Glutathione Modulators Reverse the Pro-tumour Effect of Growth Factors Enhancing WiDr Cell Response to Chemotherapeutic Agents

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Abstract. *Background: Glutathione has been implicated in growth factor-mediated chemoresistance of colon cancer cells. Materials and Methods: We evaluated the influence of hepatocyte growth factor, vascular endothelial growth factor and epidermal growth factor on the effect of 5-fluorouracil, oxaliplatin and SN-38 (the active metabolite of irinotecan) on WiDr cells. We also analysed the effect of glutathione modulators (L-buthionine-SR-sulfoximine, and L-2-oxothiazolidine-4-carboxylate) on the growth-promoting effect induced by growth factors and on the antiproliferative activity of the aforementioned drugs. Results: Exposure to growth factors reduced drug cytotoxic activity, specially in the case of 5-fluorouracil. The addition of L-buthionine-SR-sulfoximine or L-2-oxothiazolidine-4-carboxylate to the chemotherapeutic agents abrogated pro-tumour effects of the growth factors, and produced a greater antitumour activity than the drugs alone. Conclusion: Among the combinations analysed, the addition of L-2-oxothiazolidine-4-carboxylate to SN-38 was found to be the best chemotherapeutic combination, resulting in a near 70% increase in the cytotoxic activity of SN-38.*

Colorectal cancer (CRC) remains one of the major causes of cancer death worldwide (1). Remarkable and clinically relevant advances have been made in recent years in the treatment of this disease, essentially owing to the introduction of oxaliplatin and irinotecan. Oxaliplatin is a novel diaminocyclohexane platinum agent (2). Irinotecan is a semi-synthetic derivate of the natural alkaloid camptothecin which inhibits topoisomerase I. *In vivo* irinotecan is enzymatically converted by carboxylesterase to its most active cytotoxic metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38) (3). These new drugs have been able to achieve response rates in the region of 20% and median survival time of more than a year (4). These results are comparable to those obtained using bolus 5-fluorouracil/leucovorin (5-FU/LV). Moreover, the use of irinotecan or oxaliplatin in combination with 5-FU/LV can consistently achieve response rates in the region of 50% and a median overall survival time greater than 16 months (5-7).

Even with the significant improvements in traditional chemotherapy, limitations remain with this treatment. As a consequence, several novel targeted molecular compounds are being investigated both as single agents and in combination with chemotherapy. Some of the most promising targets include growth factors (GFs) and their related receptors. Since 2004, three novel agents have been approved: bevacizumab, which binds vascular endothelial growth factor (VEFG) (8), and cetuximab and panitumumab, which block the epidermal growth factor receptor (EGFR) (9, 10).

GFs are not only involved in survival signalling, cell migration, metastasis formation and angiogenesis, but also confer a reduction in the responses of tumour cells to cytotoxic compounds (11). In fact, we have previously demonstrated that GFs such as hepatocyte growth factor (HGF), VEGF and EGF decreased the antitumor effects of 5-FU (12). These results supported the notion that the inhibition of the growth-promoting action of GFs is a promising approach to sensitizing tumour cells and overcoming drug resistance. Previous studies have also demonstrated that the activation of the EGF/EGFR system by SN-38, the active metabolite of irinotecan, could contribute to resistance to this drug (13). In addition, gefitinib, a EGFR tyrosine kinase inhibitor, and irinotecan have been shown to have a synergistic effect on CRC cells (14).

An increasing amount of evidence has indicated that the intracellular redox state plays an essential role in the mechanisms underlying the action of GFs. Specifically, GFs

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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 (15). The intracellular "redox homeostasis" capacity is primarily regulated by glutathione (GSH), the most prevalent intracellular non-protein thiol (16). Since GSH plays an important role in the growth-promoting effect of GFs, as we have shown (12), and it is also involved in the protection against cellular injury caused by various anticancer agents modulating cellular susceptibility to chemotherapy (17), manipulation of GSH levels might yield a therapeutic gain for chemotherapy in the presence of GFs. Indeed, we have previously shown that GSH modulators, buthionine-(SR)-sulfoximine (BSO) (18) and L-2-oxothizaolidine-4-carboxylate (OTZ) (19-21), induce a depletion in GSH, and produce growth-inhibitory effects, inducing an increasing chemosensitivity of tumour cells. BSO is an irreversible inhibitor of the enzyme γ-glutamylcysteine synthetase (the rate-limiting enzyme in GSH synthesis) (22), while OTZ, a 5-oxoproline analogue, is metabolised by 5-oxoprolinase and coverted to cysteine, the rate-limiting amino acid for GSH synthesis (23). Moreover, we have recently demonstrated that the addition of both GSH modulators to chemotherapy with 5-FU abrogates WiDr cell GF-mediated chemoresistance and thereby enhances the therapeutic benefit of this anticancer drug significantly (12).

On the other hand, the availability of two new active agents, irinotecan and oxaliplatin, raises the question of optimal drug sequencing in first- and second-line treatments. According to a meta-analysis which studied 242 randomised trials comparing various chemotherapy regimens in patients with advanced CRC, the exact ranking of specific regimens remains uncertain (24). In view of this, it is necessary to investigate aspects of tumour biology that may predict sensitivity to particular drugs.

Given these premises, the purpose of this study was to compare the influence of HGF, VEGF and EGF on the anti-tumour activity of 5-FU, oxaliplatin and irinotecan and to analyse which of them could deliver a greater increase in the therapeutic benefit when combined with the GSH modulators in the presence 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, MI, 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 the complete medium plus FCS and a cell count was obtained with a Coulter counter (Coultronics, Margency, France). Viability, determined by trypan blue exclusion, ranged from 95% to 98%.

Chemicals. Growth factors were obtained from Sigma Chemical Company and reconstituted in agreement with their specification sheets. Oxaliplatin, BSO and OTZ were also obtained from Sigma Chemical Company. 5-Fluorouracil (5-FU) was purchased from Acofarma S.C.L. (Barcelona, Spain). For the *in vitro* experiments, drugs were dissolved in MEM at the appropriate concentration. Stock solutions of SN-38 10 mM (kindly provided by Pfizer Inc., Groton, CT, USA) were prepared in DMSO and stored frozen. Treatment solutions were made by serial dilution in growth medium such that the final concentration of DMSO was always <0.02%. This concentration has previously been demonstrated not to be toxic (25).

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 hours. The cells were then exposed to BSO for 24 hours. Subsequently, the BSO was removed and the cells were treated with one of the chemotherapeutic agents (oxaliplatin for 2 hours, or in the cases of 5-FU or SN-38 for 24 hours). In the experiments with OTZ, the cells were exposed to the cysteine prodrug for 4 hours before the addition of the cytotoxic agents. After treatment, the cells were washed free of drug and allowed to grow in growth medium alone (hereinafter referred to as schedule A of OTZ) or with OTZ (hereinafter referred to as schedule B of OTZ). These experiments were also carried out in the presence of one of HGF, VEGF or EGF, which were added at the same time as the cytotoxic drugs 5-FU, oxaliplatin or SN-38 and were maintained until the end of the experimental period. The concentration of each GF was chosen from preliminary studies to determine the maximum increase in growth (data not shown). At 24, 48 and 72 hours 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, oxaliplatin or SN-38 alone or in combination with BSO or OTZ, either in the presence or the absence of growth factors, 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:

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

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

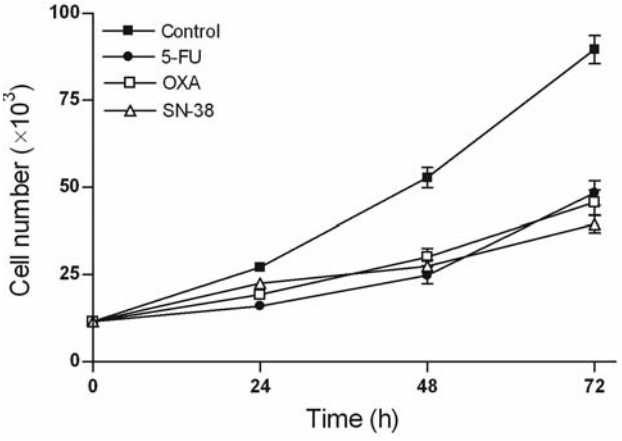

Figure 1. *Effect of 5-FU, oxaliplatin and SN-38 on the growth rate of WiDr cells. Results are expressed as the mean±standard deviation of the three independent experiments.*

Results

Effect of 5-FU, oxaliplatin and SN-38 on proliferation rate of WiDr cells. This experiment was performed to compare the effects of 5-FU, oxaliplatin and SN-38 on the proliferation rate of WiDr colon cancer cells. Based on preliminary studies (data not shown), the IC_{50} of each cytotoxic drug at 48 hours was used.

Treatment with 5-FU resulted in a 1.7- and 1.8-fold reduction ($p<0.0001$) in the growth rate of WiDr cells at 24 and 72 hours, respectively. Exposure to oxaliplatin and SN-38 also reduced significantly the growth rate of WiDr cells compared with controls at 24 hours (1.4- and 1.2-fold, respectively, $p<0.05$), and this reduction increased progressively to the maximum observed at 72 hours (2- and 2.3-fold, respectively, $p<0.0001$) (Figure 1).

Effect of HGF, VEGF and EGF on the antitumor activity of 5-FU, oxaliplatin and SN-38. In order to analyse the influence of GFs in the cytotoxic activity of these drugs, the experiments were carried out in the presence of HGF (7.5 ng/ml), VEGF (10 ng/ml) and EGF (25 ng/ml).

The presence of GFs significantly reduced the cytotoxic activity of the three active agents. Specifically, at 48 hours, 5-FU activity was reduced by 30% with VEGF and EGF, and almost totally suppressed with HGF ($p<0.001$). At 72 hours, treatment with 5-FU in the presence of any of the three GFs 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 2A. In the case of oxaliplatin, exposure to GFs resulted in approximately a 20% reduction in drug activity at 48 hours, although no significant modification in proliferation rate was observed in the presence or absence of GFs at 72 hours (Figure 2B). This

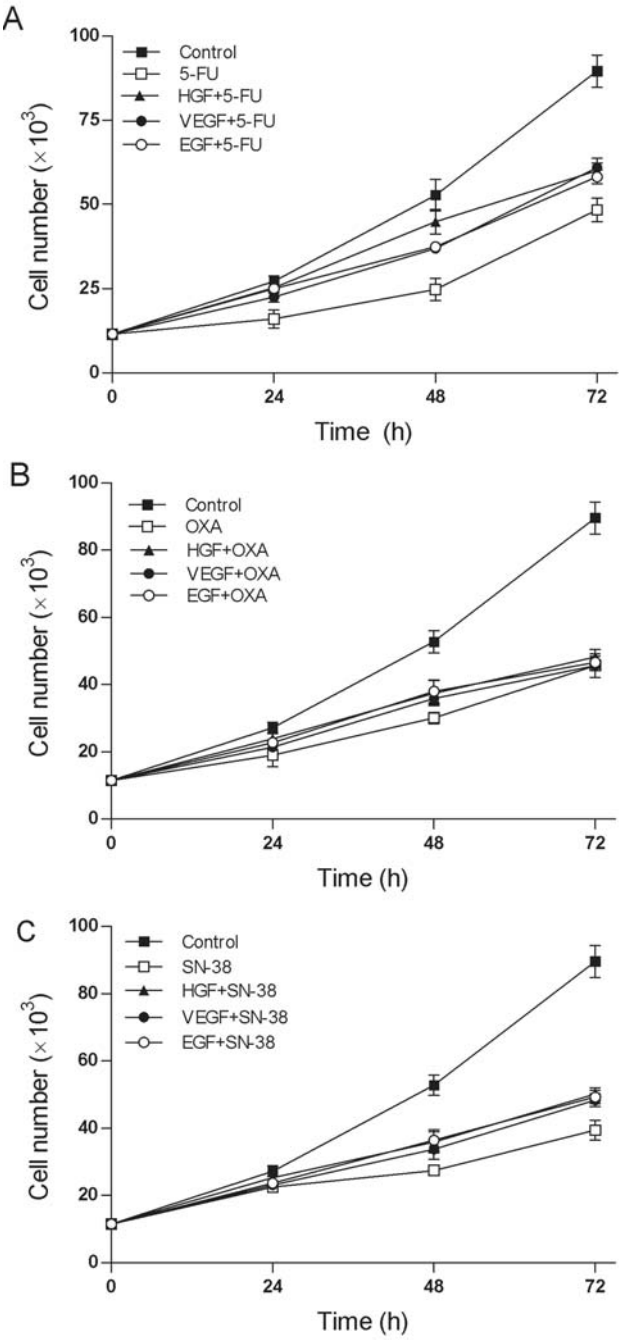

Figure 2. *Relative growth rate of WiDr cells treated with 5-FU (A), oxaliplatin (B) and SN-38 (C) in the presence or absence of GFs. Results are expressed as mean±standard deviation of the three independent experiments.*

was in contrast to SN-38 activity, which was diminished after 48 hours of incubation and the decrease was maintained until the end of the experiment (a near 20% reduction), resulting in a 2-fold reduction in growth rate with respect to untreated cells at 72 hours ($p<0.0001$) (Figure 2C).

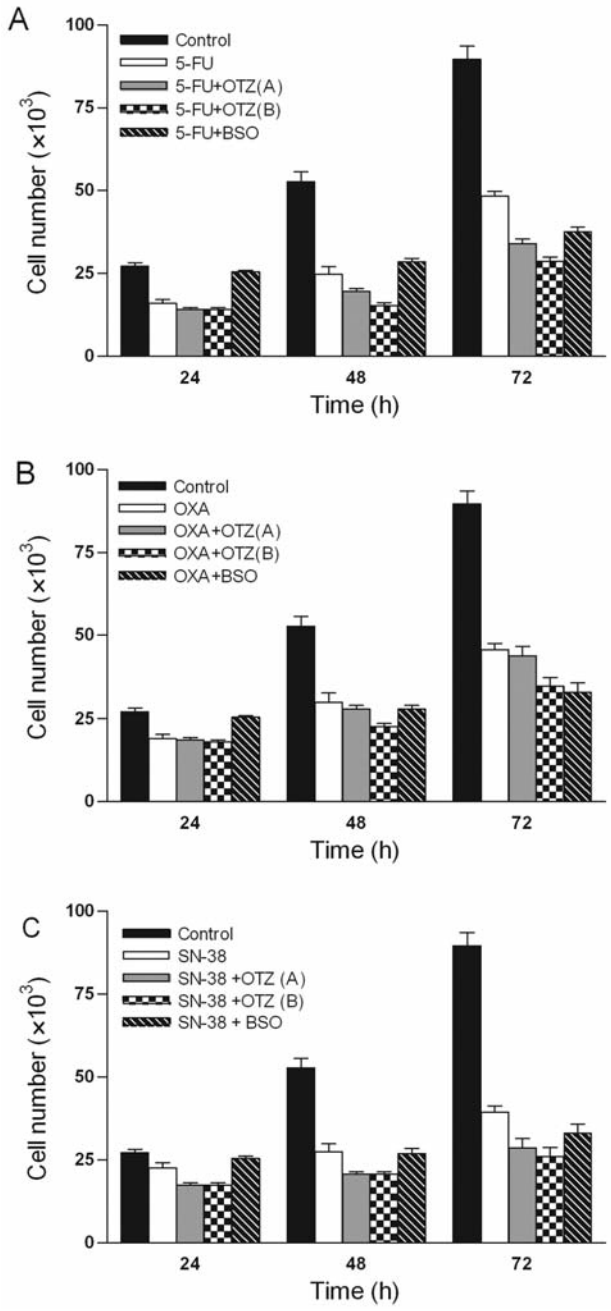

Figure 3. *Effect of 5-FU (A), oxaliplatin (B) and SN-38 (C) treatment alone and in combination with the GSH modulator agents BSO and OTZ (schedules A and B) on the proliferation rate of WiDr cells. Results are expressed as the mean±standard deviation of the three independent experiments.*

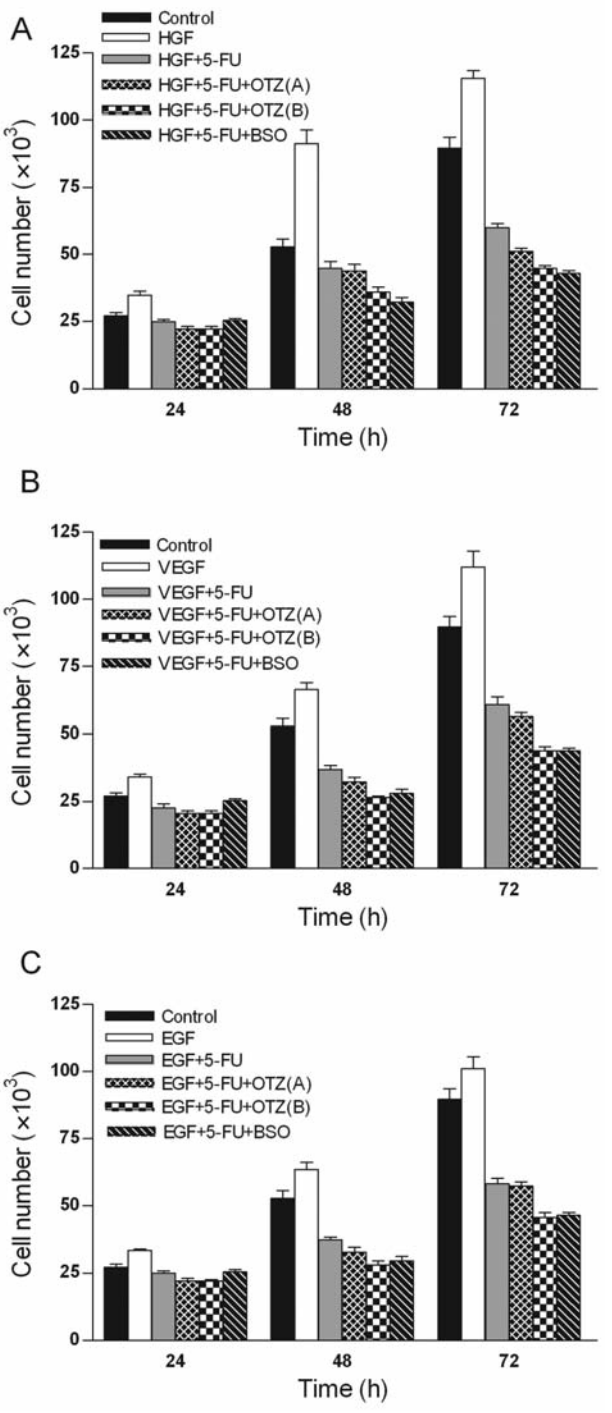

Figure 4. *Effect of 5-FU treatment alone and in combination with BSO and OTZ (schedules A and B) on the growth rate of WiDr cells in the presence of GFs. Results are expressed as the mean±standard deviation of the three independent experiments.*

Effect of BSO and OTZ on the cytotoxic activity of 5-FU, oxaliplatin and SN-38 on colon cancer cells in the presence or absence of GFs. Since the aim of the present investigation was to determine whether the GSH modulators could yield a therapeutic gain, *in vitro* studies focused on comparing the proliferation rate of tumour cells treated with active agents in combination with GSH modulators in the presence or absence of GFs.

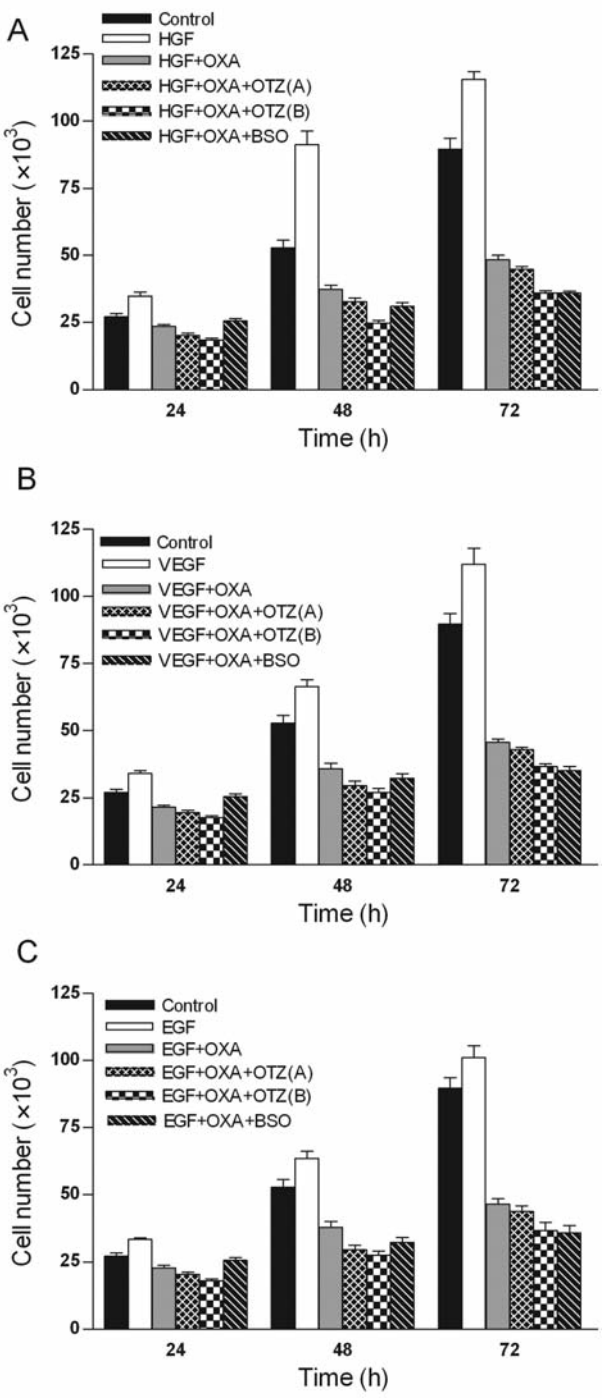

Figure 5. *Effect of oxaliplatin treatment alone and in combination with BSO and OTZ (schedules A and B) on the growth rate of WiDr cells in the presence of GFs. Results are expressed as mean±standard deviation of the three independent experiments.*

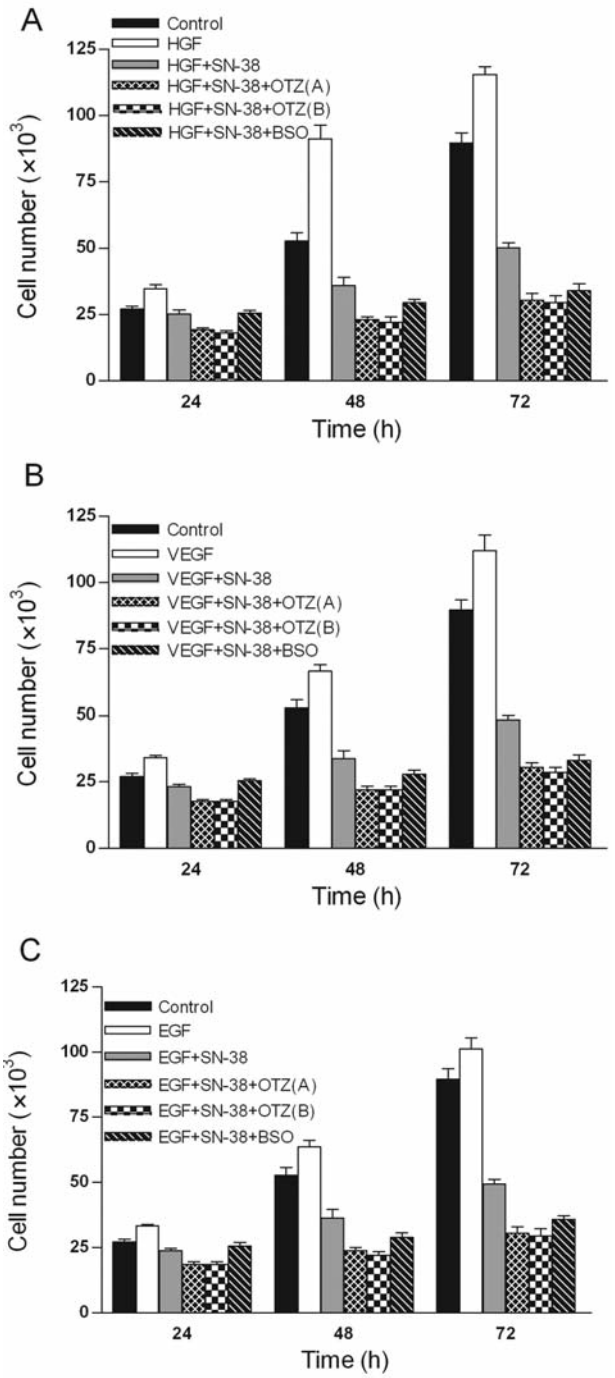

Figure 6. *Effect of SN-38 treatment alone and in combination with BSO and OTZ (schedules A and B) on the growth rate of WiDr cells in the presence of GFs. Results are expressed as mean±standard deviation of the three independent experiments.*

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.

Firstly, we analysed the effect of GSH modulators on activity of the chemotherapeutic agents in the absence of GFs. As shown in Figure 3A, the addition of BSO (100 μM) 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 hours, and resulting in a 29% increase in the cytotoxic activity of 5-FU. Similarly, the combination of OTZ (5 mM) and 5-FU produced a significantly greater anti-tumour effect than 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 hours, treatment with schedule B produced an even greater reduction (3.1-fold, $p<0.0001$). Overall, OTZ enhanced the antitumor effect of 5-FU significantly (42% and 69% increase with schedules A and B, respectively), indicating an additive effect with both schedules (DMF of 0.8 and 0.9, respectively).

With oxaliplatin, the addition of OTZ (schedule A) resulted in a non-significant improvement in antitumor activity against WiDr cells with respect to the drug alone, whereas in the case of schedule B this combination produced a 30% increase in the antitumor effect of the drug at 72 hours. Similarly, the combination of BSO and oxaliplatin resulted in a near 40% increase in the cytotoxic activity of this drug. Therefore, the effect of these combinations on growth inhibition was also additive, as represented by a DMF of approximately 0.8. As shown in Figure 3B, no significant difference in proliferation rate was observed between OTZ (schedule B) and BSO, in both combinations there was a near 2.7-fold reduction with respect to untreated cells ($p<0.001$).

In the case of SN-38, the addition of BSO resulted in an additive effect (DMF of 0.8), producing a 19% increase in the cytotoxic activity of the drug at 72 hours. At this point, the combination resulted in a 2.7-fold reduction with respect to control cells ($p<0.0001$). As shown in Figure 3C, the combination of SN-38 and OTZ produced a significantly greater anti-tumour effect than SN-38 alone at all times. Thus, the combined therapy of OTZ + SN-38 (using either schedule A or B) produced a 30% increase in the cytotoxic activity of SN-38 during the first 48 hours. However, at 72 hours, schedule A resulted in a 3.1-fold reduction ($p<0.0001$) in the growth rate compared with untreated cells, whereas treatment with schedule B produced an even greater reduction (3.5-fold, $p<0.0001$). Overall, OTZ significantly increased the anti-tumour effect of SN-38 (37.5% and 52% with schedules A and B, respectively), indicating an additive effect with both schedules (DMF of 0.8).

Secondly, we determined the effect of BSO and OTZ on drug activity in the presence of GFs. A schedule dependency was observed when cells were exposed to the combination of OTZ and 5-FU (Figure 4) or of OTZ and oxaliplatin (Figure 5). In particular, the addition of schedule A of OTZ produced no significant increase in the antitumor effect of 5-FU in the presence of VEGF and EGF, although this combination resulted in a 17% increase in the antitumor effect of the drug at 72 hours in the case of HGF (1.7-fold reduction in the proliferation rate compared with untreated cells, $p<0.0001$). In contrast, OTZ given according to schedule B or BSO added to 5-FU both produced a 2-fold reduction ($p<0.0001$) in the proliferation rate compared with controls at 72 hours. At this point, these combinations led to a near 40% increase in the cytotoxic activity of 5-FU in the presence of HGF or VEGF and to a 25% in the case of EGF. Overall, both GSH modulators pre-treatment combined with 5-FU in the presence of GFs resulted in a DMF of approximately 0.9, again suggesting an additive effect.

As can be seen in Figure 5, oxaliplatin treatment after BSO exposure resulted in an additive effect (DMF of approximately 0.9), producing a 2.5-fold reduction ($p<0.0001$) in the proliferation rate compared with untreated cells at 72 hours. At this point, although there was no significant increase in the anti-tumour effect of oxaliplatin with the addition of schedule A of OTZ, treatment with schedule B also resulted in a 2.5-fold reduction ($p<0.0001$) in the proliferation rate compared with controls (DMF of nearly 0.9). Moreover, the enhancement of oxaliplatin-induced growth inhibition produced by OTZ pretreatment was observed for the first 24 hours (approximately a 30% increase was noted with respect to drug activity in the presence of GFs). In fact, this combination resulted in a DMF of 2.0, 1.3 and 1.5, in the presence of HGF, VEGF and EGF respectively at 24 hours, indicating an apparently synergistic effect.

Finally, in contrast to oxaliplatin and 5-FU, schedule dependency was not observed for the combination of OTZ and SN-38 (Figure 6). It is important to note that the enhancement of drug-induced growth inhibition was already observed after the first 24 hours of incubation. In fact, the addition of OTZ to SN-38 therapy resulted in a synergistic effect at this point, as represented by a DMF of 3.3, 1.8 and 1.9 in the presence of HGF, VEGF and EGF respectively. Moreover, SN-38 treatment after OTZ exposure resulted in a 3-fold reduction ($p<0.0001$) in the proliferation rate compared with controls at 72 hours. Overall, the combined therapy produced a near 70% increase in the cytotoxic activity of SN-38 in the presence of GFs at 72 hours (DMF=1). Similarly, the combination of BSO and SN-38 produced a significantly greater antitumor effect than SN-38 alone. The combined therapy resulted in a 2.6-fold reduction ($p<0.0001$) in the proliferation rate compared with controls, leading to approximately a 50% increase in the cytotoxic activity of SN-38 in the presence of GFs at 72 hours. Thus, BSO pretreatment combined with SN-38 in the presence of GFs resulted in a DMF of approximately 1, once again suggesting an additive effect.

Discussion

The aim of the present study was, first, to compare the influence of GFs on the chemotherapeutic efficacy of 5-FU, oxaliplatin and SN-38, and second, to learn whether GSH

modulators could improve the efficacy of chemotherapy with these drugs in the presence of GFs.

We initially compared the anticancer effects of 5-FU, oxaliplatin and SN-38 in WiDr cells *in vitro*. Our study shows that all of them produce similar growth-inhibitory effects. However, the effects evolved differently over time. Specifically, the cytotoxic activity of oxaliplatin and SN-38 augmented progressively, resulting in SN-38 being the least active of the drugs at 24 hours but the most active after 72 hours. In contrast, treatment with 5-FU produced the greatest antiproliferative effect after the first 24 hours of incubation and then, once the drug treatment was stopped, the growth of tumour cells rebounded. This fact could be related to the recovery of GSH levels after its initial 5-FU-induced reduction as we have shown previously (12). Indeed, it has been previously described that elevated intracellular GSH levels may be a cause of acquired resistance to 5-FU, platinum agents and camptothecins (26-28).

In order to elucidate another possible mechanism of chemoresistance, we examined the effects of GFs on the chemotherapeutic efficacy of the drugs. Thus, and in accordance with current knowledge (29-31), we have shown that the presence of GFs reduces the cytotoxic activity of the three active agents significantly. Specifically, the influence of GFs on the growth-inhibiting action of the chemotherapeutic compounds was found to be maximal in the case of treatment with 5-FU. This fact could be related to the high tumour recurrence observed after partial hepatic resection of CRC liver metastases, where a significant GF release is produced in order to stimulate liver regeneration (32, 33). In contrast, the effect of GFs on oxaliplatin activity was minimal, resulting in no significant modification of the proliferation rate with respect to the drug alone at 72 hours. Taking into account that exposure to GFs resulted in a near 20% reduction of SN-38-induced growth inhibition at this point, there was no significant difference in the antitumor effect with respect to oxaliplatin in such circumstances. Furthermore, it has been proposed that EGF signalling is enhanced by irinotecan and may play a role in determining chemosensitivity to this drug (13). Indeed, the monoclonal antibody targeting EGFR, cetuximab, and the EGFR tyrosine kinase inhibitor, gefitinib, can overcome irinotecan resistance (9, 14), resulting in a synergistic inhibiting effect on CRC cells. Several mechanisms of GF-induced chemoresistance have been identified, including activation of EGFR-src-signal transducers and activators of the transcription 3 (STAT 3) oncogenic pathway, which prevents DNA damage caused by topoisomerase I inhibition (34). Additionally, it has been suggested that the GF-induced increase in intracellular GSH levels and the activation of the redox-sensitive transcription factor nuclear factor kappa B (NF-κB) could play a major role in inducible chemoresistance (35, 36).

In the light of these data, we believe that the use of GSH modulators such as OTZ or BSO could be a good strategy for reversing these GF pro-tumour effects and improving the efficacy of chemotherapy involving oxaliplatin and SN-38, as we have previously demonstrated in the case of 5-FU (12). We initially compared the ability of BSO and OTZ to increase the cytotoxic activity of the drugs in the absence of GFs, with an additive effect being demonstrated with the combined therapies. In the case of 5-FU and oxaliplatin, prolonged exposure to OTZ (schedule B) was found to be necessary. In fact, the combination of 5-FU and OTZ (schedule B) produced a near 70% increase in drug efficacy, approximately 30% higher than the enhancement of the drug-induced growth inhibition by BSO at 72 hours. For oxaliplatin, while schedule A of OTZ did not significantly increase the antitumour effect, the combination with schedule B of OTZ or BSO resulted in an enhancement of drug induced-growth inhibition of approximately 30% at 72 hours. Related to these results, it has previously been reported that GSH may modulate cytotoxicty of platinum agents, although intracellular GSH levels do not appear to influence the cell growth inhibiting activity of these compounds in cells not previously exposed to platinum complexes, suggesting that GSH is of importance in acquired resistance (27). We also found that BSO and OTZ enhanced the antiproliferative effect of SN-38 at 72 hours significantly (a 20% and 50% increase, respectively). BSO-mediated enhancement of SN-38 activity has also been previously reported by others authors in several tumour cell lines (28, 37). However, to the best of our knowledge, the current study is the first to show OTZ-induced modulation of SN-38 antitumor activity. Furthermore, it is important to note that the additive effect of the OTZ and SN-38 combination was already observed in the first 24 hours of incubation, when the drug is less active, resulting in a similar antitumur effect as 5-FU at this point (a 30% increase in drug activity). Overall, of the three active agents optimum results were obtained with the use of combinations of 5-FU and OTZ (schedule B) and with the use of SN-38 and OTZ (both schedules A and B). Considering that treatment with 5-FU and SN-38 has been reported to activate NF-κB (a GSH-dependent transcription factor), the observed enhancement in the activity of the cytotoxic agents by GSH modulators could be explained, in part, by the inhibition of drug-induced NF-κB activation. This is in accordance with previous studies which have demonstrated that targeting NF-κB can potentiate the therapeutic efficiency of 5-FU and SN-38 on colon cancer cells (38, 39).

Finally, we also evaluated the effects of adding OTZ or BSO to treatments using chemotherapeutic agents in the presence of GFs. Overall, the combination of both modulators with the three active compounds produced a significantly greater anti-tumour activity than that obtained with the use of drugs alone. Specifically, the addition of OTZ (schedule A) resulted in a non-significant improvement in 5-FU and in oxaliplatin anti-tumour activity, but, in contrast, schedule dependency was not observed with the combination of SN-38

and OTZ. For the treatment with 5-FU, OTZ (schedule B) and BSO seem to have similar effects on the proliferation rate of WiDr cells (a 2-fold reduction in the proliferation rate at 72 h) in the presence of GFs; the similar effects were obtained with 5-FU alone in the absence of GFs. With oxaliplatin, not only did both drugs OTZ (given in accordance with schedule B) and BSO abrogate the pro-tumour effects of the GFs, but the combined therapies also produced a near 25% increase in the antitumor effect with respect to the cytotoxic drug alone at 72 hours (2.5-fold reduction in the growth rate). In addition, OTZ-induced oxaliplatin activity enhancement was observed after only 24 hours of incubation, resulting in an apparently synergistic effect at this point. Furthermore, with SN-38, OTZ treatment reversed the GF-induced pro-tumour effect and produced a significantly greater antitumor activity than SN-38 alone at any point (a near 30% increase). Specifically, this combined treatment resulted in a 3-fold reduction in the growth rate of WiDr cells at 72 hours. Similarly, BSO abrogated the growth-promoting effects of GFs, and its combination with SN-38 produced similar and a 15% greater antitumor effect than the drug alone in the absence of GFs at 48 and 72 hours, respectively.

Given these results, we observe that the weakest anti-tumour effect was obtained with the combination of 5-FU and GSH modulators (approximately 50% smaller effect than that obtained with SN-38 and OTZ). This is consistent with the fact that GF-induced resistance to 5-FU was found to be the most severe. It must also be taken into account that GFs abrogate 5-FU-induced growth inhibition at 24 hours, a significant antitumor effect was observed with the cytotoxic drug alone. In contrast, the addition of OTZ to SN-38 therapy resulted in a synergistic effect at this point of minimal drug activity. Therefore, comparing all possible combinations, we found that OTZ pre-treatment combined with SN-38 produced the maximal growth inhibition in the presence of GFs. It is important to note that replacement of OTZ after drug removal is not necessary. Moreover, while BSO-mediated GSH depletion is not specific to cancer cells, OTZ is capable of inducing a selective modulation of GSH content, reducing its levels in tumour cells while simultaneously increasing them in normal tissues, as we have previously demonstrated (19-21).

In conclusion, the present study has demonstrated that GSH modulators BSO and OTZ reverse the growth-promoting effect of GFs and thereby enhance the antitumor response of WiDr cells to 5-FU, oxaliplatin and SN-38 significantly. In particular, in this tumour cell line, the addition of OTZ to chemotherapy with SN-38 was found to be the best overall therapeutic regimen of those studied. Nevertheless, the choice between these agents needs to be made on an individual basis and, thus, further research is required into determinants which could help identify those patients who may have the best response to each therapy.

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