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Low-dose taxotere enhances the ability of sorafenib to induce apoptosis in gastric cancer models

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Abstract

Despite the low efficacy of conventional antitumour drugs, chemotherapy remains an essential tool in controlling advanced gastric and oesophageal cancers. We aimed to provide a biological rationale based on the sorafenib–taxotere interaction for the clinical treatment of gastric cancer. *In vitro* experiments were performed on four human gastric cancer cell lines (GK2, AKG, KKP and NCI-N87). Cytotoxicity was evaluated by sulforhodamine B (SRB) assay, cell cycle perturbations, apoptosis and mitotic catastrophe were assessed by flow cytometric and microscopic analyses, and protein expression was studied by Western blot. In the *in vivo* experiments, nude mice xenografted with the most resistant line were treated with sorafenib and docetaxel singly or in association. Sorafenib inhibited cell growth (IG_{50} values ranged from 3.4 to 8.1 μM) and caused down-regulation of MAP-K/ERK phosphorylation and of mcl-1 and p-bad expression after a 48-hr exposure. Apoptosis induction was associated with caspase-3 and -9 activation and mitochondrial membrane depolarization. The drug combination enhanced apoptosis (up to 80%) and produced a synergistic interaction when low doses of the taxane preceded administration of the antityrosine kinase. This synergism was probably due to the induction of an anomalous multidiploid G_0-G_1 peak and to consequent mitotic catastrophe, which increased sensitivity to sorafenib. Consistent with *in vitro* results, the docetaxel–sorafenib sequence exhibited high therapeutic efficacy in NCI-N87 mouse xenografts producing tumour weight inhibition ($>$ 65%), tumour growth delay (up to 25 days) and increased mouse survival (30%). Our findings suggest the potential clinical usefulness of treatment with sorafenib and docetaxel for advanced gastric cancer.

Keywords: gastric cancer • sorafenib • apoptosis • taxotere • mitotic catastrophe

Introduction

Gastric and oesophageal cancers are among the most common tumours in the world, representing the second leading cause of cancer mortality. Furthermore, gastric tumours are more frequently diagnosed at an advanced, unresectable stage [1]. Surgical resection is currently the mainstay of treatment and can cure patients with early-stage cancer. Conversely, very few improvements in treatment efficacy have been made over time for those with advanced disease [2]. However, multimodal strategies integrating pre- and post-operative treatments have clearly improved gastric cancer prognosis when used in association with curative intent surgery. In particular, the benefit of perioperative chemotherapy for patients with gastric and gastro-oesophageal cancers was confirmed in the two large phase III MAGIC and French FFCD Group trials [3–6]. The identification of more effective chemotherapy regimens and of the optimal treatment schedule in a neoadjuvant setting, aimed at obtaining a volumetric reduction and thus increasing the number of patients with locally advanced resectable disease, remain important goals. Interesting results in this area have been obtained from the use of multiple targeted therapies in gastric cancer. Different classes of new agents directed against specific targets, including monoclonal antibodies [7–12], tyrosine kinase inhibitors [13], anti-angiogenic compounds [14, 15] and the proteasome inhibitor bortezomib [16] have shown promising activity in clinical studies on advanced gastric cancer. In particular, preliminary phase II trials have produced encouraging

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results in terms of response rates and overall survival time for the combination of cytotoxic agents with the EGFR- or VEGF-targeted monoclonal antibodies, cetuximab and bevacizumab [8–11, 15], when used as first- or second-line treatments.

Deregulation of the raf/MAP/ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, which is involved in cell proliferation, survival and differentiation, is implicated in the development of several solid tumours, including gastric cancer [17, 18]. The oral multikinase inhibitor sorafenib targets Raf serine/threonine kinases (raf-1, b-raf), vascular endothelial growth factor receptor (VEGFR)-1/-2/-3, platelet-derived growth factor receptor-β (PDGFR-β) and Flt-3, c-kit and p38 tyrosine kinases [19]. Following interesting results obtained from experimental studies, several clinical trials have shown an antitumour activity of sorafenib used in combination with cytotoxic agents in different solid tumours. Among the various combination treatments studied, the most promising evidence of antitumour activity of the multikinase inhibitor in a phase I/II clinical setting was observed when sorafenib was combined with interferon-α in renal cell carcinoma [20], with dacarbazine in melanoma [21–23], with doxorubicin in hepatocellular carcinoma [24] and with gemcitabine in pancreatic and ovarian cancer [25–27]. Experimental data on gastric cancer are lacking and the few clinical studies carried out have highlighted an efficacy of sorafenib when used in combination with taxanes [28–31].

Docetaxel has been shown to be active as a single agent in patients with tumours resistant to first-line therapy or when used in combination with cisplatin and 5-FU (DCF regimen), despite showing some toxicity [32]. The activity of the taxane in advanced gastric cancer has also been documented in neoadjuvant and perioperative regimens including radiotherapy [33, 34]. For these reasons, more tolerable docetaxel-containing regimens need to be defined, and molecular-targeted agents could be potential candidates to associate with the taxane.

In the present study we investigated the cytotoxic activity and mechanisms of action of sorafenib, used alone or in combination with docetaxel, in *in vitro* and *in vivo* gastric cancer models. Our final aim was to provide a biological rationale to use as the basis for clinical treatment planning.

Materials and methods

Cell lines

The study was performed on three cell lines (GK2, AKG, KKP) derived from human gastric adenocarcinoma (intestinal type), established and characterized in our laboratory[35, 36], and one commercial cell line obtained from a liver metastasis of a well differentiated gastric carcinoma (NCI-N87), purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained as a monolayer at 37°C and subcultured weekly. Culture medium was composed of DMEM/HAM F12 (1:1) supplemented with foetal calf serum (10%), glutamine (2 mM), non-essential amino acids (1%) (Mascia Brunelli S.p.A., Milan, Italy) and insulin (10 μg/ml) (Sigma Aldrich, Milan, Italy). Cells were used in the exponential growth phase for all of the experiments.

In vitro studies

Drugs

Sorafenib (Nexavar®, Bayer, Milan, Italy) was solubilized in dimethyl sulfoxide (DMSO) (Carlo Erba, Milan, Italy) to a final concentration of 10 mmol/l, divided into aliquots and stored at –80°C. Drug stocks were freshly diluted in culture medium immediately before use. The final DMSO concentration never exceeded 1% and this condition was used as control in each experiment. Docetaxel (Taxotere®, Sanofi-Aventis, Milan, Italy) was freshly diluted in culture medium immediately before use.

Chemosensitivity assay

The sulforhodamine B assay was used according to the method of Skehan *et al.* [37]. Briefly, cells were collected by trypsinization, counted, and plated at a density of 5000 cells/well in 96-well flat bottomed microtitre plates (100 μl cell suspension/well). Experiments were run in octuplet and each experiment was repeated thrice. The absorbance of treated cells was determined at a wavelength of 540 nm using a colorimetric plate reader.

Single drug exposure

Sorafenib was tested at 0.001, 0.1, 1 and 10 μM concentrations for 24, 48 or 72 hrs. Docetaxel was tested at 0.0001, 0.001, 0.01 and 0.1 μM concentrations for 1 hr followed by a 24-, 48- or 72-hr culture in drug-free medium (washout). Growth inhibition and cytocidal effects of drugs were calculated according to the method of Monks *et al.* [38].

Drug combination exposure

Different drug schedules were used: (*i*) simultaneous exposure to sorafenib and docetaxel for 1 hr followed by exposure to sorafenib for 48 hrs; (*ii*) sorafenib for 48 hrs → docetaxel for 1 hr → 24-, 48- or 72-hr washout and (*iii*) docetaxel for 1 hr → 24-, 48- or 72-hr washout → sorafenib for 48 hrs. In all combination experiments, sorafenib was tested at concentrations of 0.1, 1 and 10 μM, while docetaxel was used at concentrations of 0.001, 0.01, 0.1 μM for KKP and AKG cells and 0.0001, 0.001 and 0.01 μM for GK2 line.

Drug interaction analysis

The type of drug interaction was determined by the median effect principle, according to the method of Chou and Talalay [39]. The interaction between the two drugs was quantified by the combination index (CI) at increasing levels of cell kill. The CI values used for the quantification of synergism were calculated from average CI values obtained from separate experiments and at multiple effect levels (*e.g.* ED_{25}, ED_{50}, ED_{70}, ED_{90}). CI values lower than, equal to, or higher than 1 indicated synergy, additivity or antagonism, respectively.

Western blot analysis

Cells were treated according to the previously described Western blot procedure [40]. Antibodies used were anti-phospho-Bad (Ser112), anti-b-raf, anti-c-raf, anti-MEK1/2, anti-phospho-MEK1/2 (Ser217/Ser221), anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-PDGFR-β, anti-caspase-3, anti-caspase-9 (Cell Signaling Technology, Inc., Danvers, MA, USA), anti-EGFR (UpState Biotechnology, Charlottesville, VA, USA), anti-mcl-1 (BD Pharmingen, Inc., San Diego, CA, USA), anti-Flk1/VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-actin (Sigma

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Aldrich). The bound antibody was detected by enhanced chemiluminescence using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Cologno Monzese, Italy).

Mutation analysis

DNA was extracted from the gastric cell lines using the QIAamp DNA Mini Kit (Qiagen, Qiagen-Gruppe, Hilden, Germany). Exons 1 and 2 of *k-ras* gene were amplified by PCR, and DNA sequencing was performed with Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence products were analysed by 3100 Avant Sequencer (Applied Biosystems).

Flow cytometric analysis

Cell cycle

After drug treatments and washouts, cell distribution in the different cell cycle phases was evaluated by flow cytometry using a flow cytometry device (FACS) Vantage flow cytometer (Becton Dickinson, San Diego, CA, USA) as previously described [40]. Data acquisition and analysis were performed with CELLQuest software (Becton Dickinson) and ModFit 2.0 (DNA Modelling System, Verity Software House, Inc., Topsham, ME, USA).

Biparametric cyclin B1-DNA content determination

After docetaxel treatment and the different washouts, cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) at 48°C for 15 min., resuspended in ice-cold ethanol (70%), stored overnight at –20°C, washed twice in PBS and incubated in PBS containing 0.25% Triton X-100 for 5 min. at 48°C. Samples were then incubated overnight at 48°C with the anti-cyclin B1 mouse primary antibody (BioOptica, Milan, Italy) in PBS containing 1% BSA, washed once in PBS and incubated with a FITC-conjugated antimouse immunoglobulin antibody (Dako Cytomation, Glostrup, Denmark) diluted in PBS containing 1% BSA, for 1 hr at 48°C in the dark. Finally, cells were counterstained with a staining solution containing PBS, propidium iodide (5 mg/ml, MP Biomedicals, Verona, Italy) and RNAse (10 kunits/ml, Sigma Aldrich) for 2–4 hrs at 48°C in the dark before cytofluorimetric analysis.

TUNEL assay

Apoptotic cells were evaluated by flow cytometry [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay] [40]. Briefly, after treatment, cells were trypsinized, fixed, exposed to the TUNEL reaction mixture, counterstained with propidium iodide and analysed by FACS.

Mitochondrial membrane potential ($\Delta\Psi$) depolarization assay

After a 20-, 40-, 60-, 120-, 240- and 360-min exposure to 10 μM of sorafenib, mitochondrial membrane potential was evaluated by flow cytometry according to the previously described JC-1 method [41]. Data acquisition and analysis were performed with CELLQuest software. For each sample, 15,000 events were recorded.

Morphological analysis

After a 1-hr exposure to 0.01 μM of docetaxel, cells were harvested and treated as previously described [42]. Slides were mounted in Eukitt (BioOptica) and examined under a photomicroscope (Axioscope 40, Zeiss, Vienna, Austria) to visualize normal and altered mitotic figures, representative of mitotic catastrophe, chromatin condensation and/or fragmentation.

In vivo studies

Animals

Antitumour efficacy was evaluated on 6–8-week-old CD-1 male nude (nu/nu) mice weighing 22–24 g (Charles River Laboratories, Calco, Italy). All procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare No. 8, G.U., July 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

Drugs

Sorafenib (Nexavar®) was dissolved in Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 95% ethyl alcohol, Sigma-Aldrich, Milan, Italy) at 4-fold the highest dose used in the experiments, foil-wrapped, and stored at room temperature. Final dosing solutions were prepared on the day of use by diluting the stock solution to a 1× solution with water. Docetaxel (Taxotere) was obtained from Aventis Pharma S.A. (Dagenham, UK). Drugs were diluted in PBS and freshly prepared before each experiment.

Antitumour efficacy

As GK2, AKG and KKP cell lines showed low tumorigenicity when cells were injected into nude mice, *in vivo* experiments were performed on the liver metastasis-derived cell line NCI-N87. Tumour cells in the exponential phase of *in vitro* growth were suspended (3×10^6 viable cells) in 0.2 ml of serum-free medium and injected into the hind leg muscles of the mice. Each experimental group included six mice and experiments were repeated at least twice. Starting on the sixth day after tumour implant, when a mass of around 300 mg was evident, mice were treated by daily p.o. gavage with 30 mg/kg/day of sorafenib, a dose which was shown to have antitumour efficacy in a panel of human tumour xenograft models [19]. Treatment was prolonged for 14, 21 or 28 days. Docetaxel was administered i.p. at a dose of 5 mg/kg/day for three consecutive days on the basis of our previous published results [43].

In the combination experiments, the two drugs were administered 24 hrs apart. Tumour weight was calculated from calliper measurements according to the method of Geran *et al.* [44]. Antitumour efficacy of treatments was assessed using the following endpoints: (*i*) percent tumour weight inhibition (TWI%), calculated as (1 – [mean tumour weight of treated mice/mean tumour weight of controls]) × 100; (*ii*) tumour growth delay, evaluated as T-C, where T and C are the median times for treated and control tumours, respectively, to achieve equivalent size; (*iii*) increased lifespan (ILS) of mice. The animals were killed for ethical reasons when their tumours reached 3 g in weight or when they became moribund during the observation period (the time of killing was recorded as the time of death).

Statistical analysis

The statistical difference of tumour weight in untreated and treated groups was determined by a two-tailed Student's t-test for unpaired samples assuming unequal variances. Differences were considered statistically significant when $P < 0.05$. Survival curves were generated by the

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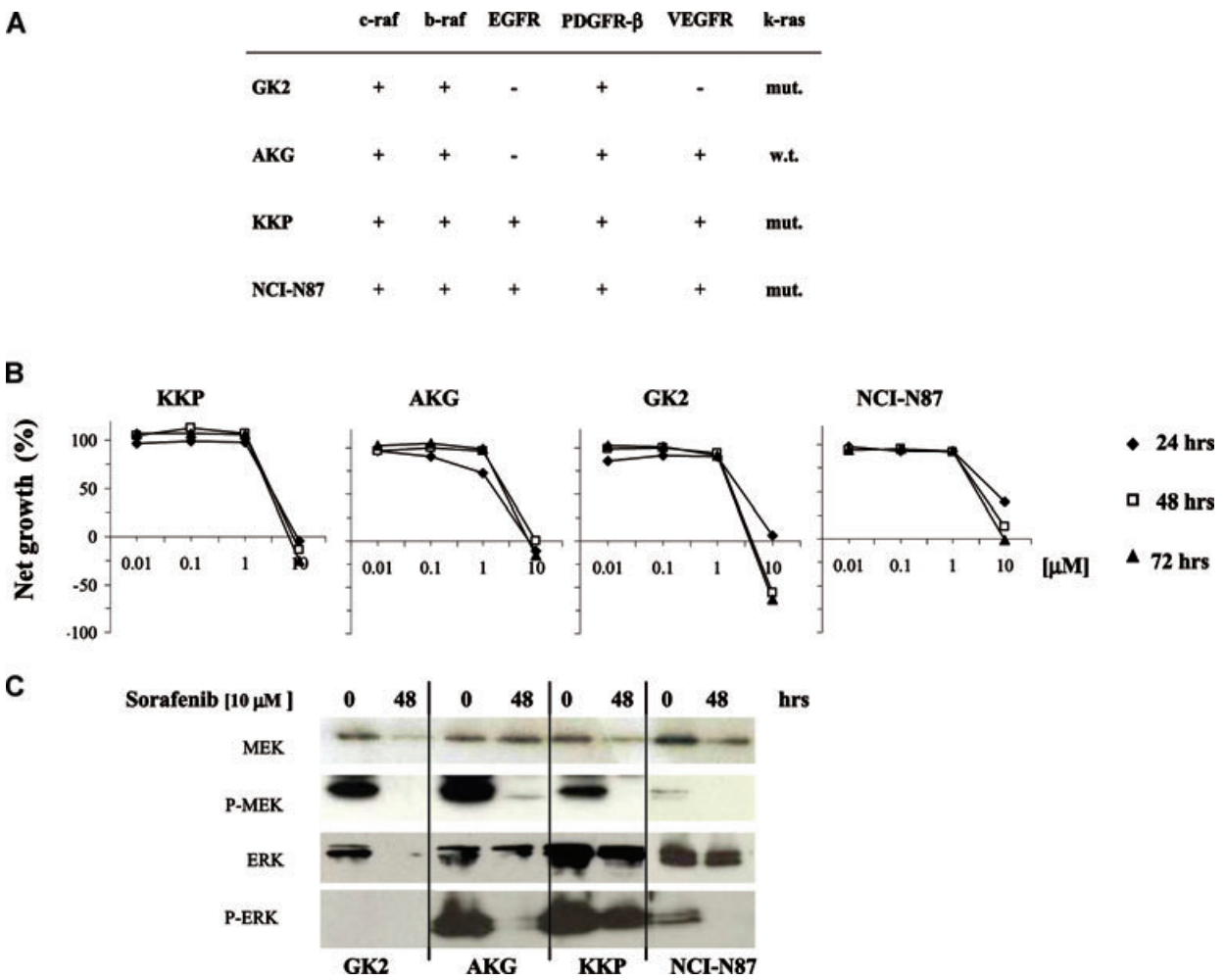

Fig. 1 (**A**) Western blot analysis of baseline expression of sorafenib targets (c-raf, b-raf, EGFR, PDGFR-β, VEGFR) in gastric cancer lines, and *k-ras* mutation analysis (mut., mutated; w.t., wild-type). (**B**) Cytotoxic activity of sorafenib after 24, 48 and 72-hr exposure in gastric cancer cell lines. (**C**) Effect of 10 μM sorafenib concentration on MAP kinase pathway after 48-hr exposure.

Kaplan–Maier product-limit estimate, and statistical differences between the various groups were evaluated by log-rank analysis with Yates correction (software Primer of Biostatistics, McGraw-Hill, New York, NY, USA).

Results

In vitro studies

Cell line molecular profiles

Cell lines were characterized by different molecular profiles. All cell lines expressed c-raf, b-raf and PDGF receptor β. VEGF receptor expression was found in AKG, KKP and NCI-N87 cells, while the expression of EGF receptor was only present in the last two. k-ras mutation analysis revealed gene alterations in GK2, KKP and NCI-N87 cells (Fig. 1A).

Cytotoxic activity of sorafenib

The activity of sorafenib in all of the cell lines after different exposure times and at different concentrations is reported in Fig. 1B. The drug produced an antiproliferative dose-related effect, independent of exposure time, starting from 1 μM in all cell lines. More specifically, the concentration inhibiting cell growth by 50% (GI_{50}) ranged from 3.4 μM in GK2 cells to 8.1 μM in NCI-N87cells. A significant cytocidal effect was also observed in GK2 cells but only after a 48- and 72-hr exposure and at drug concentrations of around 10 μM. After a 48-hr exposure to sorafenib (10 μM), inactivation of MEK/ ERK, the mitogenic signalling cascade triggered by c-raf, was observed in all cell lines, as shown

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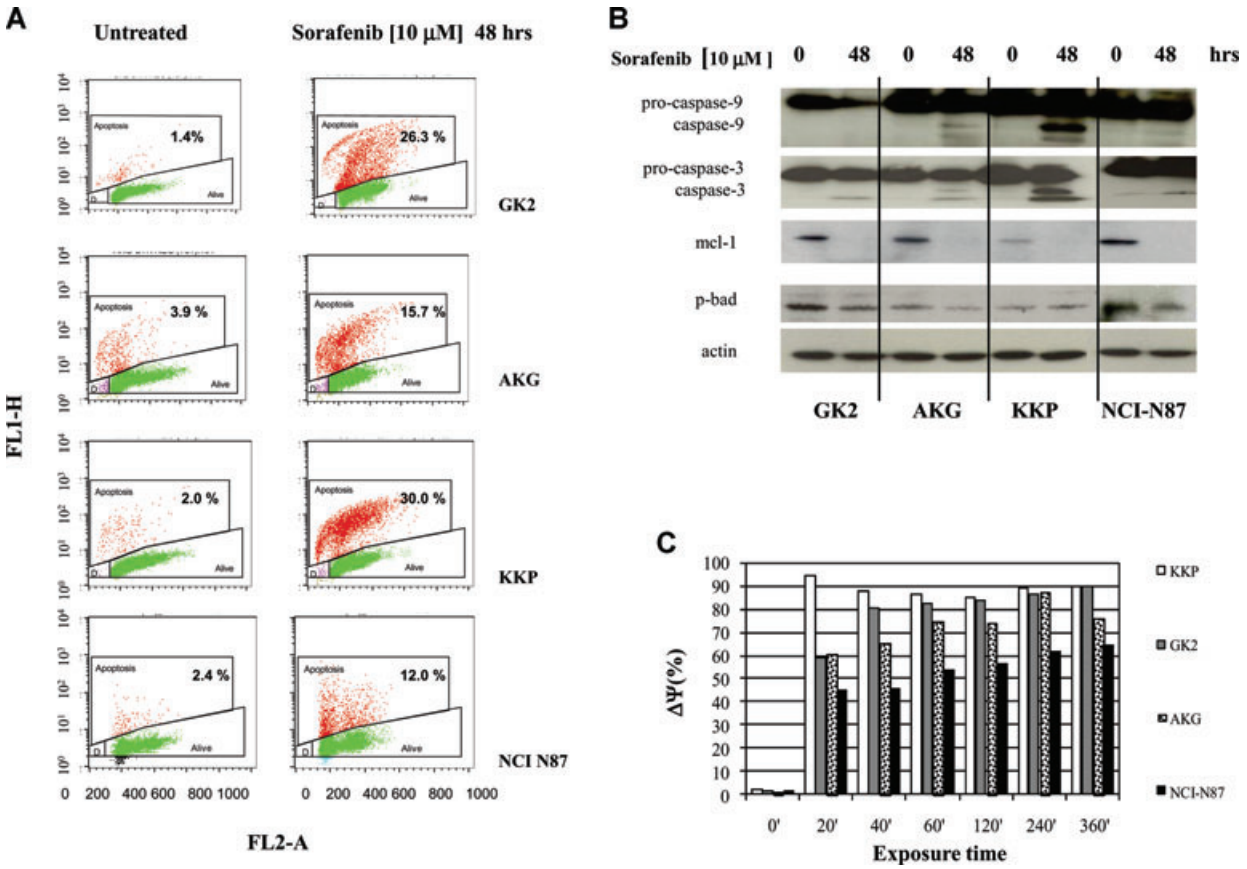

Fig. 2 (**A**) Representative images of cytofluorimetric analysis of apoptosis in GK2, AKG, KKP and NCI N87 cells after 48-hr exposure to 10 μM sorafenib concentration. (**B**) Changes in apoptotic-related markers after 48-hr exposure to 10 μM sorafenib concentration. (**C**) Percentage of mitochondrial membrane potential depolarization ($\Delta\Psi$) after different exposure times to 10 μM sorafenib concentration.

by the drastic reduction in expression of their phosphorylated forms (Fig. 1C).

Apoptosis analysis by TUNEL assay showed cell death induction in all of the cell lines ranging from 12% to 30% after a 48-hr exposure to the multikinase inhibitor (Fig. 2A). After the same exposure time, cleavage of pro-caspase-9 and -3 into their active forms was also observed, together with a reduction in the expression of the anti-apoptotic proteins, mcl-1 and p-bad (Fig. 2B). Concomitantly, strong mitochondrial membrane depolarization ($\Delta\Psi$) was observed in all four cell lines starting after only 20 min. exposure to sorafenib (Fig. 2C).

Activity of sorafenib in combination with docetaxel

Different treatment schedules were used. Drug combination studies highlighted an antagonistic interaction when exposure to sorafenib preceded that of docetaxel. Simultaneous exposure to the two drugs produced only an additive interaction in GK2, AKG and KKP cells, but a synergistic effect in NCI-N87. Conversely, a synergistic interaction was obtained in all of the cell lines when docetaxel exposure preceded that of sorafenib. This synergism would appear to be highly dependent on the time interval between drug administrations. In particular, synergism was observed in all four cell lines when a 24-hr interval was used, in three cell lines using a 48-hr interval and in two cell lines for a 72-hr interval (Fig. 3A).

Induction of apoptosis and caspase-3 expression were significantly higher when exposure to the taxane preceded that of sorafenib with respect to the inverse sequence (Fig. 3B). Furthermore, after 1-hr exposure to 0.01 μM of docetaxel followed by a 24-hr washout in GK2 cells, flow cytometry highlighted a subpeak on the left side of the G0/G1 peak (Fig. 4A). Similar results were observed in the other three cell lines (data not shown). This hypopeak is not ascribable to the presence of apoptotic cells, as confirmed by the TUNEL assay (Fig. 4B). The nature of the anomalous subpopulation was investigated using a biparametric approach based on cyclin B1 detection and propidium iodide staining to search for abnormalities in mitotic exit caused by docetaxel exposure (Fig. 4C). In GK2 cells 16 hrs after a 1-hr

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A

Treatment schedule	Type of interaction on different cell lines			
	GK2	AKG	KKP	NCI-N87
Sorafenib (48 hrs)→docetaxel (1 hr)→ 24-hr w.o.	antagonism	antagonism	antagonism	antagonism
Sorafenib (48 hrs) →docetaxel (1 hr)→ 48-hr w.o.	antagonism	antagonism	antagonism	antagonism
Sorafenib (48 hrs) →docetaxel (1 hr)→ 72-hr w.o.	antagonism	antagonism	antagonism	antagonism
Docetaxel + sorafenib (1 hr)→ sorafenib (47 hrs)	**additivity**	**additivity**	**additivity**	**synergism (CI=0.5)**
Docetaxel (1 hr)→ 24-hr w.o. → sorafenib (48 hrs)	**synergism (CI=0.9)**	**synergism (CI=0.5)**	**synergism (CI=0.9)**	**synergism (CI=0.8)**
Docetaxel (1 hr)→ 48-hr w.o. → sorafenib (48 hrs)	**synergism (CI=0.4)**	antagonism	**synergism (CI=0.9)**	**additivity**
Docetaxel (1 hr)→ 72-hr w.o. → sorafenib (48 hrs)	antagonism	antagonism	**synergism (CI=0.9)**	**synergism (CI=0.6)**

w.o., washout; CI, combination index

B

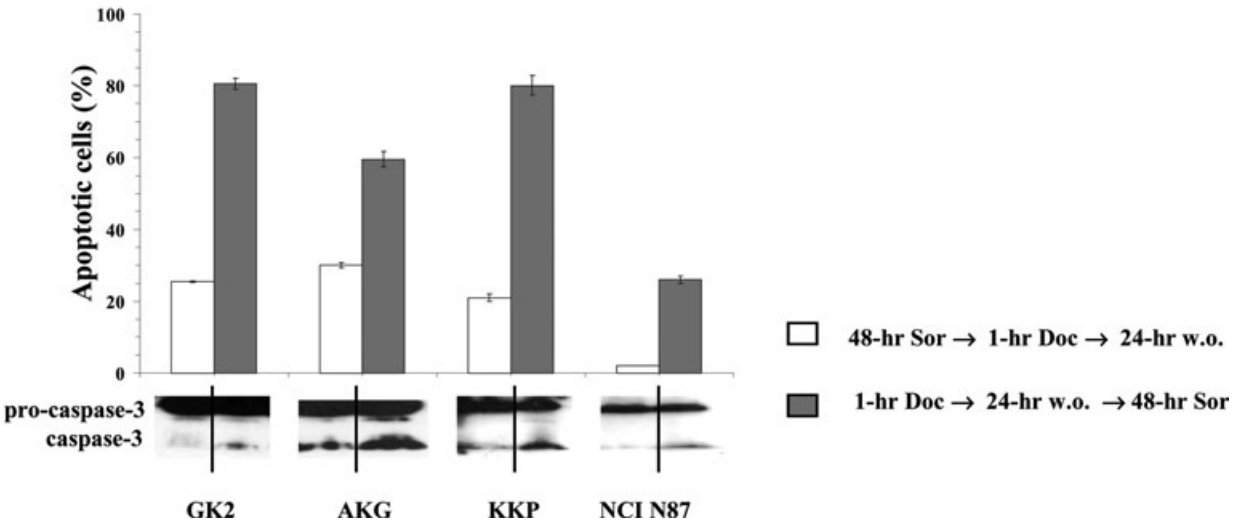

Fig. 3 (**A**) *In vitro* analysis of interaction between sorafenib and docetaxel after different treatment schedules. In all combination experiments, sorafenib was tested at concentrations of 0.1, 1 and 10 μM, while docetaxel was used at concentrations of 0.001, 0.01, 0.1 μM for KKP and AKG cells, and 0.0001, 0.001 and 0.01 μM for GK2 line. (**B**) Apoptosis induction and influence on caspase-3 activation caused by different sorafenib (Sor) – docetaxel (Doc) treatment schedules.

treatment with 0.01 μM of docetaxel, 20% of the cell population with 4N DNA content were cyclin B1-negative. This percentage doubled (42.4%) after 24 hrs. Similar results were observed in the other three cell lines (data not shown). Morphological photomicroscope analysis of cells exposed to docetaxel showed the presence of a population with aneuploid features consisting mainly of DNA condensation, abnormal mitotic figures, multinucleation and formation of large viable cells (Fig. 4D).

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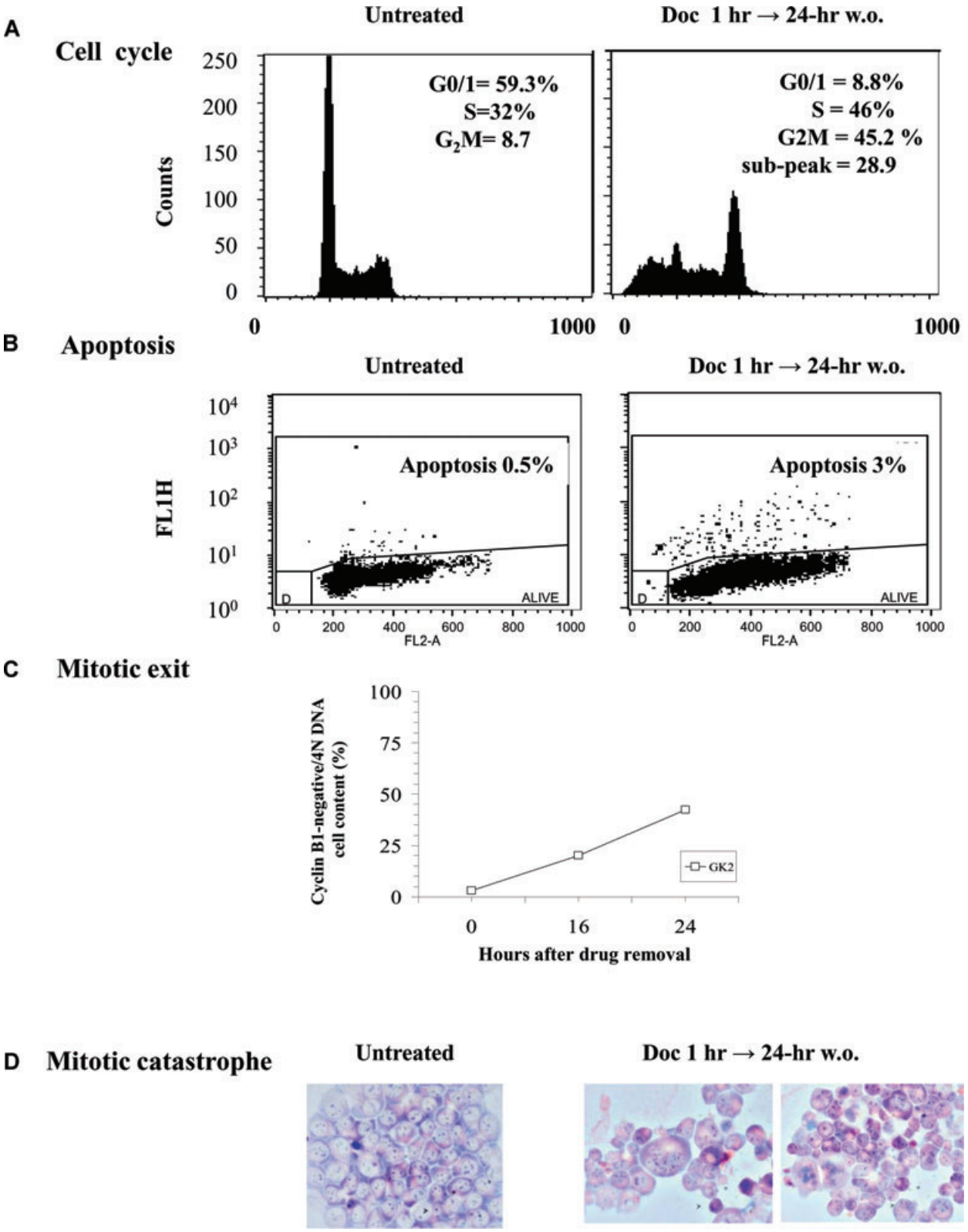

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Fig. 4 (**A**) Cell cycle perturbation induced by 0.01 μM concentration of docetaxel (Doc) after 1-hr exposure followed by 24-hr washout. (**B**) Representative images of cytofluorimetric analysis of apoptosis in untreated GK2 and after exposure to 0.01 μM of docetaxel. (**C**) Biparametric analysis of GK2 cells based on cyclin B1 detection and propidium iodide staining to highlight potential docetaxel-induced abnormalities in mitotic exit. (**D**) Representative images of morphologic microscope analysis of GK2 cells exposed to docetaxel showing cell populations with DNA condensation, abnormal mitotic figures, multinucleation and large viable cell formation.

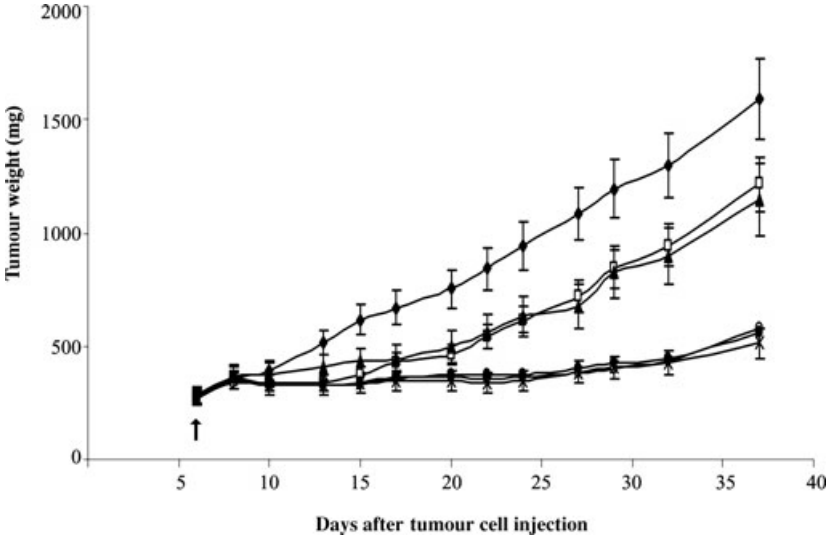

Fig. 5 Antitumour efficacy of combination of docetaxel and sorafenib in NCI-N87 tumour xenografts. Mice were injected i.m. with NCI-N87 cells and starting from sixth day after implant were treated with docetaxel and sorafenib alone or in combination. (◆), untreated; (□), docetaxel; (▲), sorafenib; (○), docetaxel followed by sorafenib, the latter given for 14 days; (∗), docetaxel followed by sorafenib, the latter given for 21 days; (●), docetaxel followed by sorafenib, the latter given for 28 days. Arrows indicate the start of treatments. Data obtained in combination treatment groups were significantly different ($P < 0.001$) from those obtained in groups treated with a single agent and from untreated groups. Similarly, results obtained in groups treated with single agent were significantly different ($P < 0.001$) from those observed in untreated groups.

Antitumour activity of docetaxel and sorafenib combination in NCI-N87 xenografts

The human gastric cancer line NCI-N87 showed high tumorigenic ability: i.m. injection of 3×10^6 cells produced tumours in all of the mice, with a measurable mass of about 300 mg evident on the sixth day after cell injection when treatment started. Treatment with single-agent docetaxel or sorafenib caused a lower reduction in tumour growth than that produced by the administration of docetaxel followed by sorafenib, which lasted for more than 3 weeks after the start of treatment (Fig. 5). In fact, while docetaxel or sorafenib alone caused about 40% TWI accompanied by a 9-day delay in tumour growth, combination docetaxel and sorafenib was highly effective in inhibiting tumour growth (about 70%, $P < 0.001$ *versus* untreated and single treatment) and delaying tumour growth (up to 25 days, $P < 0.001$ *versus* untreated and single treatment) (Table 1). The antitumour effect improved survival in mice by up to 30%, which was significantly higher ($P < 0.001$) than that of the untreated group and of the groups treated with docetaxel or sorafenib alone. Notably, a promising, albeit slight, advantage in terms of tumour growth delay and ILS was obtained by prolonging the administration of sorafenib from 21 to 28 days.

Discussion

Sorafenib is a multikinase inhibitor showing activity against the raf/MEK/ERK pathway whose inappropriate activation is a common feature in human tumours, including gastric cancer [45, 46]. Although sorafenib has proven effective in a wide variety of human cancer cell lines [47–53], its activity has not yet been investigated in gastric cancer cells. In our work, the tyrosine kinase inhibitor showed significant cytotoxic activity in all of the cell lines when used at concentrations lower than 10 μM, which corresponds to the peak plasma level reached after administration of 400 mg twice daily recommended for phase II trials [54]. Reduced MEK and ERK activation observed after sorafenib exposure supports the assumption that its antiproliferative proprieties are due mainly to the inhibition of the raf pathway, as reported for other tumour histotypes [19].

We also observed that single-agent sorafenib has important pro-apoptotic activity, triggering the mitochondrial apoptotic pathway and reducing the expression of anti-apoptotic proteins mcl-1 and p-bad. Notably, the anti-apoptotic Bcl-2 family member mcl-1, which appears to depend on ERK-mediated phosphorylation [55–58], is believed to be involved in resistance to anticancer drugs and to recurrence in gastric cancer [59, 60].

As combining drugs with different mechanisms of action may enhance antitumour activity by overcoming drug resistance mechanisms, we tested the activity of sorafenib in combination with different conventional chemotherapeutic agents used in the treatment of advanced gastric cancer (*data not shown*). Among these, the most promising results were obtained from the association of sorafenib with docetaxel, while combinations with paclitaxel and gemcitabine did not produce a synergistic effect. In particular, a synergistic effect was observed in all cell lines when exposure to

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Table 1 Therapeutic efficacy of docetaxel and sorafenib combination in NCI-N87 xenografts

Treatments*	TWI (%)†	T- C (days)§	ILS (%)#
Docetaxel for 3 days	39	9	10
Sorafenib for 28 days	38	9	14
Docetaxel → sorafenib for 14 days	66	19	24
Docetaxel→ sorafenib for 21 days	68	25	29
Docetaxel → sorafenib for 28 days	65	25	30

*Nude mice bearing NCI-N87 human gastric cancer tumours were treated i.p. with 5 mg/kg/day of docetaxel for 3 days or with 30 mg/kg/day sorafenib p.o. for 14, 21 or 28 consecutive days.
† Tumour weight inhibition calculated at the nadir of the effect.
§ Tumour growth delay, evaluated as T – C, where T and C are the median times for treated and control tumours, respectively, to achieve equivalent size.
# Increased lifespan of mice.
Statistical analysis: combination treatments (schedules c-e) were significantly different ($P < 0.001$) from single treatments (schedules a and b) and from untreated groups in terms of tumour weight inhibition, tumour growth delay and survival of mice.

docetaxel was followed by a 24-hr washout and then by treatment with sorafenib. The synergism detected was accompanied by a strong induction of apoptosis not observed at the end of the inverse sequence, which produced an antagonistic interaction. The schedule-dependent synergism observed in all cell lines was probably due to the action of low-dose docetaxel, which induced an increase in G_2-M phase in all cell lines and the appearance of a sub-G1 peak. The subpeak population was not representative of DNA fragmentation, as the TUNEL assay results suggested, but rather of an aneuploid G_0/G_1population with DNA content between 4N and 8N. These data were confirmed by biparametric analysis which showed, 16 and 24 hrs after drug removal, the development of cells lacking cyclin B1 (G_2 phase marker) and characterized by tetraploid DNA content. Furthermore, morphological analysis after exposure to docetaxel showed cells with features typical of mitotic catastrophe, *i.e.* abnormal DNA condensation, atypical mitotic figures and multinucleation. The induction of mitotic catastrophe by low-dose docetaxel in gastric cancer cell lines confirmed results previously reported by our group on a prostatic cancer model [42]. Furthermore, such docetaxel-induced cell damage provided a suitable target for the pro-apoptotic action of sorafenib, as seen by the dramatic increase in cell death at the end of the treatment schedule.

Our *in vivo* results demonstrated the high antitumour efficacy of the docetaxel and sorafenib combination in mice bearing NCI-N87 xenografts. A slightly stronger antitumour activity was obtained when the administration of sorafenib was prolonged to 28 days. These results suggest that modulating the treatment schedule by increasing the overall duration of sorafenib administration or by using multiple treatment cycles could improve response to therapy. Finally, all the treatments were well tolerated by animals as no body weight loss or toxic deaths were observed.

In conclusion, our investigations provide evidence of the promising activity of sorafenib as a single agent in gastric cancer and of its potential clinical usefulness when used in association with docetaxel for the treatment of patients with advanced disease.

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