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Research Paper

Chemotherapy Regimen GOLF Induces Apoptosis in Colon Cancer Cells through Multi-Chaperone Complex Inactivation and Increased Raf-1 Ubiquitin-Dependent Degradation

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ABSTRACT

The multi-drug combination of oxaliplatin (OXA), 5-Fluorouracil (5-FU) and leucovorin (LF) is currently considered as the gold standard treatment for metastatic colorectal carcinoma. In previous studies, we have studied a chemotherapy regimen containing gemcitabine (GEM), OXA, LF, and 5-FU (named GOLF regimen) that has shown a good safety profile and highly significant anti-tumor activity. In the present study, we have investigated on the anti-tumour mechanisms of GOLF in human colon cancer HT-29 and WiDr cell lines. We have found that GOLF induced growth inhibition that was largely caused by apoptosis differently from other combinations. Moreover, the different drugs composing GOLF were highly synergistic in inducing growth inhibition. Apoptosis induced by GOLF combination was paralleled by PARP cleavage and caspase 9 and 3 activation that were not recorded in the other combinations. An about 85% decrease of the activity of Erk and Akt was found in GOLF-treated cells. These effects were likely due to decreased expression of the upstream activator Raf-1 and of Akt itself, respectively. The intracellular levels of these signalling components can be post-translationally regulated by ubiquitin-dependent degradation through proteasome. Therefore, we have evaluated the expression of some chaperone components and we have found that GOLF did not affect the expression of both heat shock protein (HSP) 90 and 27 but induced an about 90% increase of HSP70 levels suggesting the inactivation of the multi-chaperone complex. Moreover, an about 4-fold increase of the ubiquitination of Raf-1 was also found and the addition for 12 h of 10 μM proteasome inhibitor lactacystin caused an accumulation of the ubiquitinated isoforms of Raf-1. In conclusions, GOLF was a combination highly synergistic in inducing both growth inhibition and apoptosis of colon cancer cells. These effects likely occurred through the disruption of critical survival pathways and the inactivation of multi-chaperone complex.

ABBREVIATIONS

OXA, oxaliplatin; 5-FU, 5-fluorouracil; LF, leucovorin; IRI, CPT-11; GEM, gemcitabine; GOLF, GEM-OXA-LF-5-FU; OLF, OXA-LF-5-FU; ILF, CPT-11-LF-5-FU; GILF, GEM-CPT-11-LF-5-FU; HSP, heat shock protein; MAbs, monoclonal antibodies; RBD, Ras Binding Domain; ECL, chemiluminescence's technique; GSK3b, Glycogen synthase kinase-3b; CI, combination index; DRI50, Dose reduction index50; CTR, Control; SD, standard deviation; PI, propidium iodide; Erk, extracellular signal regulated kinase.

INTRODUCTION

Colorectal carcinoma is the second leading cause of cancer death in the Western countries with almost 50% of the patients dying for cancer related problems and with a dismal 5-year survival rate.[1] 5-fluorouracil (5-FU), alone or in combination with biomodulators such as leucovorin (LF), levamisol, interferon-α, using different schedules and modalities of administration is the only systemic treatment in adjuvant setting and advanced stage of disease.[2-6] More recently, new drugs such as the topoisomerase I inhibitor, CPT-11 (IRI) and the novel platinum derivative oxaliplatin (OXA), have shown a significant anti-tumor activity against colon cancer patients.

In the last five years, a number of clinical trials have demonstrated that both IRI and OXA may be successfully combined with 5-FU in the first line treatment of metastatic colo-rectal carcinoma leading to a very high rate of response and effectively improving the overall survival of these patients.[5,6]

As a results of its good tolerability and efficacy the combination of OXA with LF and infusional 5-FU known as oxali-FUFA (FOLFOX-4) is currently recommended by the American Food and Drug Administration as the first-line treatment for advanced colorectal carcinoma.

Previous studies have shown that the gemcitabine (GEM) (difluoro-2',2'-deoxycytidine) synergistically interacts with either 5-FU[7-10] as well as with OXA,[11-14] in terms of anti-tumor activity in a number of different tumor models in vitro. These results have provided the rationale to perform several clinical trials in a number of different malignancies.

In previous studies, we have tested both the in vitro and in vivo activity against human colon cancer of a chemotherapy regimen containing GEM, OXA, LF, and 5-FU (named GOLF regimen). The results showed that GOLF induced far greater anti-tumor activity (super-additive) than those obtainable with all of the other possible combinations of the four drugs, including the OXA-LF-5-FU (OLF) or the IRI-LF-5-FU (ILF) regimen, which are currently considered as efficacious treatments for metastatic colorectal cancer.[6] The GOLF combination has also been used in the treatment of advanced colon and gastric cancer patients and shown a good safety profile and highly significant anti-tumor activity.[15-17] The favorable anti-tumor activity of GOLF regimen could be explained by changes of the pharmacokinetics and pharmacodinamics of both 5-FU and OXA induced by GEM (refs. 8, 9 and unpublished results). The GOLF regimen compared to the above mentioned combinations showed also the unique ability to kill colon cancer cells by inducing above all apoptosis, a feature that was only partially shared by OLF.[17]

Apoptosis, or programmed cell death, is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage.[18] The balance between survival and death responses determines either tumor development or cell death/apoptosis. Cancer cells may survive in an environment in which they would not normally exist. This is accomplished by alterations in the expressions or functions of genes controlling both survival and apoptotic signalling pathways. Survival pathways are, in fact, generally upregulated in cancer cells. Such pathways involve the activation of cell surface receptors, serine threonine kinases, transcription factors as well as other molecules.[19] Well known survival pathways, that can be over-activated in cancer cells, are the ras-Raf-1-Erk-dependent and the Akt-mediated signallings. In fact, Raf-1 and MEK are components of the ras → extracellular signal regulated kinase (Erk)-dependent signal transduction pathway that is involved in regulation of both proliferation and apoptosis.[20] In details, Raf-1, stimulated after steric interaction with Ras, phosphorylates and activates a MKK whose main component is Mek-1. Mek-1 phosphorylates the MAPKs Erk 1 and 2 that translocate to the nucleus and phosphorylate gene transactivators, such as the serum response factor-1.[21,22] A second important anti-apoptotic pathway involves signaling via Akt/PKB.[23,24] In fact, it has been demonstrated that Akt can be activated concomitantly or independently from Ras → ERK-1/2 signalling by growth factors.[25-27] Additionally, it has been reported that PC12 cells display a protective anti-apoptotic pathway in response to hypoxic stimuli.[28] The protection from apoptosis by Akt could be due to the regulation of mitochondrial physiology since Akt is involved in the regulation of bcl-related proteins such as Mcl-1.[25] However, the requirement of Akt for the protection from apoptotic events is highly variable depending upon the experimental model used.[26,27,29]

The overexpression of survival factors and the consequent activation of their pathways can lead to deregulated cell growth and cancer. The increased expression of such proteins can be dependent either by their increased synthesis (transcription and/or translation) or decreased degradation (post-translational regulation).

Protein degradation can be driven by the proteasome-dependent pathway following the covalent addition to the proteins of several small molecules of 14 KDa, called ubiquitin.[30,31] This event leads to the subsequent delivery of the protein to a macromolecular complex called proteasome that determines the final proteolysis and degradation of the protein.[31] On the other hand, mechanisms that prevent the unfolding and the consequent degradation of intracellular proteins exist. One of these mechanisms is regulated by the so-called heat shock proteins (HSPs) that could be involved as molecular chaperones to rescue damaged proteins and subtracting them from the proteasome-dependent degrading pathway.[32-35] HSP90 is one of the most abundant cellular chaperone proteins and functions in a multi-component complex of chaperone proteins that may include p60/Hop, p50Cdc37, HSP-40/HDJ2, p23, BAG-1, HSP70 and one of a variety immunophilins.[36] It forms the basis of a super-chaperone machine that promotes the proper folding of client proteins so that they can respond to a stimulus or bind ligand. However, the machine is in constant flux and cycles between two HSP90 conformations, determined by ATP or ADP binding, which in turn specify which set of cochaperones associate with the chaperone complex.[37] In fact, binding of HSP70 to the multi-chaperone complex favors the ATP hydrolysis and makes the client protein susceptible to ubiquitination and delivery to the proteasome (where it is degraded).[37]

Several protein kinases, including Raf-1, ErbB-2, Akt and Bcr-Abl depend upon the HSP90/multi-chaperone for proper function and stability and this is likely the way by which HSP90/multi-chaperone is involved in the regulation of apoptotic processes.[23,24,38-41]

On these bases, we have investigated in an in vitro colon carcinoma model, whether GEM may enhance the anti-tumor and apoptosis-inducing activity of the Oxali-FUFA combination. The effects on apoptosis and proliferation were also compared with those induced by other pharmacological combinations containing either OXA or IRI. In these experimental conditions, we have evaluated the effects of the different combinations on both the expression and activity of the components of ras-Raf-1 and Akt-dependent survival signallings. Finally, we have studied the involvement of the HSP90/multi-chaperone complex in the regulation of the expression of these survival factors.

MATERIALS AND METHODS

Materials. DMEM, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ). Rabbit antisera raised against α–tubulin and ubiquitin, Erk-1/2 K-23, Raf-1 E-10 monoclonal antibody (MAb) C-14 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt Mab and the relative activity evaluation kit was purchased by Cell Signalling. Anti-pan-Ras MAb clone 10 were purchased from Calbiochem. Anti-HSP70 C92F3A-5 and anti-HSP90 AC88 monoclonal antibodies (Mab) were from StressGen Biotech. Co. (Victoria, BC, Canada) and anti-HSP-27 G3.1 MAb from Affinity Bioreagents (Neshanic Station, NJ). Rabbit antiserum raised against PARP and anti-phosphatidylinositol-3-kinase (PI3K) UB93-3 MAb were purchased by Upstate Biotech., Lake Placid, NY. Anti-pErk E10 MAb and rabbit anti-Pro-caspase 9 antiserum were purchased from Cell Signalling, Beverly, MA. Anti-Pro-caspase 3 MAb 31A1067 was purchased form Alexis (Lausen, Switzerland).

Cell cultures. The WiDr and HT29 colon carcinoma cell lines were purchased from American Type Cell Cultures, MD, USA (ATCC). WiDr cells were maintained in Rosewell Park Modified Iscove medium (RPMI)-1640 and HT29 in DMEM containing 10% heath-inactivated foetal bovine serum, 5% l-glutamine, 20% HEPES buffer solution, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Evaluation of synergistic conditions. The following drugs were used in this study: GEM (Eli Lilly), OXA (Sanofi-Synthelabo), LV (Lederle), 5-FU (Roche), and CPT-11 (IRI; Aventis Pharma). For the study of the synergism between the different drugs contained in GOLF on cell growth inhibition of HT29 and WiDr, the cells were seeded in 96-multiwell plates at the density of 5 x 10^3 cells/well. The medium was harvested after 24 hours of incubation at 37°C and 5% CO_2. Subsequently, 100 μl of fresh medium containing GEM at the final concentrations of 0, 25, 50, 100, 150, 200, 300 or 500 μg/ml were added to the wells. After 30 minutes of incubation, the medium was replaced with 100 μl of a fresh medium or medium containing 10^{-4}M FA. After a further 30 minutes of incubation, the medium was withdrawn and replaced with 100 μl of fresh medium containing 5-FU at the final concentrations of 0, 10^{-6}, 5 x 10^{-6}, 10^{-5}, 5 x 10^{-5}, 10^{-4}, 5 x 10^{-4}, 10^{-3} or 5 x 10^{-3} M. After six hours, fresh medium was added containing OXA at the concentrations of 0, 10^{-7}, 5 x 10^{-7}, 10^{-6}, 5 x 10^{-6}, 10^{-5}, 5 x 10^{-5}, 10^{-4}, 5 x 10^{-4} or 10^{-3} M. After a further four hours, the medium was withdrawn and fresh medium was added containing no drugs or 5-FU at the same concentrations as those described above, and the cells were incubated for another 38 hours. The effects of cytotoxic drugs were evaluated by means of a colorimetric assay (MTT). Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration.[42] Assessment of synergy was performed quantitating drug interaction by Calcusyn computer program (Biosoft, Ferguson, MO). Combination index (CI) values of < 1, 1 and > 1 indicate synergy, additivity, and antagonism, respectively.[42]

Drug combinations and Western blot analysis. The most effective conditions to obtain the maximal cytotoxic and pro-apoptotic effects of the drug combination were extrapolated from the previous experiments. HT29 subconfluent cells were trypsinized, counted, and seeded in appropriate culture disks. The medium was harvested after 24 h of incubation at 37°C and 5% CO2. Subsequently, fresh medium was added to the tumor cells used as control (CTR), whereas fresh medium containing GEM at final concentrations of 50 μg/ml for GEM-treated (GEM), GEM-IRI-LF-treated (GILF) and GEM-OXA-LF (GOLF) treated groups, or IRI at final concentrations of 100 μg/ml for IRI-LF-treated (ILF) group or OXA at final concentrations of 10^{-5} M/ml for OXA-LF-treated (OLF) group was added to the tumor cells. After 30 min of incubation for GEM, GILF and GOLF and 4 h of incubation for ILF and OLF, the medium was replaced with fresh medium (for CTR, and GEM) or medium containing 10^{-4} M LV (for ILF, GILF, OLF and GOLF). After a further 30 min of incubation, the medium was withdrawn, and replaced fresh medium (for CTR and GEM) or fresh medium containing 5-FU at final concentrations of 10^{-3} M for all other groups. After 24 h, fresh medium (for CTR and GEM), or fresh medium containing 100 μg/ml IRI (GILF) or OXA at the concentration of 10^{-5} M (for group GOLF) was added to the wells. After 4 h, the medium was withdrawn, and fresh medium containing no drugs was added to the samples in CTR and GEM, whereas medium containing 10^{-3} M 5-FU was added to the samples in all other groups. Thereafter, the cells were incubated for 24 h before being detached. For cell extract preparation, the cells were washed twice with ice-cold PBS/BSA, scraped, and centrifuged for 30 min at 4°C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 NaCl, 1mM EDTA, pH 7.5, 10 mM Na2HPO4, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 units/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE. The proteins on the gels were electro-transferred to nitrocellulose and reacted with the different MAbs or anti-sera.

Affinity precipitation of Ras. HT-29 cells were grown for 48 h with the different drugs and the relative concentrations at 37°C as described above. The cells were lysed in the Mg^{2+} buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA and 2% glycerol. Then, 10 μl Ras Binding Domain (RBD) conjugated to agarose was added to 1 mg of cell lysate and the mixture was incubated at 4°C for 1 h. The agarose beads were collected by microcentrifugation at 14,000 x g for 5 sec. and washed three times with Mg^{+2} buffer. The agarose beads were boiled for 5 min in 2X Laemmli sample buffer and collected by a microcentrifuge pulse. The supernatants were run on 12% SDS-PAGE, then the proteins were electrotransferred on a nitrocellulose film. The nitrocellulose was incubated overnight with 1μg/ml of anti-Ras Mab, clone RAS10 and with a secondary Mab, a goat α-mouse HRP conjugated IgG, for 1.5 h. The film was washed with PBS/0,05% Tween 20 and detected by ECL, chemiluminescence's technique, (Amersham).

AKT kinase assay. Colon cancer cells were treated with the different drug combination as described above. At the time of processing 1 ml ice-cold Cell Lysis Buffer (20 mM TRIS, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptine, 1 mM PMSF) was added to cells that were incubated on ice for 10 min. The cells were collected and transferred to microcentrifuge tubes and centrifuged at 1,200 g for 10 min at 4°C. The supernatants were collected and precipitated with 20μl of IgG1 anti-Akt monoclonal antibody immobilized with agarose beads (Cell Signaling Technology, MA, USA) by o/n incubation with gentle rocking at 4°C. The resulting immunoprecipitates were then incubated for 30 minutes at 30°C with 1μg Glycogen synthase kinase-3β (GSK3β) fusion protein (Cell Signaling Technology) in the presence of 200 μM ATP and Kinase Buffer (25 mM TRIS, pH 7.5, 5 mM βglycerophosphate, 2 mM dithiotreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). The reaction was terminated with the addition of 20 μl 3X SDS sample buffer. The supernatants were boiled for 5 minutes and electrophorased by 12% SDS-PAGE and the protein electro-transferred on a nitrocellulose film. Phosphorylation of GSK3 was detected using as probe an anti-Phospho-GSK3α/β (Ser21/9) rabbit polyclonal antibody (diluted 1:1000) and then with a secondary anti-rabbit HRP-conjugated monoclonal antibody, (diluted 1:2000). The film was washed with TBS 1X-0,05% Tween 20 buffer and the specific reactivity was detected by ECL (Amersham).

Statistical Analysis. Each experimental point was performed in triplicate, and each experiment was repeated three times. All of the data are shown as mean values ± standard deviations. The statistical significance of all the differences in mean values was determined by using a two-tailed student's t-test.

Evaluation of apoptosis by DNA-flow cytometry. The cells were centrifuged and directly stained in a propidium iodide (PI) solution (50 μg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) overnight at 4°C in the dark. The flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) interfaced with a Hewlett Packard computer (mod.310) for data analysis. To evaluate cell apoptosis, PI fluorescence was collected as FL2 (Log scale) by the CellFIT software (Becton Dickinson). The intracellular DNA content was evaluated by analysing at least 10,000 events for each point in at least three different experiments in order to obtain a standard deviation of less than 5%.

Study of the ubiquitination of Raf-1. HT-29 were seeded and treated with different combinations and/or 10 μM lactacystin for the indicated times. At the time of the assay cells were washed three times with PBS and cell proteins were extracted as described above. For the determination of Raf-1 ubiquitination the supernatants were subjected to immunoprecipitation with anti-Raf-1. The different proteins were precipitated from 300 μg of cell lysates using 5 μg of MAb for 12 h at 4°C and 50 ml of Protein A Sepharose (Sigma, Milan, Italy) 1:1 suspension for 12 h at 4°C. Immunoprecipitated samples were washed four times with lysis buffer supplemented with 0.1% SDS, boiled in 20 μl Laemmli Buffer for 5 min and electrophorased by 10% SDS-PAGE. Proteins were then electroblotted and probed with the anti-ubiquitin rabbit antiserum (diluted 1:500) FL-76 (Santa Cruz Biotech., CA). The specific bands for ubiquitin were detected with goat anti-rabbit (Santa Cruz, CA) conjugated with peroxidase and subsequent ECL reaction (Amersham, Milan, Italy).

Fluorescence microscopy. After washing in PBS, cells were treated with in situ detection kit, according to manufacturers (SantaCruz Biotechnology CA). In details, cells were incubated with PI and a FITC-conjugated antibody raised against annexin V for at 37°C at the dark. Then, cells were observed under fluorescent microscope using a dual filter set for FITC and rhodamine. The images were acquired with a dedicated software.

Table 1 **Synergistic interaction between drugs that compose GOLF chemotherapy regimen on cell proliferation of colon cancer cells evaluated with Calcusyn (Biosoft)**

Cell Line	GEM ED_{50} (μg/ml)	OXA ED_{50} (μM)	5-FU ED_{50} (μM)	Ratio GEM/OXA/ 5-FU	CI_{50}	DRI_{50}	Interpretation
HT-29	0.0083	0.83	0.83	1:100:100	0.02	GEM: 657 OXA: 580 5-FU: 700	Very strong synergism
WIDR	0.0092	0.92	0.92	1:100:100	0.03	GEM: 367 OXA: 380 5-FU: 320	Very strong synergism

*CI_{50} were calculated for 50% cell survival (ED_{50}) by isobologram analyses performed with CalcuSyn software. **DRI_{50} represents the order of magnitude (fold) of dose reduction obtained for ED_{50} effect in combination setting as compared to each drug alone.

RESULTS

Effects of the different combinations on colon cancer cell proliferation and apoptosis. We have evaluated the effects of the different pharmacological combinations, described in Materials and Methods section, on cell growth inhibition of two colon carcinoma cell lines, HT-29 and WiDr. Moreover, we have studied the contribution of apoptosis in determining such effect in the same experimental conditions. In details, we have evaluated the growth inhibition induced by different combinations of GEM, OXA and 5-FU at 72 h on HT-29 and WiDr cells. We have performed these experiments with MTT assay and the resulting data were elaborated with the dedicated software Calcusyn,[42] see also Materials and Methods. With this mathematical model synergistic conditions occur when the combination index (CI) is below 1.0. When CI is less than 0.5 the combination is highly synergistic. We have found that the combination of GEM, OXA and 5-FU was highly synergistic when GEM, OXA and 5-FU were used at equimolar concentrations while overwhelming concentrations of each drug were not (Table 1 and data not shown, respectively). In synergistic drug combination the CI_{50s} (the combination index calculated for 50% cell survival by isobologram analysis) were 0.02 and 0.03 for HT29 and WiDr cells, respectively (Table 1). Therefore, the combined use of the three agents was highly synergistic on the growth inhibition of both cell lines. Dose reduction $index_{50}$ (DRI_{50}) represents the magnitude of dose reduction obtained for the 50% growth inhibitory effect in combination setting as compared to each drug alone. In our experimental conditions the DRI_{50} was equal to 657 and 367 for GEM, to 580 and 380 for OXA and 700 and 320 for 5-FU in HT29 and WiDr, respectively (Table 1). Thereafter, we have evaluated the effects of the different combinations in inducing both cell growth inhibition, evaluated with a spectrophotometric assay, and apoptosis, assessed at FACS analysis after labelling with PI. We have found that GOLF induced an about 65% growth inhibition that was mostly due to apoptosis on both HT-29 and WiDr cell lines (Fig. 1). On the other hand, ILF, GILF and OLF induced an about 40, 45 and 50% growth inhibition, respectively, that was paralleled by only an about 20% apoptosis on both cell lines (Fig. 1). Interestingly, OLF was the most effective combination in inducing apoptosis. FL and GEM treatments were poorly effective in inducing both growth inhibition and apoptosis. In fact, FL induced an about 10% growth inhibition and 5% apoptosis while GEM caused about 15% growth inhibition and 5% apoptosis on both cell lines (Fig. 1). Moreover, we have evaluated the in situ apoptosis with double fluorescence microscopy based on the evaluation of both PI and annexin V labelling in HT-29 cells. We have found again that GOLF was able to induce a strong apoptotic effect while OLF caused apoptosis only in a smaller number of cells and ILF induced predominantly necrosis (Fig. 2). These data confirmed that GOLF was the only combination able to induce apoptosis that appeared largely responsible for growth inhibition. Moreover, GOLF was the most active combination in inducing anti-proliferative effects on both cell lines.

Effects of the different combinations on caspase activation and PARP fragmentation. On the basis of the previous data we have evaluated if the

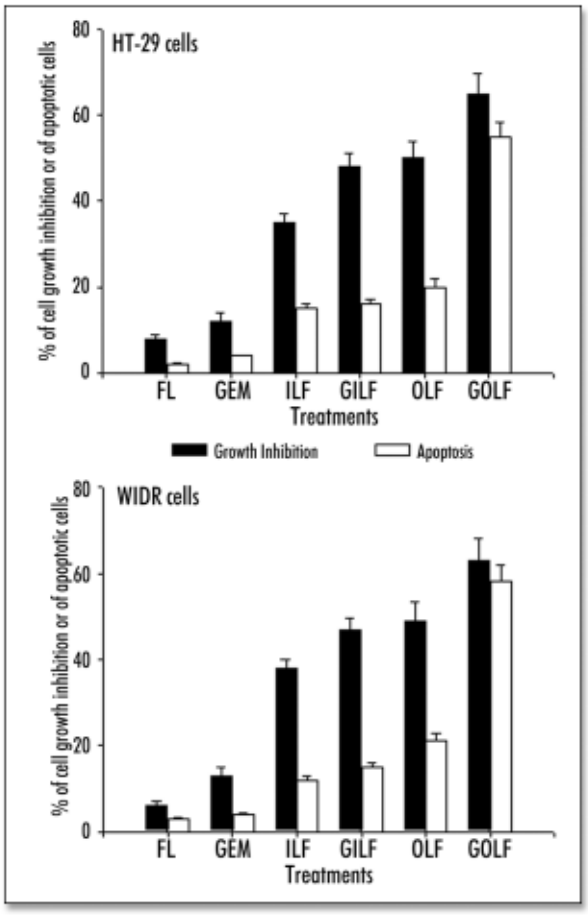

Figure 1. Effects of the different combinations on cell growth inhibition and apoptosis of colon cancer cells. The cytotoxic (black columns) and apoptotic (white columns) effects of the different pharmacological combinations were tested on HT-29 (upper panel) and WiDr (lower panel) colon carcinoma cells by means of MTT assay after 48 hours exposure to: FL, GEM, ILF, GILF, OLF, GOLF (see Material and Methods). The results are expressed as % of cell growth inhibition ± standard deviation (SD). The experiments were performed three times and SDs were always less than 10%. Bars, SDs. Apoptosis was evaluated at FACS analysis after PI labelling as described in "Materials and Methods" and was expressed as % of apoptotic cells. The experiments were performed three times and SDs were always less than 10%. Bars, SDs.

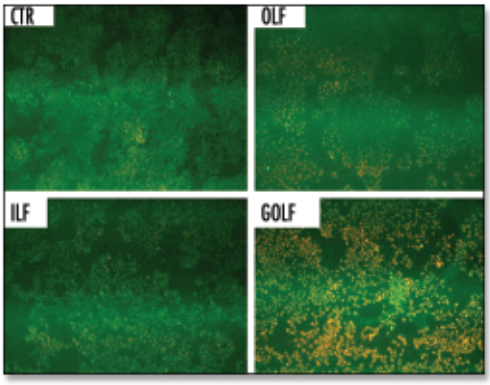

Figure 2. Pro-apoptotic effects of the GOLF combination on colon carcinoma cell lines in vitro. HT-29 cells were seeded and exposed to the different pharmacological combinations as described in "Materials and Methods". Subsequently, we have evaluated the apoptotic effects of the combinations on HT-29 cells at fluorescence microscopy after labelling with PI and anti-annexin V antibody. Then, cells were observed under fluorescent microscope using a dual filter set for FITC and rhodamine. The images were acquired with a dedicated software. CTR, control cells; for OLF, ILF and GOLF see Materials and Methods. The experiments were performed at least three times and the results were always similar. Red and green fluorescent cells were apoptotic.

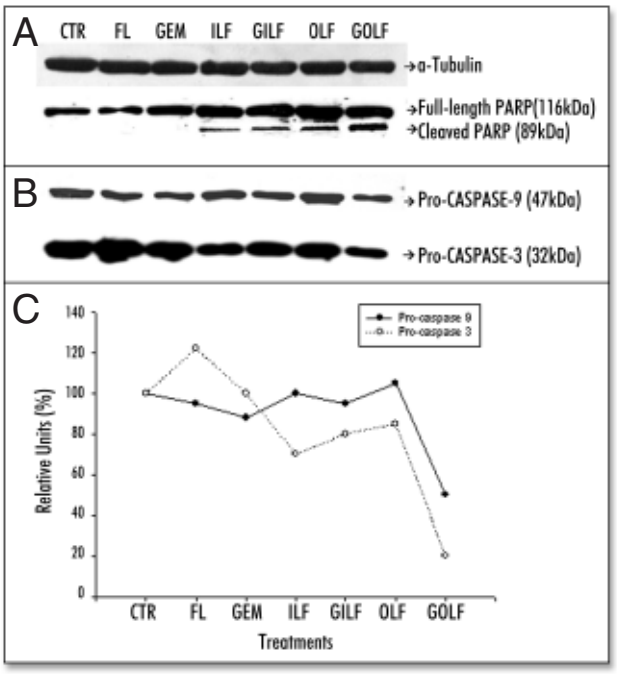

Figure 3. Effects of the different combinations on PARP cleavage and caspase activation. HT-29 cells were seeded and exposed to the following pharmacological combinations: control (CTR), FL, GEM, ILF, GILF, OLF, GOLF as described in "Materials and Methods". At the time of the experiments, the cells were detached and the proteins were extracted to be subjected to SDS-PAGE electrophoresis. Then, the proteins were electrotransfered on nitrocellulose film and probed with anti-α-tubulin or anti-PARP (A) or anti-pro-caspase 9 or anti-pro-caspase 3 (B) antibodies as described in "Materials and Methods". Then the specific bands were immunodetected with a secondary HRP-conjugated antibody and the specific reactivity was detected by ECL (Amersham). The experiments were performed at least three times and the results were always similar. (C) Scan of the bands associated with pro-caspase 3 and 9 expression was performed with a dedicated software and expressed as % relative units considering control as 100% (mean of three different experiments).

different combinations induced the activation of mediators of the execution phase of apoptosis. We have firstly evaluated the effects of the different combination on PARP cleavage, that occurs in the final stages of apoptosis, as evaluated with a western blotting using an antibody which recognizes both PARP fragments. We have found that both FL and GEM did not cause PARP fragmentation while this effect was minimal in ILF and GILF-treated HT-29 cells and more evident in OLF-treated cells (Fig. 3A). However, only GOLF treatment induced the appearance of a large fragment at 89 kDa, a clear feature of PARP cleavage (Fig. 1A). Moreover, we have evaluated the effects of the different combinations on the cleavage and the consequent activation of both caspase 9 and 3 with western blotting using specific antibodies that recognize only the intact forms of the two enzymes. We have found that the only combination that reduced the pro-caspase 9 levels was GOLF (Fig. 3B). In fact, GOLF induced an about 60% reduction of pro-caspase 9 expression while the other treatments did not significantly change the levels of the enzyme (Fig. 3C). On the other hand, ILF, GILF and OLF were all able to cause an about 40% reduction of pro-caspase 3 with the maximal effect recorded in ILF-treated cells (Fig. 3B and C). However, GOLF caused a more pronounced effect on caspase 3 activation since pro-caspase 3 levels were 80% reduced in GOLF treated cells (Fig. 3B and C). These effects suggest that the apoptosis induced by GOLF was largely due to caspase 9 activation, as upstream caspase, and by caspase 3 stimulation, as terminal caspase. Therefore, apoptosis occurrence in GOLF-treated cells was likely paralleled by the activation of a mitochondrial apoptotic pathway. On the other hand, the small caspase 3 activation recorded in ILF, GILF and OLF was likely not induced by caspase 9 and suggest the stimulation of alternative apoptotic pathways.

GOLF regimen induces decreased expression of Raf-1 and Akt affecting their downstream signallings. Since apoptosis onset in eukaryotic cells is substantially regulated by the balance between apoptotic and survival pathways we have evaluated the effect of the different combinations on both expression and activity of the components of the ras → Erk and Akt-dependent pathways. We have found that all the different treatments have only slight effects on ras expression that was never reduced by the different combinations (Fig. 4A). The activity of ras was also unmodified by the treatments with ILF and GOLF as evaluated by the precipitation for affinity of ras with the RBD of Raf-1 (Fig. 4B). These data confirmed that the different treatments had no effects on both ras expression and activity. On the other hand, GOLF caused an about 90% reduction of Raf-1 expression while GEM, ILF, GILF and OLF an about 20% decreased Raf-1 expression (Fig. 4A). FL did not induce any change of Raf-1 levels (Fig. 1A). All the treatments did not affect the expression of Erk-1 and 2 proteins while ILF, GILF and OLF caused a slight decrease of Erk-1 and 2 activity that was, on the other hand, almost abolished in HT-29 cells treated with GOLF (Fig. 4A). These data suggested that the reduction of the activity of the downstream effector kinases Erk-1 and 2 was likely due to the reduction of the expression of the upstream activator Raf-1 being also both the activity and expression of ras unmodified by the different treatments. Similarly, ILF, GILF and OLF induced an about 50% decrease of Akt protein expression that was almost absent in GOLF-treated HT-29 cells while the other treatments were ineffective (Fig. 4C). Consequently, Akt activity was 50% reduced in ILF-treated cells while it was almost absent (10% of control values) in HT-29 cells exposed to GOLF (Fig. 4C). The expression of the upstream kinase PI3K was unaffected by both ILF and GOLF (Fig. 4D). These data again suggested that the decreased activity of Akt was likely due to its decreased expression induced at different extents by the various treatments. Accordingly with our results, only GOLF was able to almost completely abolish the activity of both survival enzymes Erk-1/2 and Akt likely through the reduction of the expression of Raf-1 and Akt itself, respectively.

GOLF regimen inactivates the HSP90 multichaperone complex and increases Raf-1 ubiquitination and degradation. The previous results suggested that GOLF induced Erk-1/2 and Akt activity reduction through the

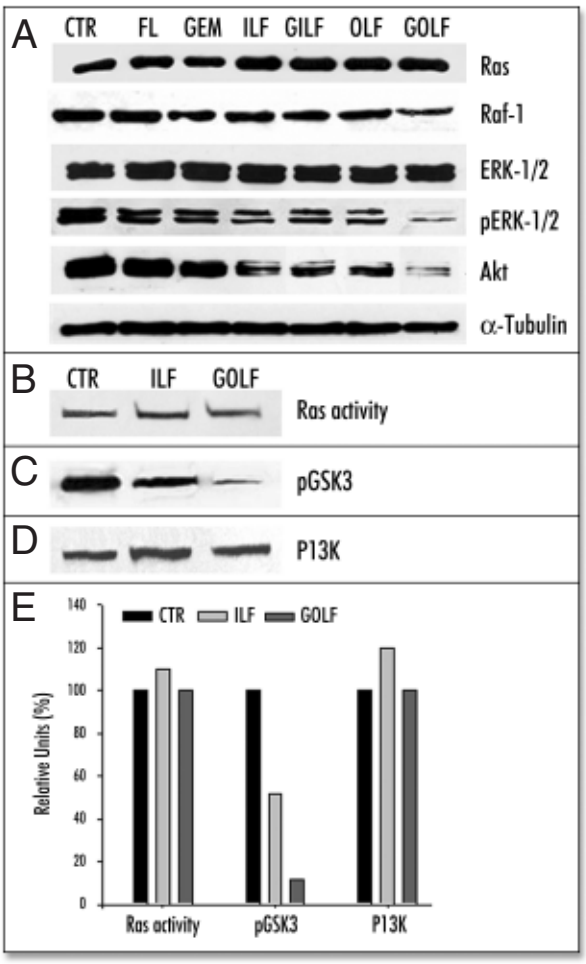

Figure 4. Effects of the different combinations on the expression and activity of components of intracellular survival signallings. (A) HT-29 cells were seeded and exposed to the following pharmacological combinations: control (CTR), FL, GEM, ILF, GILF, OLF, GOLF as described in Materials and Methods. At the time of the experiment, the cells were detached and the proteins were extracted to be subjected to SDS-PAGE electrophoresis. Then, the proteins were electrotransfered on nitrocellulose film and probed with the different primary antibodies as described in Materials and Methods. Then the specific bands associated with Ras, Raf-1, Erk-1/2, the phopshorylated isoforms of Erk-1/2, Akt and α-tubulin were immunodetected with a secondary HRP-conjugated antibody and the specific reactivity was detected by ECL (Amersham) as described in "Materials and Methods". The experiments were performed at least three times and the results were always similar. (B) HT-29 cells have been seeded and treated as described above. Affinity precipitation of ras was performed with the RBD of Raf-1 conjugated with agarose for the evaluation of ras activity as described in "Materials and Methods". Western blotting for ras was performed as described above. The experiments were performed at least three times and the results were always similar. (C) HT-29 cells have been seeded and treated as described above. Akt activity was evaluated incubating immunoprecipitated Akt with its substrate GSK3β and ATP. Consequently, the reaction of phosphorylation of Akt was allowed and the reaction mixture was run in SDS-PAGE electrophoresis and blotted on nitrocellulose film. Then, the film was probed with specific anti-pGSK3β antibody. The specific band was immunodetected with a secondary HRP-conjugated antibody and the specific reactivity was detected by ECL (Amersham) as described in Materials and Methods. The experiments were performed at least three times and the results were always similar. (D) HT-29 cells have been seeded and treated as described above. PI3K was determined with western blotting using a specific antibody as described in Materials and Methods. (E) Scan of the bands associated with activated ras, phosphorylated GSK3β and PI3K expression was performed with a dedicated software and expressed as percent relative units considering control as 100% (mean of three different experiments).

decreased expression of Raf-1 and Akt. It has been reported that HSP90/multi-chaperone complex prevents proteasome-mediated degradation of several signalling molecules including Raf-1 and Akt.[43,44] On the bases of these considerations, we have studied the effects of the different treatments on the expression of HSP90, 27 and 70 in HT-29 cells by western blot assay. We have found that HSP27 was upregulated by the exposure of HT-29 cells to both FL and GEM with a maximal 60% increase while it was unchanged by all the other treatments (Fig. 5A and B). HSP70 was up-regulated only by the exposure of HT-29 cells to GOLF with a 90% increase (Fig. 5A and B). Also in this case all the other treatments were unable to modulate the expression of HSP70. All the combinations were also ineffective on the expression of HSP90 (Fig. 5A and B). These results suggested that GOLF was the only combination that increased the expression of HSP70 in the multi-chaperone complex remaining unchanged the levels of both HSP27 and 90. This effect could switch the multi-chaperone complex in the inactive status allowing the ubiquitin-dependent degradation of client proteins by proteasome.[37] On the basis of these considerations, we have evaluated if the reduction of the expression of the components of the survival signalling induced by GOLF could be due to an increase of their degradation via a proteasome-dependent pathway. We have found that the ubiquitination of Raf-1 was 4-fold and 0.2-fold increased in HT-29 cells exposed to GOLF and GILF, respectively, as evaluated with western blotting for ubiquitin after Raf-1 immunoprecipitation (Fig. 5C). In the same experimental conditions OLF was not able to affect Raf-1 ubiquitination. This effect was again paralleled by a 2-fold decrease of the expression of the protein in GOLF-treated cells (data not shown). Subsequently, HT-29 cells have been exposed to OLF or GOLF or GILF and/or the specific proteasome inhibitor lactacystin in order to evaluate the effects on Raf-1 ubiquitination. The addition of 10 μM lactacystin for 12 h, inhibiting proteasome-dependent degradation of ubiquitinated Raf-1, caused an accumulation of the ubiquitinated form of the enzyme (Fig. 5C). The synchronous treatment of the cells with lactacystin and all three different treaments potentiated the accumulation of the ubiquitinated isoforms of Raf-1 induced by lactacystin (Fig. 5C). However, the effect was again more evident in GOLF-treated cells likely due to the inactivation of the multi-chaperone complex occurring in these specific experimental conditions. These data suggest that Raf-1 was ubiquitinated and degraded by a proteasome-dependent pathway in HT-29 cells and that the exposure to GOLF increased poly-ubiquitination and, consequently, degradation of Raf-1 in colo-rectal cancer cells.

DISCUSSION

The small number of chemotherapeutic agents with activity against colorectal cancer reflects the intrinsic resistance of colon cancer cells in vitro and in vivo. Very few drugs are, in fact, able of inducing cytotoxic activity in colon cancer cells at doses potentially achievable in humans without the occurrence of unacceptable toxicity.[5,6] Since its introduction by Heidelberger et al. in 1957, 5-FU has been one of the main components of treatments for colorectal, breast, head and neck, and pancreatic carcinomas. The majority of the studies testing 5-FU, alone or in combination with biomodulators like LF, reported low rates of objective responses and minimal impact on survival[45-46] in patients with metastatic colo-rectal carcinoma. OXA is a novel diaminocycloetane platinum agent that mainly acts by causing inter- and intra-strand DNA crosslinks.[47-50] It has a wide range of anti-tumour activity and promising effects in the treatment of colo-rectal carcinoma (alone or in combination with 5-FU and LF).[50-54] The efficacy of oxaliplatin plus LF (Oxali-FUFA) as first line of chemotherapy has been largely demonstrated in the last years

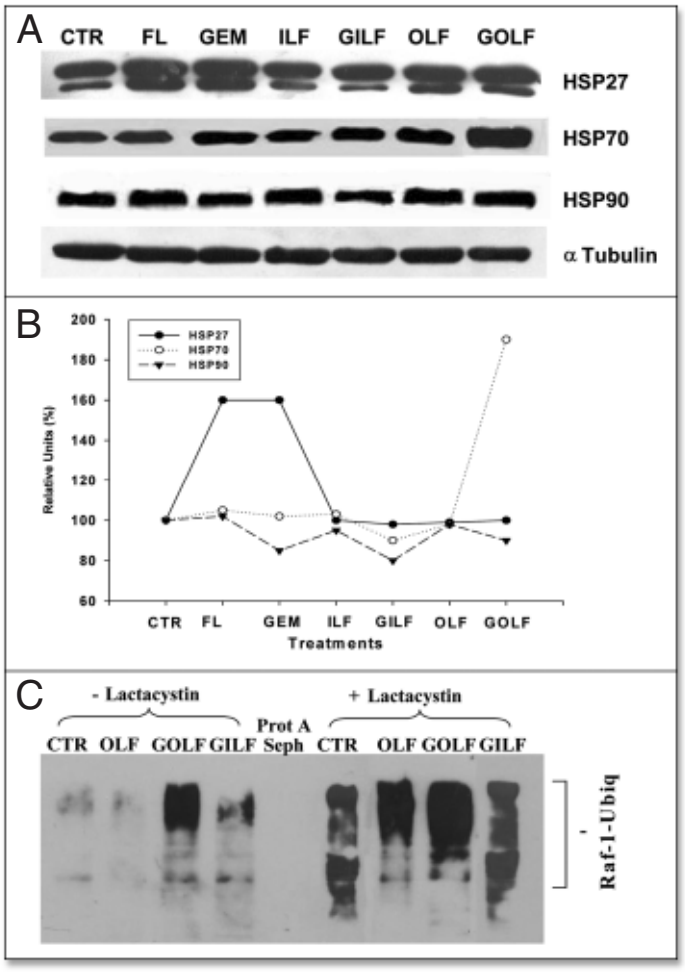

Figure 5. Effects of the different combinations on the expression of HSP70, 90 and 27 and on the ubiquitination of Raf-1. (A) HT-29 cells were seeded and exposed to the following pharmacological combinations: control (CTR), FL, GEM, ILF, GILF, OLF, GOLF as described in Materials and Methods. At the time of the experiment, the cells were detached and the proteins were extracted to be subjected to SDS-PAGE electrophoresis. Then, the proteins were electrotransfered on nitrocellulose film and probed with the different primary antibodies as described in Materials and Methods. Then the specific bands associated with HSP27, HSP70, HSP90 and α-tubulin were immunodetected with a secondary HRP-conjugated antibody and the specific reactivity was detected by ECL (Amersham) as described in Materials and Methods. The experiments were performed at least three times and the results were always similar. (B) Scan of the bands associated with HSP27, HSP70 and HSP90 expression was performed with a dedicated software and expressed as % relative units considering control as 100% (mean of three different experiments). C) HT29 cells have been cultured for 12 hrs in the presence or absence of 10 μM lactacystin (specific proteasome inhibitor) and exposed to OLF or GOLF or GILF as described in Materials and Methods. Then cellular proteins were extracted and immunoprecipitated with an anti-Raf-1 MAb as described in "Materials and Methods". The immunoprecipitated was subsequently run in SDS-PAGE and immunoblotted for ubiquitin. The expression of total ras Raf-1 was also determined with western blotting assay using specific antibodies as previously described in Materials and Methods. The experiments were performed at least three different times and the results were always similar. –Lactacystin, cells unexposed to lactacystin; +Lactacystin, cells exposed to 10 μM lactacystin for 12 h; Prot A Seph, Lane containing protein A sepharose in the absence of cell lysate; CTR, Control cells.

in a large number of different clinical trials performed in patients with metastatic colo-rectal carcinoma.[51,55] More recently, it has been shown that Oxali-FUFA (following the FOLFOX 4 schedule proposed by De Gramon) is better tolerated, and leads to a higher objective response rate, a longer time to progression, and a longer overall survival than the IRI, LF, and 5-FU combination (ILF) proposed by Salts et al. in patients with advanced colorectal carcinoma.[56] GEM (difluoro-2',2'-deoxycytidine) is a difluorinated analogue of deoxycytidine that requires activation through the synthesis of its phosphorylated metabolites to cause DNA damage and antiproliferative effects.[57,58] The early preclinical and clinical studies of GEM showed that it has significant activity against a number of human solid tumors.[58-60]

In a previous in vitro study, we showed that the GEM + 5-FU combination has supra-additive anti-tumor activity against colon carcinoma cells. This was attributed to the pharmacological interaction of the two drugs insofar GEM enhances 5-FU uptake in target cells and its consequent transformation into active metabolites.[61] We also found that GEM enhances the intracellular concentration of 5-fluoro-uridin-triphosphate (5-FUTP), which is capable of damaging mRNA and inhibiting glycosyl transferases in cancer cells. Furthermore, studies by other groups using colon carcinoma cells and other models have shown that OXA can have supra-additive anti-tumor activity when used in combination with GEM.[11-14] On the basis of these data, we have added GEM to the conventional OLF (FOLFOX4) generating a new chemotherapy regimen called GOLF. The GOLF combination has been already used in the treatment of advanced colon and gastric cancer patients and showed a good safety profile and highly significant anti-tumor activity.[15-17]

In the present paper, we have evaluated the biological and biochemical effects of GOLF on human colon cancer cells in the attempt to give a mechanistic explanation to its promising clinical activity. Our data demonstrated that GEM was able to enhance the cytotoxic and the pro-apoptotic activity of the Oxali-FUFA multidrug combination in colon carcinoma cell lines in vitro. Moreover, GOLF combination was highly synergistic in inducing growth inhibition on two colon cancer cell lines as evaluated with a dedicated software derived form the median effect model by Chou and Talalay. These findings appear of major interest considering that the observed effects occurred at doses potentially achievable in cancer patients without the appearance of significant side effects.

Our findings suggest that GEM is able to enhance the anti-tumor activity of Oxali-FUFA by inducing a large amount of apoptosis in the target cells and very likely by enhancing the processes of programmed cell death individually activated by 5-FU and oxaliplatin. In this regard, recent studies in various models have shown that clinical response and patient survival are correlated with the occurrence of apoptosis during the first two cycles of chemotherapy.[62] In our experimental conditions, apoptosis occurring in GOLF-treated cells was paralleled by the activation of caspases 9 and 3 and by PARP cleavage (a conventional substrate of caspase 3) suggesting the involvement of a mitochondrial pathway in the generation of programmed cell death.[63] Interestingly, all the other combinations had no effects on caspase 9 activation and, moreover, ILF, GILF and OLF caused caspase 3 cleavage, but at a lower extent, suggesting the involvement of different pathways in the induction of apoptosis. On the other hand, apoptosis occurrence is determined by the balance between apoptotic and survival signallings.[64] Therefore, we have investigated on the activity and expression of some components of

critical survival pathways. We have found that GOLF decreased the expression of Raf-1 without affecting both the expression and activity of the upstream enzyme ras. This effect was paralleled by the reduction of the activity of Erk-1/2 whose expression was again unchanged. Therefore, the reduced activity of these pro-survival enzymes was likely due to the decreased expression and consequent activity of the upstream kinase Raf-1. Similarly, also the expression and activity of Akt was strongly reduced in GOLF-treated cells without changes of the upstream activator PI3K. All these effects were not or minimally induced by all the other combinations.

Raf-1 and Akt intracellular levels are both regulated by the balance between synthesis and degradation. Mechanisms that prevent the unfolding and the consequent degradation of intracellular proteins exist and they can induce the increase of the expression of these proteins independently from their synthesis. One of these mechanisms is regulated by HSPs that could be involved as molecular chaperones to rescue damaged proteins and subtracting them from the proteasome-dependent degrading pathway.[32-35] HSP90 is one of the most abundant cell chaperone proteins. It functions in a multi-component complex of chaperone proteins that may include p60/Hop, p50Cdc37, HSP40/HDJ2, p23, BAG-1, HSP70 and one of a variety immunophilins.[36] It forms the basis of a super-chaperone machine that promotes the proper folding of client proteins so that they can respond to a stimulus or bind ligand. However, the machine is in constant flux and cycles between two HSP90 conformations, determined by ATP or ADP binding, which in turn specify which set of cochaperones associate with the chaperone complex.[37] Cycling of this machine is driven by ATP hydrolysis. Although HSP90 is a weak ATPase, its activity is regulated by cochaperones and dramatically enhanced by client protein binding. The binding of HSP70 to the multi-chaperon complex favors the ATP hydrolysis and makes the client protein susceptible to ubiquitination and delivery to the proteasome (where it is degraded).[37] HSP90 has been specifically involved in the maintainance of the correct conformation of several intracellular proteins (named HSP90 clients) and much of them are kinases involved in the control of cell proliferation and survival, such as Raf-1 and Akt.[65] On the basis of these considerations, we have evaluated the effects of the different combinations on the expression of HSP27, 70 and 90. We have found that GOLF did not induce any significant change in the expression of both HSP90 and 27, but it caused an about 80% increase of HSP70 expression. All the other combinations caused again little effects on the expression of both HSP90 and 70. These results were paralleled by the increase of the ubiquitination of Raf-1 in GOLF-treated cells that was enhanced by the concomitant treatment of HT-29 with the specific proteasome inhibitor lactacystin. The effects of GILF and OLF on Raf-1 ubiquitination were poor and they slightly potentiated the effects of lactacystin. These data suggested that the decreased expression of Raf-1 in GOLF-treated cells could be due to the increase of its degradation via a proteasome-dependent pathway. The enhanced ubiquitination and consequent degradation of Raf-1 in cells exposed to GOLF could be explained on the basis of the increased expression of HSP70 that could determine the switch of the multi-chaperone complex in an inactive status. On the basis of these data, the use of specific inactivators of multi-chaperone complex in combination with chemotherapy can be hypothesized in order to potentiate the biological, biochemical and anti-cancer effects of GOLF regimen. In fact, phase I/II clinical trials with the specific multi-chaperone inhibitor 17-allylamino-17-demethoxygeldanamycin are now available on patients affected by several neoplasms and in one of these the analysis of pharmacodynamic markers in peripheral blood mononuclear cells (PBMCs), showed a reduction in the expression of Raf-1 between 24–48 h and HSP70 induction at 24–48 h[36,66] in agreement with our in vitro findings.

In conclusion, our results show that GEM in combination with OLF had synergistic effects on the induction of growth inhibition on two colon cancer cell lines. These effects, differently from those induced by the other combinations, were largely due to the activation of caspase 9 and 3-dependent apoptosis. Moreover, GOLF decreased the expression of both Raf-1 and Akt inhibiting the survival and anti-apoptotic activity of their downstream enzyme targets. Finally, the decreased expression of Raf-1 could be due to the inactivation of multi-chaperone complex and to its consequent increased degradation through a proteasome-dependent pathway.

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