). The dose modification factor (DMF), representing the degree of enhancement of drug-induced growth inhibition by BSO or OTZ, was calculated as follows:

$$\text{DMF} = \frac{\%\ \text{inhibition by modulator agent + drug}}{\%\ \text{inhibition by modulator agent} + \%\ \text{inhibition by drug}}$$

GSH determination. Intracellular GSH content was determined using the Cytofluor-2350 system. After drug exposure, the medium was removed from the cultures and the cells were washed twice with PBS. For the GSH assay, the cells were stained with 100 μM monochlorobimane (Molecular Probes, Eugene, OR, USA) for 60 min at 37˚C. The intracellular GSH content was proportional to the fluorescence intensity determined with 360 nm excitation using a 460 nm emission filter, at high and intermediate sensitivity settings.

Corrections for autofluorescence were made by subtracting fluorescence measured in unstained cells to obtain a normalized fluorescence index. Each assay was repeated three times and all experiments were performed in sextuplicate wells.

Statistical analysis. Statistical analysis was performed using GraphPad™ (GraphPad software, San Diego, CA, USA). The test of significance was carried out using Student's *t*-test and factorial analysis of variance (ANOVA) as appropriate. The values were considered to be statistically different from controls when $p<0.05$.

Results

Effect of HGF, VEGF and EGF on the intracellular GSH levels and proliferation rate of colon cancer cells. Treatment of cells with GFs initially reduced the GSH levels and subsequently significantly increased them. Exposure to VEGF (10 ng/ml) and EGF (25 ng/ml) resulted in a 16% and 18% reduction, respectively, after the first hour of incubation, with an increase at 2 h (55 and 48%, respectively; $p<0.0001$) and reversion to control levels at 10 h. In the case of HGF (7.5 ng/ml), the initial reduction was higher (31% at 2 h) and the increase was observed at 4 h (44%) and maintained until 8 h ($p<0.0001$), before reverting to control levels at 10 h (Figure 1A).

Increased cellular GSH content induced by each of the three GFs was accompanied by an increase in the proliferation rate compared with control cells. Specifically, VEGF and EGF exposure produced a 1.2-fold increase ($p<0.05$) in the proliferation rate compared with control cells at 48 and 72 h (Figure 1B). However, the increase was maximal in the case of HGF which produced a 1.7-fold and 1.3-fold increase at 48 and 72 h, respectively ($p<0.05$).

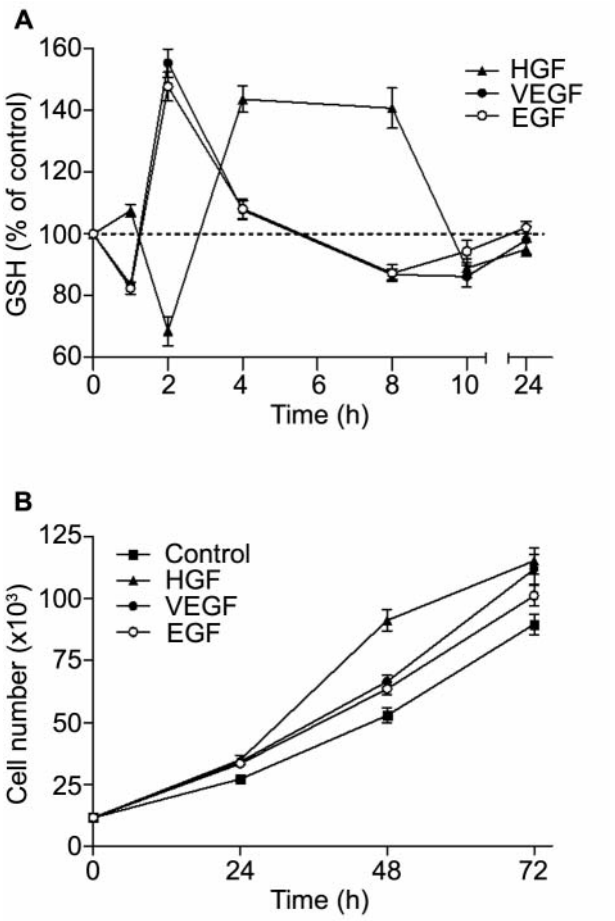

Figure 1. *Effect of GFs on intracellular GSH levels (A) and on the growth rate of WiDr cells (B). Results are expressed as mean±SD of three independent experiments.*

Effect of HGF, VEGF and EGF on the antitumour activity of 5-FU. Treatment of cells with 0.5 μg/ml 5-FU produced a 34% reduction in GSH levels after 2 h of incubation, following which levels were observed to recover and rise to 20% above control levels after 4 h ($p<0.01$), finally returning to the control levels at 10 h (Figure 2). Exposure to 5-FU resulted in a 1.7-, 2.1- and 1.8-fold reduction ($p<0.0001$) in the growth rate of WiDr cells at 24, 48 and 72 h, respectively. However, the presence of GFs reduced the cytotoxic activity of 5-FU. Specifically, at 48 h, whereas exposure to VEGF and EGF resulted in a 30% reduction of drug activity, no significant modification in proliferation rate of 5-FU treated cells in presence of HGF was observed with respect to control cells. At 72 h, treatment with 5-FU in the presence of any GF produced a 1.5-fold reduction ($p<0.001$) in the growth rate compared with untreated cells (20% reduction in drug activity), as shown in Figure 3.

Effect of BSO and OTZ on the pro-tumour activity of GFs in colon cancer cells. Tumour cells were exposed to the modulator agents BSO or OTZ. In order to exclude cytotoxic effects during the assays, only concentrations of the test compounds giving at least 90% viable cells were used in the experiments.

Treatment with 100 μM BSO produced a 51.9% reduction ($p<0.0001$) in GSH levels at 24 h of incubation. Exposure of WiDr cells to 5 mM OTZ reduced intracellular GSH levels, with the maximal reduction being obtained at 4 h (46.2% reduction, $p<0.0001$). Although proliferation was not significantly modified after 24 h of incubation with BSO, this treatment resulted in a 1.3-fold reduction ($p<0.01$) in the growth rate of WiDr 24 h after BSO was removed. In contrast, the proliferation rate with OTZ was already significantly lower (1.3-fold, $p<0.01$) after the first 24 h of incubation. However, both produced the same reduction in the proliferation rate at 72 h (1.3-fold, $p<0.01$) (Figure 4).

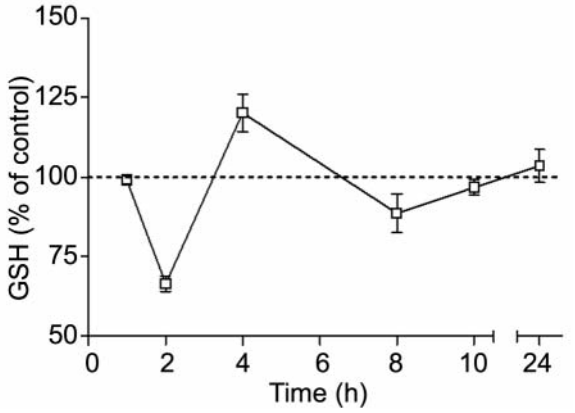

Figure 2. *Effect of 5-FU on the GSH levels of WiDr colon cancer cells, expressed as a percentage of control values.*

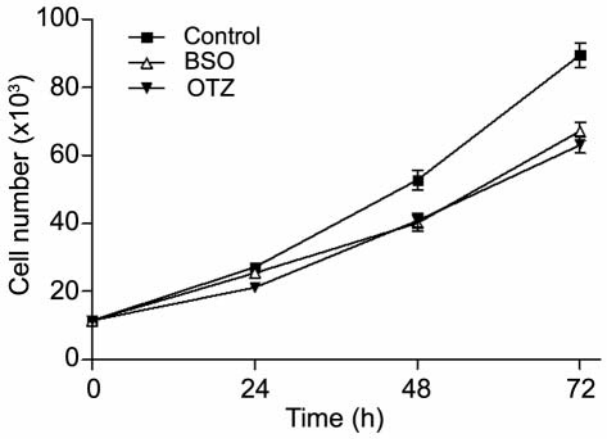

Figure 4. *Effect of BSO and OTZ on the proliferation rate of WiDr cells. Results are expressed as mean±SD of three independent experiments.*

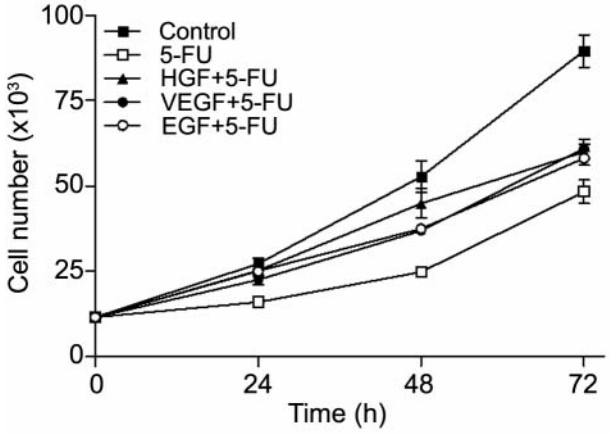

Figure 3. *Relative growth rate of WiDr cells treated with 5-FU in the presence or absence of GFs. Results are expressed as mean±SD of three independent experiments.*

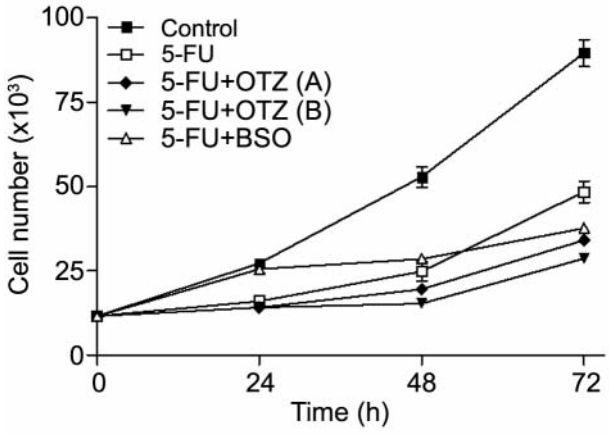

Figure 5. *Effect of GFs on the growth rate of WiDr cells previously exposed to BSO or OTZ. Results are expressed as mean±SD of three independent experiments.*

Both GSH modulators abrogated the pro-tumour effects of GFs. Specifically, at 24 h no significant modification in proliferation rate compared with untreated cells was observed and a 1.2-fold reduction ($p<0.01$) in the proliferation rate was obtained at 72 h (Figure 5).

Effect of BSO and OTZ on the cytotoxic activity of 5-FU on colon cancer cells in the presence or absence of GFs. Firstly, we analysed the effect of GSH modulators on the activity of the chemotherapeutic agent in absence of GFs. As shown in Figure 6, the addition of BSO to 5-FU therapy resulted in an additive effect (DMF of 0.8), producing a 2.4-fold reduction ($p<0.0001$) in the proliferation rate compared with untreated cells at 72 h. Overall, the combined therapy of BSO + 5-FU produced a 29% increase in the cytotoxic activity of 5-FU. Similarly, the combination of OTZ and 5-FU produced a significantly greater antitumour effect than did 5-FU alone. However, the effect was different depending on the schedule. Whereas schedule A resulted in a 2.6-fold reduction ($p<0.0001$) in the growth rate compared with untreated cells at 72 h, treatment with schedule B produced an even greater reduction (3.1-fold, $p<0.0001$). Overall, OTZ significantly increased the antitumour effect of 5-FU (42 and 69% with schedules A and B, respectively), indicating an additive effect with both combinations (DMF of 0.8 and 0.9, respectively). Therefore, of the two GSH modulators, the maximal enhancement of drug-induced growth inhibition was observed with OTZ given according to schedule B (31% greater than with BSO).

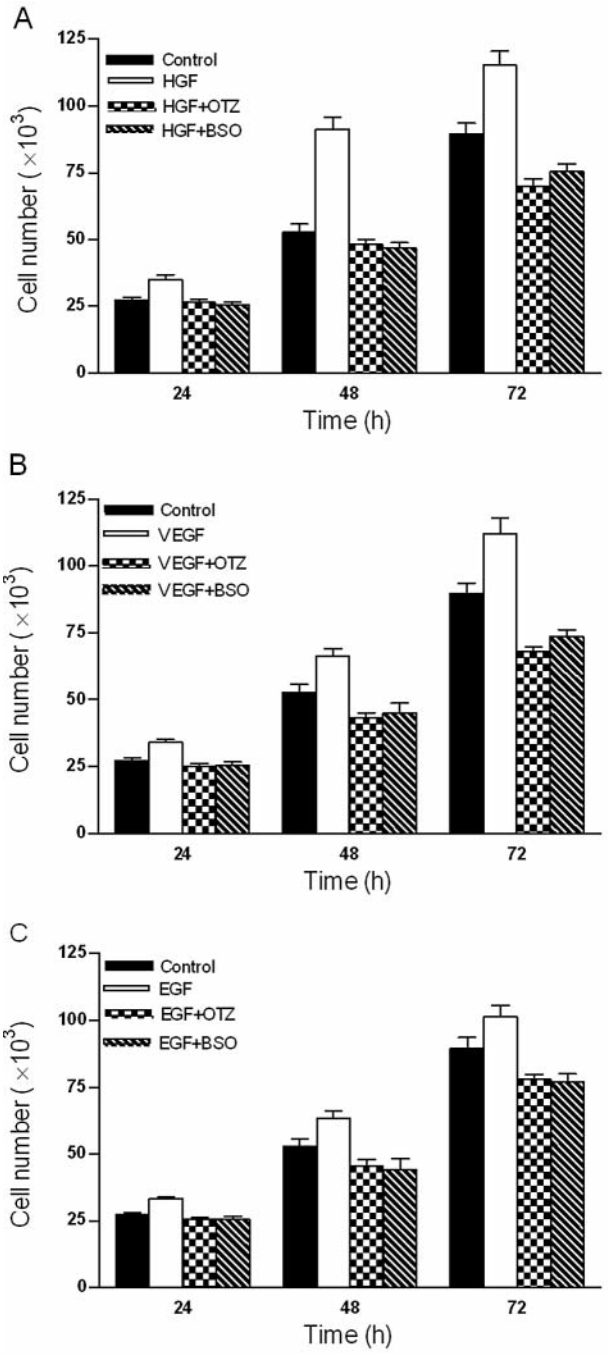

Figure 6. *Effect of 5-FU treatment alone and in combination with GSH modulator agents BSO or OTZ (schedule A and B) on the proliferation rate of WiDr cells. Results are expressed as mean±SD of three independent experiments.*

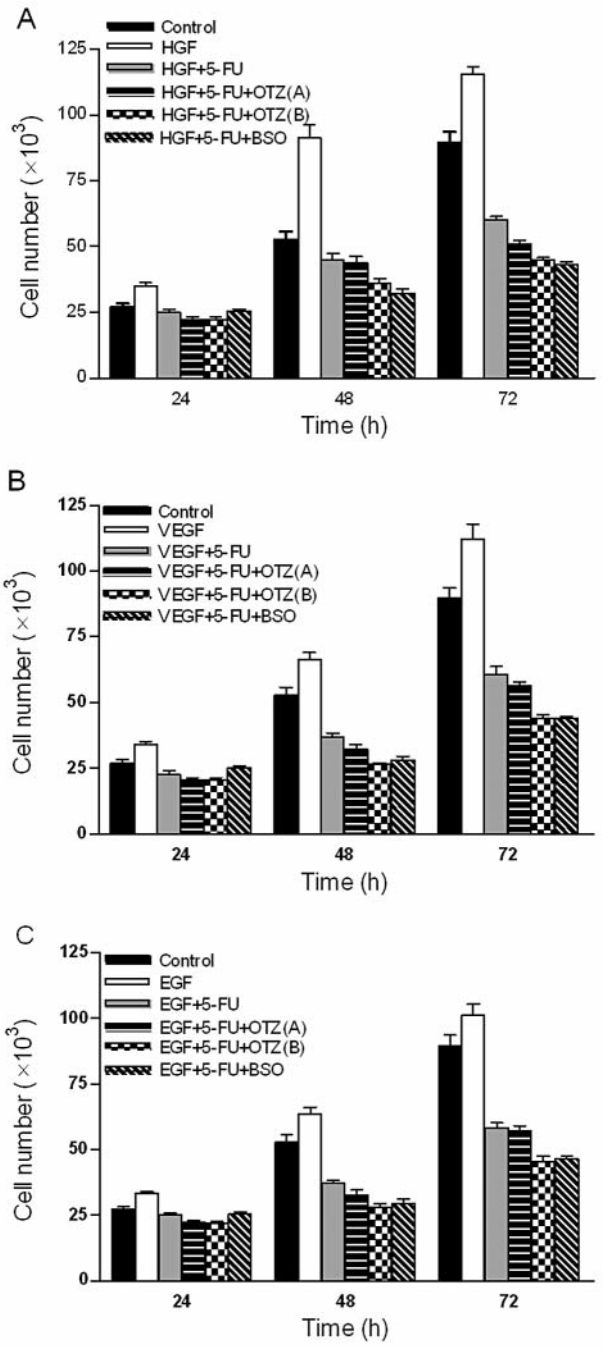

Figure 7. *Effect of 5-FU treatment alone and in combination with BSO or OTZ (schedule A and B) on the growth rate of WiDr cells in presence of GFs. Results are expressed as mean±SD of three independent experiments.*

In the presence of GFs, 5-FU treatment after BSO exposure resulted in a 2-fold reduction ($p<0.0001$) in the proliferation rate compared with controls. These combinations led to a 40, 39 and 23% increase in the cytotoxic activity of 5-FU in the presence of HGF, VEGF and EGF, respectively, at 72 h. Overall, BSO pretreatment combined with this cytotoxic drug in the presence of GFs resulted in a DMF of approximately 1, suggesting an additive effect. As shown in Figure 7, whereas there was no significant increase in the antitumour effect of 5-FU with the

addition of schedule A of OTZ in the presence of VEGF or EGF, in the case of HGF this combination produced a 17% increase in the antitumour effect of the drug at 72 h (1.7-fold reduction in the proliferation rate compared with untreated cells, $p<0.0001$). On the other hand, the combined therapy of 5-FU and OTZ given according to schedule B produced a nearly 2-fold reduction ($p<0.0001$) in the growth rate of WiDr cells at 72 h in the presence of any GF. Therefore, treatment with OTZ produced a 34, 40 and 25% enhancement of 5 FU-induced growth inhibition after 72 h of exposure to HGF, VEGF and EGF, respectively. The effect of this combination on growth inhibition was additive, as represented by a DMF of approximately 0.9.

Discussion

Resistance to chemotherapy is believed to be one of the major causes of treatment failure in CRC. Thus, it is necessary to explore alternative therapeutic modalities to overcome drug resistance. To understand one of the mechanisms of resistance to chemotherapy in colon cancer cells, we examined the effects of GFs on WiDr cell proliferation and on the chemotherapeutic efficacy of 5-FU. Overall, we found that GFs have a growth-promoting effect on WiDr cells and increase intracellular GSH levels in a time-dependent manner, suggesting that the increase in GSH is important for the mitogenic effect of GFs. This pro-tumour effect was maximal after the exposure to HGF, which could be related to the longer increase in the GSH content induced by this factor. What is more, high GSH levels are known to contribute to increased DNA synthesis (22). In addition, several authors have confirmed that HGF, VEGF and EGF play a crucial role in tumour growth and development of metastases in CRC (23). Indeed, bevacizumab, a monoclonal antibody to VEGF, is used in combination with 5-FU regimens as a first-line agent for metastatic CRC (24), and cetuximab, an antibody to the EGF receptor, is approved for the second-line treatment of metastatic CRC (25). With respect to the influence of GFs on GSH levels, some of the apparent discrepancies in the literature may be due to different times of measurement, showing levels increased or decreased with EGF depending on the study (26, 27). On the other hand, HGF has been reported to increase cell GSH levels only under subconfluent density, which could explain why HGF only acts as a mitogen under low cell density (28) and could also be related to the partial loss of activity we found after 72 h of HGF incubation. Moreover, accumulating data suggest that HGF may function as an antioxidant factor able to protect against oxidative stress-mediated death through modulation of intracellular GSH levels (29). Indeed, HGF protects cancer cells from DNA-damaging agents, decreasing sensitivity to chemotherapeutic agents (30). To the best of our knowledge, the current study is the first to show HGF abrogates 5-FU-induced growth inhibition in colon cancer cells over 48 h. Taking into account that HGF could be one of the main factors involved in the tumour-enhancing effect after partial liver resection, the use of this drug in such situations could be related, in part, with the high tumour recurrence. In addition, according with previous studies in other tumour models (31), we have shown that EGF and VEGF reduce the cytotoxic effect of 5-FU. It has been proposed that 5-FU-induced EGFR activation conferred protection against chemoradiation in colon cancer cells, through activation of DNA repair (32) and in relation to this, inhibition of EGFR tyrosine kinase has been investigated for reversing resistance to fluoropyrimidine treatment (33). Moreover, GFs promote cell survival *via* the induction of anti-apoptotic proteins such as Bcl-XL, which has an important role in the resistance of CRC cells to current treatment modalities (34). It has also been proposed that the EGFR-src-signal transducers and activators of transcription 3 (STAT 3) oncogenic pathway play a central role in tumorigenesis and are involved in tumour escape from genotoxic treatments (35). On the other hand, GFs are also known to activate the redox sensitive transcription factor nuclear factor kappa B (NF-κB), which may play a major role in inducible chemoresistance (36). In addition, treatment with 5-FU activates NF-κB and inhibition of NF-κB enhances the cytotoxic effect of 5-FU in colon cancer cells (37). Another possible mechanism of GF-induced drug resistance is the increased intracellular GSH levels. In fact, several authors have confirmed that GSH status is a determining factor of cellular susceptibility in host and tumour tissues to the activity of chemotherapeutic agents (12). Furthermore, it is known that CRC is particularly resistant to cytotoxic chemotherapy, which could be related to the high GSH levels (14). Indeed, we observed that with 5-FU after GSH levels decreased, they recovered and then increased, which could also be related to cellular resistance through a mechanism of "adaptation to the oxidative stress" in response to 5-FU-induced ROS generation (5). This is consistent with the fact that once the drug treatment has been finished, the growth of tumour cells rebounds, as we have observed.

In the light of these data, we believe that treatment with BSO or OTZ could be a good strategy for improving the chemotherapy involving 5-FU in the presence of GFs. We initially compared the ability of BSO and OTZ as anticancer agents. In agreement with the literature (20, 21), our study shows that both induce a decrease in the intracellular GSH level (approximately 50%) and produce a significant growth-inhibitory effect. However, while OTZ impaired activity at an early stage, BSO treatment produced GSH depletion at 24 h but did not reduce proliferation until 24 h after it was removed. Both GSH modulators abrogate the growth-promoting effects of GFs on WiDr cells by depressing GSH levels. Previous studies have also shown

that BSO interfered with the proliferation induced by GFs in other cell types (27, 28), although the effect of OTZ has not been reported prior to the present study. It is important to note that GF-induced signal transduction mechanisms are redox sensitive, and alterations in cellular GSH content may thus affect the growth of GF-sensitive cells. In fact, GF-stimulated ROS generation plays a role in these complex signalling cascades (38, 39). However, ROS are well recognised for playing a dual role because their overproduction, which might be initiated by two successive triggering agents such as GSH modulators and GFs, results in oxidative stress, a process that can damage cell structures, including lipids and membranes, proteins and DNA (11, 40). Moreover, the fact that NF-κB is involved in GF-dependent proliferation and that the transcription factor activity might also be subject to regulation by GSH (41) means that it is possible to speculate that depletion of cellular GSH content could interrupt NF-κB functions and consequently lead to growth inhibition, as has also been suggested by other authors in the case of tumour necrosis factor-alpha-induced NF-κB activation (42).

Secondly, an additive effect was also demonstrated with the combined therapies of 5-FU with BSO or OTZ. Schedule dependency was observed with OTZ. Overall, replacement of OTZ after 5-FU removal (schedule B) was found to be the best therapeutic schedule. In fact, the optimum results were obtained with this combination (nearly 70% increase in 5-FU efficacy), approximately 30% higher than the enhancement of the drug-induced growth inhibition by BSO. Previous studies have also shown that BSO treatment enhanced the cytotoxic activity of 5-FU in colon cancer cells (43, 44). In other studies, high-dose therapy with antioxidants such as pyrrolidine dithiocarbamate (PDTC) and *N*-acetylcysteine (NAC) is reported to increase the efficacy of 5-FU (45), although we have previously demonstrated that the increased intracellular GSH levels induced by NAC are not specific to normal cells and moreover NAC was also found to enhance the proliferation rate of cancer cells (18).

Finally, we observed that the addition of OTZ or BSO to 5-FU therapy in the presence of GFs produced a significantly greater antitumour activity than that obtained with 5-FU alone. Both GSH modulators, OTZ (given according to schedule B) or BSO in combination with 5-FU seem to have similar effects on the proliferation rate of WiDr in the presence of GFs, the same as obtained with 5-FU alone in absence of GFs. However, we (16) and others (20) have previously shown that BSO enhances the cytotoxic effects of drugs against tumours, but also produces a simultaneous increase in the toxicity of these chemotherapeutic drugs in normal tissues. Clearly, an ideal cancer treatment would combine protection of normal tissues against toxicity and sensitisation of tumours to anticancer drugs. In relation to this, we have previously observed that OTZ increases the sensitivity of melanoma cells to the cytotoxic action of acrolein by depressing GSH levels, and protects peripheral blood mononuclear cells (PBMCs) by increasing them (17-19). This has been related to a significantly lower level of 5 oxoprolinase in tumours than in normal tissues (46). Hence, the selective inhibition of GSH with OTZ could be a promising approach, allowing dose escalation of the chemotherapeutic agents.

In summary, the addition of OTZ or BSO to chemotherapy with 5-FU is an option worth considering to abrogate WiDr cell GF-mediated chemoresistance and to thereby significantly enhance the therapeutic benefit of the anticancer drug in such circumstances. Moreover, taking into account the role of GFs in the development of metastasis and in the tumour-stimulating effect secondary to hepatectomy, this strategy could be crucial in the treatment of metastatic disease and to prevent the recurrence of liver metastases following curative surgery.

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References

1 Benson AB 3rd: Epidemiology, disease progression, and economic burden of colorectal cancer. J Manag Care Pharm *13*: 5-18, 2007.

2 Meta-analysis Group in Cancer: Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. Meta-analysis Goup in Cancer. J Clin Oncol *16*: 301-308, 1998.

3 Thirion P, Michiels S and Pignon JP: Meta-analysis Group in Cancer. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer. An updated meta-analyisis. J Clin Oncol *22*: 3766-3775, 2004.

4 Grothey A and Sargent D: Overall survival of patients with advanced colorectal cancer correlates with availability of fluorouracil, irinotecan, and oxaliplatin regardless of whether doublet or single-agent therapy is used first line. J Clin Oncol *23*: 9441-9442, 2005.

5 Hwang IT, Chung YM, Kim JJ, Chung JS, Kim BS, Kim HJ, Kim JS and Yoo YD: Drug resistance to 5-FU linked to reactive oxygen species modulator 1. Biochem Biophys Res Commun *359*: 304-310, 2007.

6 Melisi D, Troiani T, Damiano V, Tortora G and Ciardiello F: Therapeutic integration of signal transduction targeting agents and conventional anticancer treatments. Endocr Relat Cancer *11*: 51-68, 2004.

7 Kammula US, Kuntz EJ, Francone TD, Zeng Z, Shia J, Landmann RG, Paty PB and Weiser MR: Molecular coexpression of the *c-Met* oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome. Cancer Lett *248*: 219-228, 2007.

8 García-Alonso I, Palomares T, Alonso A, Echenique-Elizondo M, Caramés J, Castro B and Méndez J: Effect of liver resection on the progression and growth of rhabdomyosarcoma metastases in a rat model. J Surg Res *148*: 185-190, 2008.

9 Jong KPM, Sloof MJM and Vries EDM: Effect of partial liver resection on tumour growth. J Hepatol *25*: 109-121, 2003.

10 Thannickal VJ and Fanburg BL: Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol *279*: 1005-1028, 2000.

11 Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M and Telser J: Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol *39*: 44-84, 2007.

12 Balendiran GK, Dabur R and Fraser D: The role of glutathione in cancer. Cell Biochem Funct *22*: 343-352, 2004.

13 Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga-Koda L and Sulkowska M: Lipid peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol *11*: 403-406, 2005.

14 Kanbagli O, Ozdemirler G, Bulut T, Yamaner S, Aykac-Toker G and Uysal M: Mitochondrial lipid peroxides and antioxidant enzymes in colorectal adenocarcinoma tissues. Jpn J Cancer Res *91*: 1258-1263, 2000.

15 Knoll N, Ruhe C, Veeriah S, Sauer J, Glei M, Gallagher EP and Pool-Zobel BL: Genotoxicity of 4-hydroxy-2-nonenal in human colon tumor cells is associated with cellular levels of glutathione and the modulation of glutathione-*S*-transferase A4 expression by butyrate. Toxicol Sci *86*: 27-35, 2005.

16 Palomares T, Bilbao P, del Olmo M, Castro B, Calle Y and Alonso-Varona A: *In vitro* and *in vivo* comparison between the effects of treatment with adenosine triphosphate and treatment with buthionine sulfoximine on chemosensitization and tumour growth of B16 melanoma. Melanoma Res *9*: 233-242, 1999.

17 Bilbao P, del Olmo M, Alonso-Varona A, Castro B, Bilbao J and Palomares T: L 2 Oxothiazolidine-4-carboxylate reverses the tumour growth-promoting effect of interleukin-2 and improves the antitumour efficacy of biochemotherapy in mice bearing B16 melanoma liver metastases. Melanoma Res *12*: 17-26, 2002.

18 del Olmo M, Alonso-Varona A, Castro B, Calle Y, Bilbao P and Palomares T: Effects of L-2-oxothiazolidine-4-carboxylate on the cytotoxic activity and toxicity of cyclophosphamide in mice bearing B16F10 melanoma liver metastases. Melanoma Res *10*: 103-112, 2000.

19 Olmo M, Alonso-Varona A, Castro B, Bilbao P and Palomares T: Cytomodulation of interleukin-2 effect by L-2-oxothiazolidine-4-carboxylate on human malignant melanoma. Cancer Immunol Immunother *55*: 948-957, 2006.

20 Bailey HH: L-S, R-Buthionine sulfoximine: historical development and clinical issues. Chem Biol Interact *111*: 239-254, 1998.

21 Rose DM, Hochwald SN, Harrison LE and Burt M: Selective glutathionine repletion with oral oxothiazolidine carboxylate (OTZ) in the irradiated tumor-bearing rat. J Surg Res *62*: 224-228, 1996.

22 Higuchi Y: Glutathione depletion-induced chromosomal DNA fragmentation associated with apoptosis and necrosis. J Cell Mol Med *8*: 455-464, 2004.

24 Lesko E and Majka M: The biological role of HGF-MET axis in tumor growth and development of metastasis. Front Biosci *13*: 1271-1280, 2008.

25 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R and Kabbinavar F: Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. J Clin Oncol *23*: 3502-3508, 2005.

26 Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santero A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I and Van Cutsem E: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med *351*: 337-345, 2004.

27 Musallam L, Éthier C, Haddad PS and Bilodeau M: EGF mediates protection against Fas-induced apoptosis by depleting and oxidizing intracellular GSH stocks. J Cell Physiol *198*: 62-72, 2004.

28 Carmona-Cuenca I, Herrera B, Ventura JJ, Roncero C, Fernández M and Fabregat I: EGF blocks NADPH oxidase activation by TGF-beta in fetal rat hepatocytes, impairing oxidative stress, and cell death. J Cell Physiol *207*: 322-330, 2006.

29 Yang H, Magilnick N, Xia M and Lu SC: Effects of hepatocyte growth factor on glutathione synthesis, growth, and apoptosis is cell density-dependent. Exp Cell Res *314*: 398-412, 2008.

30 Arends B, Slump E, Spee B, Rothuizen J and Penning LC: Hepatocyte growth factor improves viability after H_2O_2-induced toxicity in bile duct epithelial cells. Comp Biochem Physiol C Toxicol Pharmacol *147*: 324-330, 2008.

31 Chen JT, Huang CY, Chiang YY, Chen WH, Chiou SH, Chen CY and Chow KC: HGF increases cisplatin resistance *via* down-regulation of AIF in lung cancer cells. Am J Respir Cell Mol Biol *38*: 559-565, 2008.

32 Zhang L, Hannay JA, Liu J, Das P, Zhan M, Nguyen T, Hicklin DJ, Yu D, Pollock RE and Lev D: Vascular endothelial growth factor overexpression by soft tissue sarcoma cells: implications for tumor growth, metastasis, and chemoresistance. Cancer Res *66*: 8770-8778, 2006.

33 Hiro J, Inoue Y, Toiyama Y, Miki C and Kusunoki M: Mechasnism of resistance to chemoradiation in p53 mutant human colon cancer. Int J Oncol *32*: 1305-1310, 2008.

34 Stebbing J, Harrison M, Glynne-Jones R, Bridgewater J and Propper D: A phase II study to determine the ability of gefitinib to reverse fluoropyrimidine resistance in metastatic colorectal cancer (the INFORM study). Br J Cancer *98*: 716-719, 2008.

35 Schulze-Bergkamen H, Ehrenberg R, Hickmann L, Vick B, Urbanik T, Schimanski CC, Berger MR, Schad A, Weber A, Heeger S, Galle PR and Moehler M: Bcl-x(L) and myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells. World J Gastroenterol *14*: 3829-3840, 2008.

36 Hbibi AT, Lagorce C, Wind P, Spano JP, Des Guetz G, Milano G, Benamouzig R, Rixe O, Morere JF, Breau JL, Martin A and Fagard R: Identification of a functional EGF-R/p60c-src/STAT3 pathway in colorectal carcinoma: analysis of its long-term prognostic value. Cancer Biomark *4*: 83-91, 2008.

37 Fan S, Gao M, Meng Q, Laterra JJ, Symons MH, Coniglio S, Pestell RG, Goldberg ID and Rosen EM: Role of NF-kappaB signaling in hepatocyte growth factor/scatter factor-mediated cell protection. Oncogene *24*: 1749-1766, 2005.

38 Voboril R, Hochwald SN, Li J, Brank A, Weberova J, Wessels F, Moldawer LL, Camp ER and MacKay SL: Inhibition of NF-kappa B augments sensitivity to 5 fluorouracil/folinic acid in colon cancer. J Surg Res *120*: 178-188, 2004.

39 Jagadeeswaran S, Bindokas VP and Salgia R: Activation of HGF/c-Met pathway contributes to the reactive oxygen species generation and motility of small cell lung cancer cells. Am J Physiol Lung Cell Mol Physiol *292*: 1488-1494, 2007.

40 Ushio-Fukai M and Nakamura Y: Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. Cancer Lett *266*: 37-52, 2008.

41 Mazière C, Floret S, Santus R, Morlière P, Marcheux V and Mazière JC: Impairment of the EGF signaling pathway by the oxidative stress generated with UVA. Free Rad Biol and Med *34*: 629-636, 2003.

42 Liu TZ, Hu CCA, Chen YH, Stern A and Cheng JT: Differentiation status modulates transcription factor NF-κB activity in unstimulated human hepatocellular carcinoma cell lines. Cancer Lett *151*: 49-56, 2000.

43 Tsou TC, Yeh SC, Tsai FY, Chen JW and Chiang HC: Glutathione regulation of redox-sensitive signals in tumor necrosis factor-alpha-induced vascular endothelial dysfunction. Toxicol Appl Pharmacol *221*: 168-178, 2007.

44 Chen MF, Chen LT and Bouce HW Jr: 5-Fluorouracil cytotoxicity in human colon HT-29 cells with moderately increased or decreased cellular gluthatione level. Anticancer Res *15*: 163-167, 1995.

45 Meurette O, Lefeuvre-Orfila L, Rebillard A, Lagadic-Gossmann D and Dimanche-Boitrel MT: Role of intracellular glutathione in cell sensitivity to the apoptosis induced by tumor necrosis factor (alpha)-related apoptosis-inducing ligand/anticancer drug combinations. Clin Cancer Res *11*: 3075-3083, 2005.

46 Bach SP, Williamson SE, Marshman E, Kumar S, O'Dwyer ST, Potten CS and Watson AJ: The antioxidant *N*-acetylcysteine increases 5-fluorouracil activity against colorectal cancer xenografts in nude mice. J Gastrointest Surg *5*: 91-97, 2001.

47 Chen X, Schecter RL, Griffith OW, Hayward MA, Alpert LC and Batist G: Characterization of 5-oxo-L-prolinase in normal and tumour tissues of humans and rats: a potential new target for biochemical modulation of glutathione. Clin Cancer Res *4*: 131-138, 1998.

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