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Tesi

Molecular pathways involved in the mechanisms of cytoprotection by aspirin in cancer cells.

Coordinatore del Corso **Candidato**

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UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

Dipartimento di Biologia e Patologia Molecolare e Cellulare "L. Califano"

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INTRODUCTION

Colorectal cancer is the third most common cancer in the world, and the second most common cause of cancer related death. A large body of evidence from epidemiological studies and clinical trials in patients with the hereditary colon cancer syndrome, familial adenomatosus polyposis coli (FAP), indicates that aspirin and related drugs, known as nonsteroideal antiinflammatory drugs (NSAIDs), share the property of inhibiting the ciclooxygenase (COX) enzyme, hinder the development of colon cancer and perhaps other cancer as well. COX is the rate limiting enzyme for the synthesis of eicosanoids, such as prostaglandins, from arachidonic acid. Two Cox isoforms have been identified: COX-1 constituvely expressed and COX-2, inducible form. COX-1 gene is an housekeeping gene and has an important role in protecting the gastroduodenal mucosa. COX-2 gene, an immediate early response gene, is rapidly induced in response to tumour promotes, cytokines, and growth factors (Du Bois et al. 1994, Di Popolo et al. 2000). COX-2, but not COX-1 expression was found to be increased in colorectal cancer (Eberhart et al. 1994). Numerous studies have shown that the chemopreventive effect of NSAIDs on colon cancer is mediated through inhibition of cell growth and induction of apoptosis. For these effects NSAIDs have been empolyed as sensiting agents in chemotherapy.

Aspirin, a widely used non-steroidal anti-inflammatory drug is a non-selective inhibitor of cyclo-oxigenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) (Taketo.1998). High doses of salicylates (4 to 10 g per day), including sodium salicylate and aspirin, have been used to treat inflammatory conditions such as rheumatic fever and rheumatoid arthritis. There are several actions displayed by high doses of aspirin independent from its ability to inhibit cyclooxygenases. These includes: i)inhibition of the activation of nuclear factor-kappaB (NF-kB) by prevention the phosphorylation and degradation of the inhibitory subunit Ik□(Yin et al. 1998) **;** ii) modulation of gene transcription (XU et al. 1999)**;** iii) modulation of several

protein kinases and other molecular signaling pathways as well (Pillinger et al. 1998, Dong et al. 1997). Several effects have been demostrated *in vitro*: aspirin treatment inhibits cell growth (Ricchi et al. 1997, Redondo et al. 2003) and protects against a variety of toxic stimuli such as H_2O_2, free radicals, hypoxia and chemical toxic (Grilli et al.1996, Mattie et al. 2001). In a previous work we have shown that aspirin treatment may affect the proliferation, differentiation and apoptosis of the human colon adenocarcinoma Caco-2 cells; in particular, we observed that, depending on the doses, aspirin treatment may induce apoptosis and a significant DNA synthesis inhibition associated with a modification in the level of the insulin-like growth factor II (IGF-II) (Ricchi et al, 1997), an autocrine growth factor for this cell line (Zarrilli et al, 1994; Zarrilli et al 1996).In addition, we have demonstrated that NS-398, a Cox-2 selective inhibitor, also affects Caco-2 DNA synthesis and apoptosis (Di Popolo et al, 2000).

Previously we have shown (Di Popolo et al.; 2000) that COX2 expression and PGE_2 synthesis are up-regulated by an IGFII/IGFI receptor autocrine pathway in CaCo2 colon carcinoma cells. Up-regulation of COX2 expression by IGFII is mediated through activation of IGFI receptor. The blocked of the PI3kinase, that mediates the proliferative effect of IGFI receptor in CaCo2 cells, inhibits IGFII dependent COX2 upregulation and PGE_2 synthesis. Then, the induction of proliferation and tumor progression of colon cancer cells by the IGFII/IGFII receptor pathway may depend on the activation of COX2 related events.We analyzed,also, if the inhibition of proliferation and apoptosis by NS398 and by aspirin was prostaglandins dependent In these experimental conditions aspirin is able to inhibit cell proliferation, to decrease the levels of IGFII, but these effects are not mediated by Cox2 inhibition, on the contrary the inhibition by NS398 was reverted by exogenous addition of PGE2.

Furthermore, several of the observed effects of NSAIDs in vitro are cell specific and not dependent from the inhibition of prostaglandin biosynthesis and probably not relevant for prevention in clinical trials (Aas et al. 1995). In fact, a series of studies have evaluated “in vitro” the possibility to increase anticancer drugs-induced toxicity and apoptosis through the use of older NSAIDs and new coxibs (Sairam et al. 2003 Mattie et al. 2001). But the role of NSAIDs as anticancer agent alone and in combination with conventional chemotherapeutic is becoming promising also (in vivo) in murine and rat model of cancer (Hundal et al. 2002, Vartiainen et al. 2003). In particular, several studies have been focusing on the increase in activity and in the changing of toxicity profile by combining a conventional anticancer agent with COX-2 inhibitors (Ricchi et al. 2002, Srivastava et al. 1986). Thus during these four years of my Phd program the first aim of project has been focused to demostrate:

1)the effect of aspirin on cell cycle and apoptosis alone and in combination with conventional anticancer drugs schedules.

2)the molecular mechanisms responsible for the effect of aspirin on cell cycle and survival

3)the occurrence of this molecular mechanism and this effect of aspirin also in vivo system.

RESULTS

The effect of aspirin on cell cycle and apoptosis alone and in combination with conventional anticancer drugs schedules

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We have previously shown that Caco-2 cells synthesize and secrete IGF-II during their replicative phase when this peptide stimulates cell growth by an autocrine loop (Zarrilli *et al.,* 1994). When cells reach confluence and stop growing, the expression of IGF-II is strongly reduced and Caco-2 cells start to differentiate (Pignata *et al.,* 1994; Zarrilli *et al.,* 1994). Constitutive IGF-II expression obtained through transfection of Caco-2 cells with rat IGF-II cDNA under the control of a viral promoter persistently stimulates cell growth and interferes with the enterocyte-like differentiation of this cell line (Zarrilli *et al.,* 1996). To clarifyfurther the mechanisms involved in the inhibition of Caco-2 cell replication induced by aspirin, we investigated the modifications in IGF-II mRNA expression following drug treatment and found that the effect of aspirin on cell growth was associated with a dose-dependent inhibition of IGF-II. This finding suggests that a decrease in IGF-II synthesis might be involved in aspirin-induce inhibition of cell growth, even though the parallel decrease in IGF-II mRNA expression and cell proliferation does not establish cause and effect. Accumulating evidence suggest that interference with cell cycle and/or with the intracellular growth factor (receptor)-activate signal transduction pathways are key modulators of cellular response to chemotherapeutic agents (Donaldson et al, 1994; Ciardiello et al, 1996; Tortora et al, 1997; Chen et al, 1997; De Luca et al, 1997; Lin et al, 1998). Because aspirin and NS-398 are able to inhibit cell growth, I asked whether they might modify sensitivity to anticancer drugs. Because cell killing induced by the topoisomerase poisons irinotecan (CPT 11) and etoposide (VP-16) is strictly cell cycle dependent (Nitiss and Beck, 1996; Goldwasser et al, 1995) and ultimately activates a pathway of programmed cell death, I decided to use these anticancer drugs to evaluate this hypothesis. Irinotecan (CPT 11) was selected also because it is one of the most active anticancer agent in colon cancer (Rougier et al, 1998).

Our previous data indicated that a 24 h aspirin treatment on Caco-2 cells at doses ranging from 1 to 10 mM determined a dose-dependent inhibition of DNA synthesis and a significant increase in levels of apoptosis (Ricchi et al, 1997). In the present work, I confirmed these results on cell growth for a 72 h treatment (data not shown) and evaluated again the effects on apoptosis by analysing the percentage of sub-G1 population at flow cytometry. Treatment with aspirin for 72 h, caused a dose-dependent increase in apoptosis starting from the concentration of 5 mM aspirin; the percentage of apoptotic cells was approximately 6% in control cells and 5, 9 and 12% in cells treated with aspirin at 2, 5 and 10 mM, respectively (data not shown).The above data suggest that aspirin acts in Caco-2 cells as anticancer agent with cytostatic properties and ability to induce apoptosis at high dosages. Therefore I decided to select these antiproliferative, apoptotic dosages of drugs to study whether they could modify Caco-2 sensitivity to anticancer drug treatments. To determine the effect of anticancer drugs on apoptosis, Caco-2 cells were exposed for 48 h at different concentrations of VP-16 and CPT 11 and DNA ploidy was analysed by flow cytometry. The effects on apoptosis in Caco-2 cells are shown in Figure 1. Both Vp-16 and CPT 11 dose-dependently increased the levels of apoptosis in Caco-2 cells: Vp-16 increased apoptosis from 8% at 4.25 mM to 20% at 34 mM (Figure 1A) and CPT 11 increased apoptosis from 9% at 5 mM to 16% at 15 mM (Figure 1B).

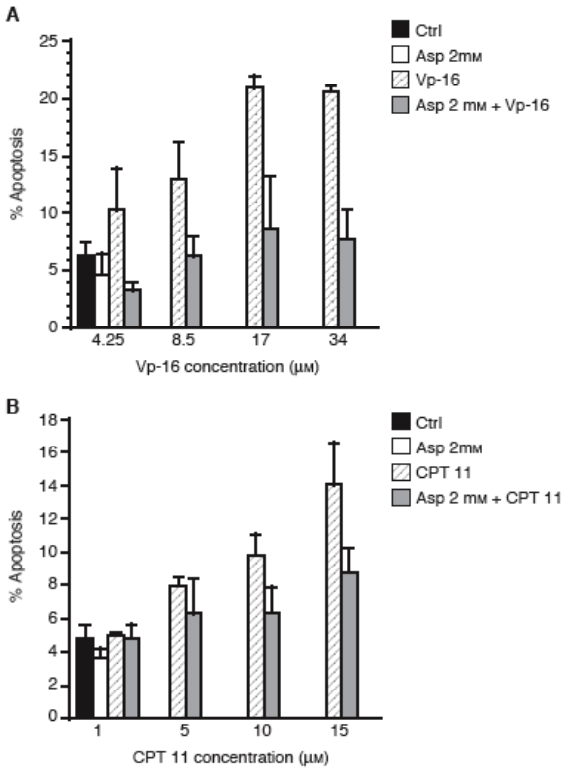

Figure 1 Effect of 2 mM aspirin co-treatment on VP-16-(**A**) and CPT 11(**B**)-induced apoptosis in Caco-2 cells. Cells at day 3 were incubated with aspirin for 24 h, then anticancer drugs at the indicated concentrations were added in presence of 2 mM aspirin for 48 h. Apoptosis was calculated as the percentage of cells showing a sub-diploid DNA peak as described in Materials and Methods. Data are expressed as mean ± s.d.

These data suggest that apoptosis may have a role in drug-induced Caco-2 cells cytotoxicity especially at high concentrations of the drugs. To evaluate whether aspirin may affect cell

killing by topoisomerase drugs, I tested a schedule of administration in which the effects of aspirin treatment on Caco-2 cell growth were present at moment of anticancer-drug exposure: cells were pre-treated with aspirin for 24 h and subsequently exposed for 48 h to the afore mentioned ranges of drugs in the continuous presence of NSAIDs (co-treatment). I evaluated the interference of 2 mM aspirin with anticancer drug induced apoptosis through the analysis of the sub-diploid DNA peak at flow cytometry. As shown in Figure 1, the levels of apoptosis were reduced for each concentration of both anticancer drugs when administered in the presence of aspirin compared with single agent treatment. Aspirin-co-treated Caco-2 cells were particularly resistant to etoposide-induced apoptosis: in fact the percentage of apoptosis in cells co-treated with aspirin and Vp-16 at 8.5 and 17 mM decreased from 14 to 7% and from 21 to 9%, respectively. The effect of aspirin on drug induced apoptosis was similar when aspirin and anticancer drugs were simultaneously administered (data not shown). These results indicate that aspirin-dependent inhibition of proliferation is associated with a reduced ability of the drug to induce apoptosis. To quantify the effects of aspirin co-treatment on Vp-16- induced apoptosis and to determine whether this effect was present at concentration in which aspirin was already apoptotic by itself, I used annexin V-FITC/propidium iodide staining assay. In fact this assay allows to clearly distinguish viable cells from those undergoing different stages of apoptosis or necrosis. The effect of aspirin at 2 and 5 mM concentration on Vp-16-induced toxicity in Caco-2 cells is analysed in Figure 2.

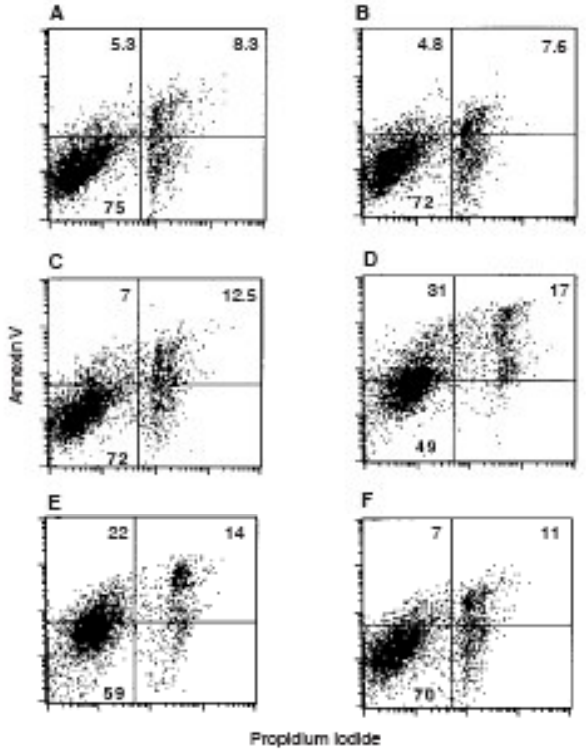

Figure 2 Effect of aspirin co-treatment on annexin V-FITC/PI staining of Caco-2 cells. Cells at day 3 were incubated with aspirin for 24 h, then anticancer drugs were added in presence of aspirin for 48 h. Four distinct phenotypes become distinguishable: (i) viable cells (lower left quadrant); (ii) cells at early stage of apoptosis (upper left quadrant); (iii) cells at late stage of apoptosis (upper right quadrant); (iii) necrotic cells (lower right quadrant). (**A**) untreated cells; (**B**) cells treated with 2 mM Aspirin; (**C**) cells treated with 5 mM Aspirin; (**D**) cell treated with 17 μM Vp-16; (**E**) cells co-treated with 2 mM aspirin and 17 μM Vp-16; (**F**) cells co-treated with 5 mM aspirin and 17 μM Vp-16. Data represent one of three similar experiments.

Dot plots of green (Annexin V-FITC) vs red (PI) fluorescence showed four separate clusters: viable cell (lower left quadrant), cells at early stage of apoptosis, (upper left quadrant), cell at late stage of apoptosis (upper right quadrant), necrotic cells (lower right quadrant). Because of the high sensitiveness of this procedure, we detected higher levels of apoptosis as compared to that obtained at the analysis of subdiploid peak. Vp-16 at 17 mM increased the percentage of early and late apoptotic cells from 13 to 48% and decreased the percentage of viable cell from 75 to 49% (Figure 2A,D). Aspirin cotreatment at 2 and 5 mM dose-dependently decreased the percentage of early and apoptotic cells from 48 to 36 and 18%, respectively, (Figure 2D vs E,F) and increased the percentage of viable cells from 49 to 59 and 70% (Figure 2D vs E,F); similar data were obtained for Vp-16 at 8.5 mM (data not shown) and CPT 11at 10 mM (Figure 3). Not significant change in the levels of necrosis was observed.

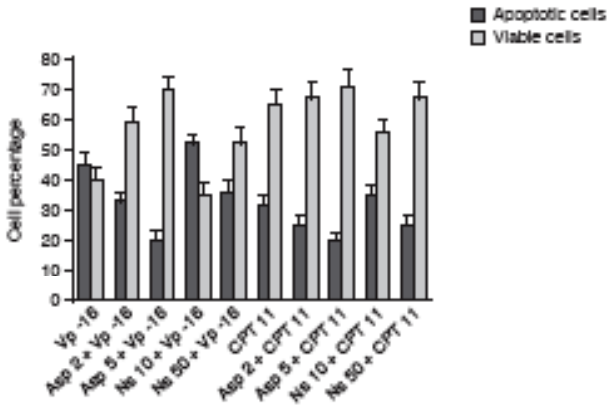

Figure 3 Effect of aspirin and NS-398 co-treatment on 17 μM Vp-16- and 10 μM CPT 11- induced apoptosis of Caco-2 cells. The percentage of viable and apoptotic cells were calculated as reported in Figure 2. Apoptotic bars are the sum of percentage of cells at early and late stages of apoptosis. Data are expressed as mean ± s.d. Data points represent the mean of triplicate experiments.

I also evaluated whether the effects of aspirin co-treatment on anticancer drug responsiveness were unique of Caco-2 cells or were obtainable in other colon cancer cells showing different Cox
isoform expression profile. I selected the colon cancer cell line SW480 being Cox-1 positive, but Cox-2 negative (Smith et al, 2000). SW480 cells were co-treated with aspirin and topoisomerase
inhibitors under the above mentioned experimental conditions.Aspirin co-treatment at 2 and 5 mM decreased both CPT 11- and VP-16-induced apoptosis. The effect was dose-dependent and
in the same range of magnitude of that obtained in Caco-2 cells (data not shown).
Data from our laboratory indicated that Caco-2 cells express Cox-2 but not Cox-1 and NS-398, a COX-2 selective inhibitor, inhibited DNA synthesis and induced apoptosis in Caco-2 cells (Di Popolo et al, 2000). To evaluate whether the effects of aspirin co-treatment on Caco-2 cells were dependent on the inhibition of Cox-2 activity, I analysed the effect of NS-398 co-treatment on anticancer drug induced apoptosis. I firstly evaluated the effect on apoptosis of NS-398 72 h treatment at different concentration using annexin V-FITC/propidium iodide staining assay. As shown in Figure 4, 10 and 50 mM NS-398 treatment induced higher level of apoptosis compared with 2 and 5 mM aspirin treatments in Caco-2 cells (22 and 25% compared with 12 and 20%, respectively).

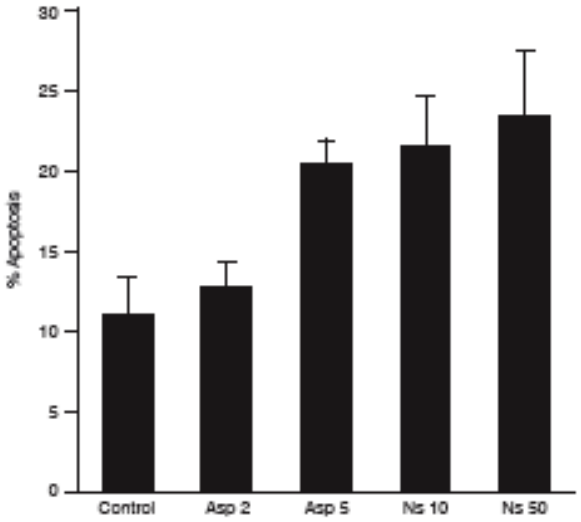

Figure 4 Effect of 2 and 5 mM aspirin and 10 and 50 μM NS-398 72 h treatment on apoptosis of Caco-2 cells. The percentage of Caco-2 cells at early and late stages of apoptosis was quantified as reported in figure 2. Data points represent the mean of triplicate experiments ± sd.

Then I evaluated the effect of NS-398 co-treatment on Vp-16- and CPT 11-induced toxicity. The percentage of cells in early plus late apoptosis vs viable cells induced by co-treatments with aspirin or NS-398 and Vp-16 or CPT 11 are given in Figure 3.

Aspirin at 2 and 5 mM dose-dependently decreased both Vp16- and CPT 11-induced apoptosis and increased cell viability. NS 398 at 10 mM increased both 17 mM Vp-16- and 10 mM CPT 11-induced apoptosis (P50.05 and P40.05, respectively), while NS 398 at 50 mM decreased 17 mM Vp-16- and 10 mM CPT 11-dependent apoptosis (P40.05 and P50.05, respectively). NS 398 at 10 mM did not significantly alter the percentage of viable cells following treatment with Vp-16- or CPT 11 co-treated cells, while NS 398 at 50 mM increased cell viability of Vp-16-treated cells from 40 to 52% (P50.05). No effects were obtained on anticancer drug induced-apoptosis with NS 398 at 1 mM (data not shown). I examined whether aspirin and NS-398 treatments had any distinct effect on cell cycle parameter under the same experimental conditions. The distribution of cells in the phases of the cell cycle is presented in Table 1.

Table 1 Cell cycle distribution (per cent) of control and Aspirin- or NS 398-treated Caco-2 cells

Treatment	G0-G1	S	G2-M
Control	58	33	9
Asp 2 mM	51	46	3
Asp 5 mM	50	48	2
NS 398 10 μM	62	29	9
NS 398 50 μM	64	29	7

DNA cell cycle analysis was performed by propidium iodide labelling as described in Material and Methods.

Aspirin treatment was associated with a modest dose-dependent increase in the proportion of cells in the S phase, while NS-398 did not significantly alter cell cycle parameters with respect to control cells. I also asked whether the different effect of aspirin and NS-398 on survival could be determined by a selective interference with anticancer drug-induced cell

cycle arrest. In fact, Vp-16 arrests cells in the pre-mitotic phase of the cell cycle leading to accumulation of the cells in the late S or G2 phase (Fearnhead et al, 1994; Downes et al, 1994); while CPT 11 causes S phase slowing (Shao et al, 1997; McDonald and Brown, 1998). Effect of Vp-16 and CPT 11 treated, Vp-16 and CPT 11 plus aspirin or plus NS-398 co-treated cells under previously described experimental conditions are shown in Table 2.

Table 2 Cell cycle distribution (per cent) of Caco-2 cells after anticancer drug treatments and anticancer drug plus aspirin or NS-398 co-treatments

Treatment	G0-G1	S	G2-M
Control	58	33	9
Vp-16 17 μM	9	0	91
Asp 2 mM+Vp-16 17 μM	15	0	85
Asp 5 mM+Vp-16 17 μM	37	0	63
NS 10 μM+Vp-16 17 μM	8	0	92
NS 50 μM+Vp-16 17 μM	15	0	85
CPT 11 10 μM	22	70	8
Asp 2 mM+CPT 11 10 μM	22	78	0
Asp 5 mM+CPT 11 10 μM	35	65	0
NS 10 μM+CPT 11 10 μM	15	85	0
NS 50 μM+CPT 11 10 μM	32	68	0

DNA cell cycle analysis was performed by propidium iodide labelling as described in Materials and Methods.

I found that Vp-16, from lowest concentration of drug tested, increased the distribution of Caco-2 cells at the G2 phase of the cell cycle. In particular, VP-16 at 8.5 mM and 17 mM treatments were able to trap 85 and 91% of Caco-2 cells in the G2 phase of cell cycle, respectively. Whereas CPT 11 at 10 and 15 mM treatments accumulated 70 and 85% of Caco-2 cells in the S phase of cell cycle, respectively (Table 2 and data not shown). Aspirin and NS-398 co-treatment
had different effect on anticancer drug induced cell cycle distribution: in cells that have been co-treated with Vp-16 at 17 mM, aspirin dose-dependently increased the percentage of Caco-2 cells at G0 –G1 phase of cell cycle. NS-398 at 10 mM had no effect on Vp-16-induced accumulation at the G2 phase of cell cycle, while at 50 mM increased the percentage of cells at G0 –G1 phase from 9 to 15%. In cells that have been co-treated with aspirin and CPT 11 at 10 mM there was an increase in the percentage of cells at G0 –G1 phase similar to that observed in Vp-16 co-treated cells. NS-398 only at 50 mM concentration increased the percentage of cells at G0 –G1 phase from 22 to 32% (Table 2).
Several reports indicate that cancer cells after a genotoxic treatment can take hours to many days before dying (Vidair et al, 1996; Han et al, 1997). To better evaluate the cytoprotective effect of aspirin, also with respect to potential delayed toxicity of Vp-16, we measured overall cell viability by performing a plating efficiency assay following 8.5 mM and 17 mM etoposide treatments and cotreatments with either 2 or 5 mM aspirin (Figure 5A,B). All replated control Caco-2 cells started to divide after a lag period of 48h (Figure 5A); they entered into the exponential phase of cell growth at day 4, and reached the stationary phase at day 14 (data not shown). Etoposide treatments at both concentrations produced an approximately 0.7-fold decrease of the cell number at day 4 after replating; the fraction of surviving cells entered into the logarithmic phase of cell growth only at day 8. On the contrary, aspirin co-treated cells more efficiently replated and started the exponential phase of cell growth at day 6, 5 mM aspirin co-treated cells showing the best profile of cell growth. Thus, aspirin co-treated cells appeared to be more viable and less sensitive to anticancer delayed toxicity. Etoposide at 34 mM was such a toxic treatment that cell restarted to divide only at day 11; nevertheless, cells cotreated with aspirin and etoposide also at this concentration, continued to show an advantage in cell growth (data not shown).

The molecular mechanisms responsible for the effect of aspirin on cell cycle and survival

From previous studies I demonstrated that aspirin treatment at millimolar concentration significantly prevented apoptosis and G2/M phase of cell cycle accumulation induced by the topoisomerase inhibitors etoposide and irinotecan (Ricchi et al., 2002). These effects were observed both in Caco-2 cells and in SW 480 cells independently from their COX's profile of expression, thus suggesting that aspirin acted through a COX-independent mechanism (Ricchi et al., 2002).In the second year of my PHD program I try to investigate the molecular mechanisms Cox2 indipendent responsible for the effect of aspirin on cell cycle and survival.To valuate the molecular pathway by aspirin induced , I analyzed the citoprotection of aspirin on apoptosis by serum deprivation. The effect on apoptosis was first evaluated by analyzing the percentage of sub-G1 population at flow cytometry. Spontaneous apoptosis of Caco-2 cells at day 4 of culture in complete culture medium was in the average 6% (data not shown); the percentage of apoptotic cells was approximately 8, 20, and 30 after serum deprivation for 48, 96, and 168 h, respectively (Fig. 5).

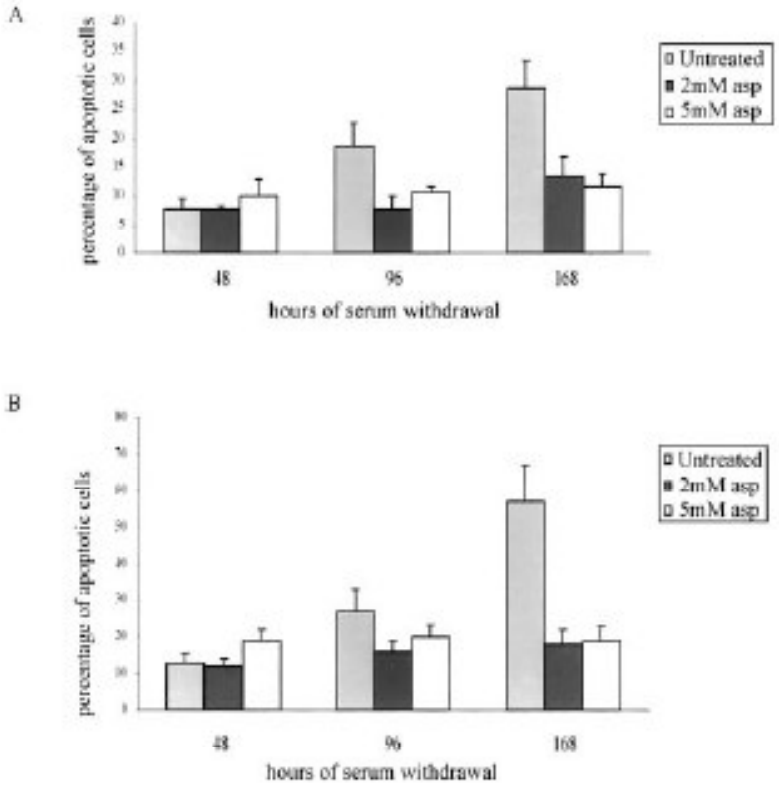

Fig. 5. Effect of 2 and 5 mM aspirin treatment on apoptosis induced by serum deprivation in Caco-2 cells. After 72 h of culture in complete medium, cells were serum deprived in the absence or presence of aspirin. After 48, 96, and 168 h of serum withdrawal, apoptosis was calculated as the percentage of cells showing a subdiploid DNA peak (A) and as the percentage of cells positive at annexin V-FITC staining (B), as described under *Materials and Methods*. Data are expressed as mean ± S.D.

The effect of 2 and 5 mM aspirin treatment on serum withdrawal induced apoptosis was first evaluated through the analysis of the subdiploid DNA peak at flow cytometry. Aspirin inhibited apoptosis in a statistically significant manner (p 0.05) at all time points tested compared with untreated cells (Fig.5A). Aspirin (2 mM) treatment caused a 60% reduction in

apoptosis compared with untreated cells with the maximal effect at 96 h of serum deprivation. The percentage of apoptosis increased up to 11% after treatment with 5 mM aspirin but was unmodified during the starvation time. Similar results were obtained when the percentage of apoptotic cells was evaluated by annexin staining (Fig. 5B); however, because of the high sensitiveness of this procedure, higher levels of apoptosis were observed in untreated cells compared with those obtained at the analysis of subdiploid peak (Fig.5A).We also analyzed in the same experimental conditions the distribution of cells in the phases of the cell cycle. We did not find any relevant modification in cell cycle distribution between starved Caco-2 cells and cells cultured in complete medium in the presence or absence of aspirin for 48 h (data not shown). On the contrary, prolonged serum deprivation caused a time-dependent decrease in the proportion of cells in the G1 phase of cell cycle and increase in the proportion of cells in the G2/M phase of cell cycle (Fig.6).

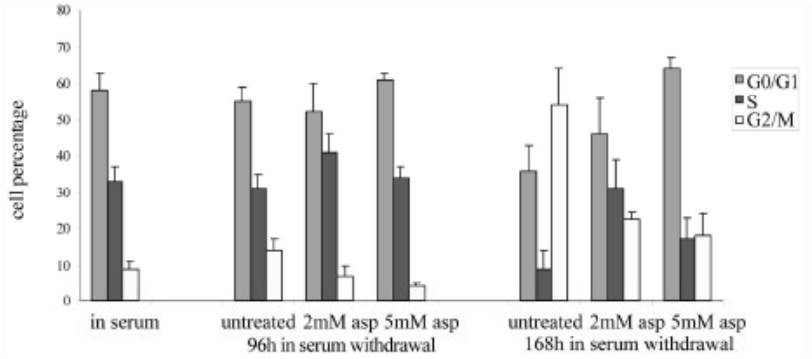

Fig. 6 Effect of 2 and 5 mM aspirin treatment on cell cycle distribution observed after Caco-2 cells serum withdrawal. After 72 h of culture in complete medium, cells were serum deprived in the absence or presence of aspirin for 96 and 168 h. DNA cell cycle analysis was performed by propidium iodide labeling as described under *Materials and Methods*. A data point representative for Caco-2 cells grown in complete medium for 168 h is also shown. Data points represent the mean of triplicate experiments ± S.D.

Aspirin (5 mM) treatment and to a lesser extent 2 mM aspirin treatment counteracted the G1 decrease and G2/M increase in cell cycle distribution induced by prolonged serum starvation leading to a profile of cell cycle similar to that of cells cultured in serum for 168 h (Fig. 6, data point "in serum" versus 96- and 168-h serum withdrawal). Data from our laboratory indicated that Caco-2 cells expressed COX-2 but not COX-1 (Di Popolo et al., 2000). To evaluate whether the effects of aspirin treatment on Caco-2 cells cycle and survival were dependent on the inhibition of COX-2 activity, I analyzed the effect of NS-398 treatment, a COX-2-selective inhibitor, on survival during serum deprivation. To address this issue, cells were starved in the presence of 10 m NS-398, a concentration that completely inhibits the biosynthesis of prostaglandin E2 in Caco-2 cells. NS-398 treatment at this concentration did not cause any significant modifications on apoptosis and cell cycle parameters with respect to starved cells (data not shown). These results, therefore, suggested that aspirin interfered with apoptosis and cell cycle modification induced by serum withdrawal in Caco-2 cells in a COX-independent manner. We have previously shown that an autocrine IGF-II/IGF-Ir pathway sustains cells proliferation and survival of Caco-2 colon cancer cells (Zarrilli et al., 1994, 1996). In particular, we and others have demonstrated that the PI3-kinase pathway preferentially, with respect to the MAP kinase pathway, delivered an antiapoptotic and proliferative signal in Caco-2 cells (Di Popolo et al., 2000; Gauthier et al., 2001). Thus, it was of interest to evaluate whether aspirin treatment had any effect on MAP kinase and PI3-kinase pathways during Caco-2 serum deprivation.

To address these questions, I focused on the activation status of ERK1 and ERK2, two major components of the MAP kinases cascade, and of the AKT protein that is a downstream effector of the PI3-kinase. Lysates from cells serum deprived for 96 h in the presence or absence of 2 and 5 mM aspirin were probed for phosphorylated AKT at Ser 473 and phosporylated ERK1/2 kinases. To evaluate the PI3-kinase/ AKT-dependent pathway in serum cultured cells and to compare the effects of aspirin in both culture conditions, lysates of

cells cultured in complete medium and treated for 96 h with 2 and 5 mM aspirin were also collected. The bands were quantified and normalized to total AKT and ERK2 protein kinases, respectively. As shown in Fig.7, aspirin treatment dose dependently induced activation of AKT protein and ERK1/2 protein with respect to untreated cells.

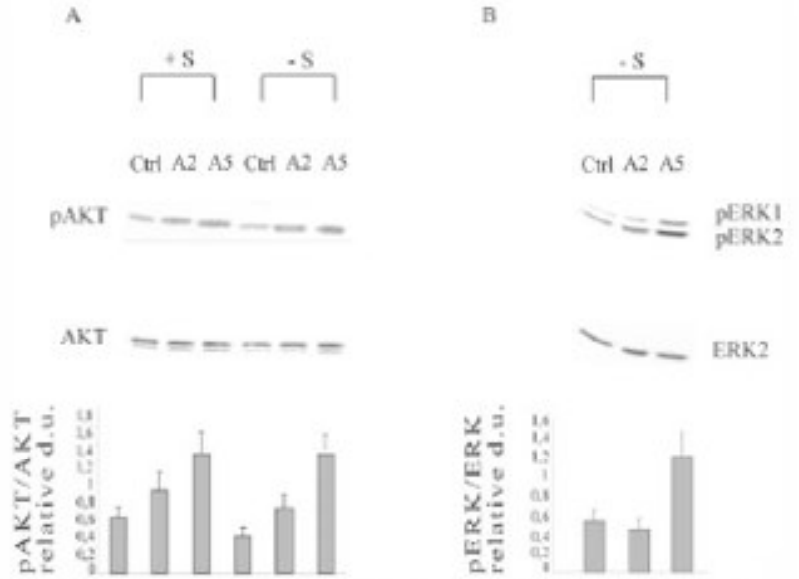

Fig. 7 Effect of 2 and 5 mM aspirin treatment on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from cells cultured in the presence (+S) and in absence (−S) of serum for 96 h (ctrl), after treatment with 2 mM (A2) or 5 mM aspirin for 96 h (A5). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 protein. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

The relative densitometric analysis showed 1.5- and 2.7-fold increase for AKT protein phosphorylation status after aspirin treatment at 2 and 5 mM, respectively (Fig. 7A) and 2.7-fold increase for ERK1/2 protein phosphorylation status after aspirin treatment at 5 mM in serum-deprived cells (Fig. 7B). On the other hand, serum deprivation slightly reduced the activation status of Akt with respect to serum-cultured cells (Fig. 7A), but aspirin treatment, although to a lesser extent than in serum withdrawal, still induced phosphorylation of Akt (Fig. 7A).

To directly demonstrate the activation of PI3-kinase by aspirin treatment in serum-starved cells, we analyzed the PI3-kinase activity in the pTyr immunoprecipitate from 96-h serum-deprived Caco-2 cells in the absence (control) or presence of 2 and 5 mM aspirin. As shown in Fig. 8aspirin treatment dose dependently increased the levels of three phosphate phosphatidylinositols.

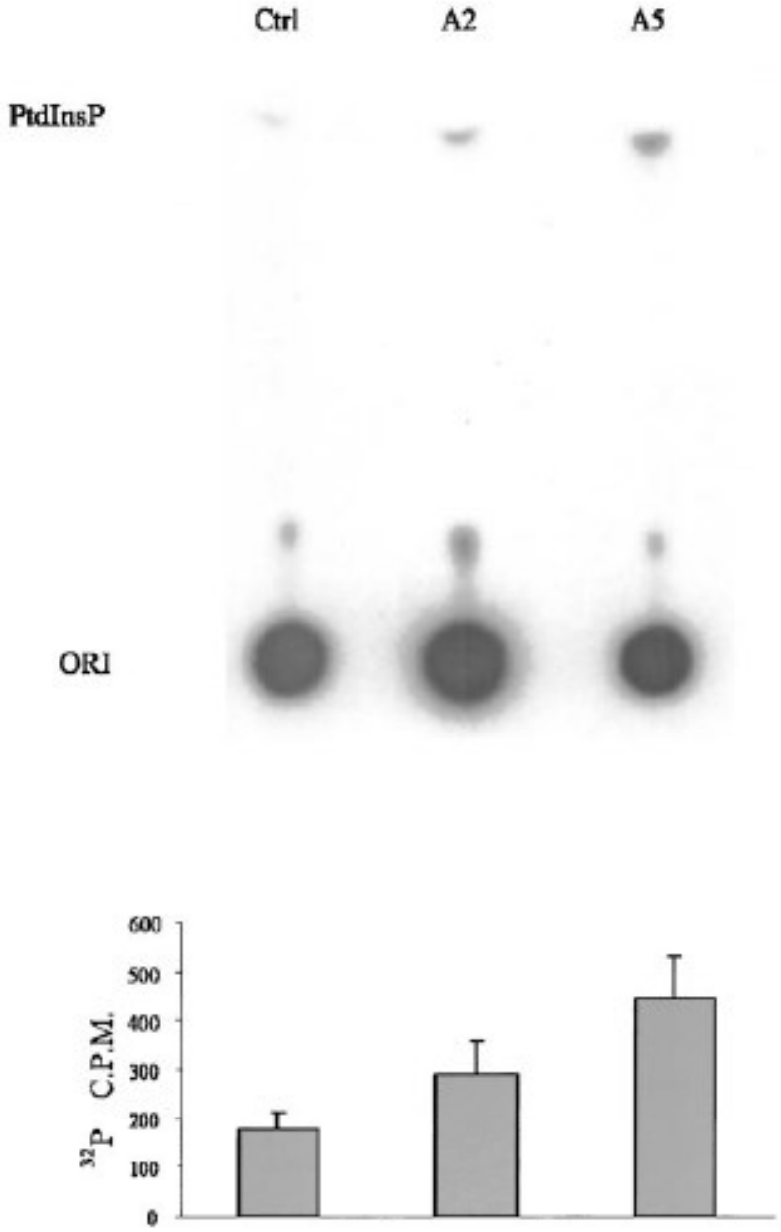

Fig. 8 Effect of 2 and 5 mM aspirin treatment on PI3-kinase activity in 96-h serum-starved cells. Cells extracts were immunoprecipitated with a pTyr monoclonal antibody, and the precipitate assayed for the presence of a PI3-kinase activity using phosphatidylinositol as a substrate as described under *Materials and Methods*. TLC analysis of the PI3-kinase reaction shown corresponds to a representative experiment that was repeated two additional times. PtdInsP, phosphatidylinositol 3-phosphate; ORI, origin. Histograms with S.D. bars represent the counts per minute of [^{32}P]phosphoinositol 3-phosphate of the three experiments.

Because the molecular effects of aspirin have been observed after prolonged exposure to drug, we asked whether these effects could be related to a direct activation of PI3- kinase by aspirin. To address this issue, we evaluated the effect on AKT and ERK phosphorylation status after aspirin acute stimulation. After 24 h of serum deprivation, Caco-2 cells were

treated with aspirin at 5 mM and cell lysates were collected after 5, 10, 15, and 30 min of drug addition. As shown in Fig.9, 5 mM aspirin treatment transiently inducedactivation of AKT and ERK1/2 proteins with respect to serum- deprived cells after 10 min of exposure.

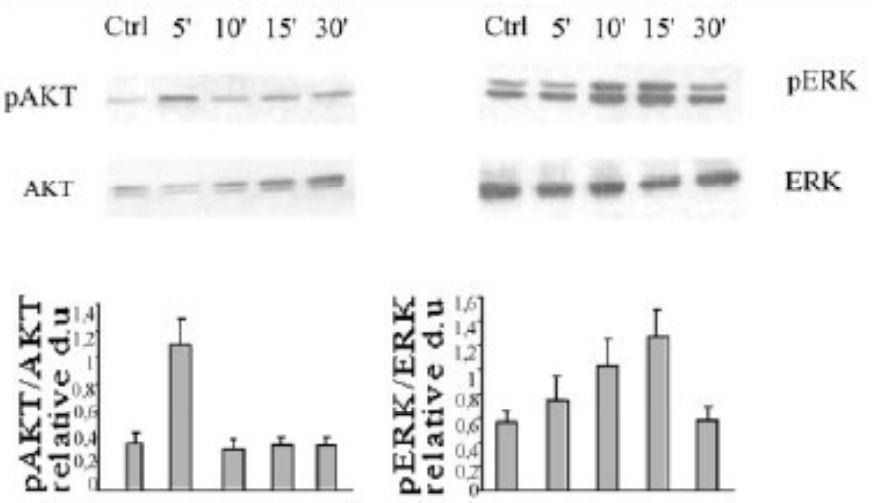

Fig. 9 Effect of 5 mM aspirin treatment on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from cells cultured in the absence of serum for 24 h (ctrl) and from cell treated with 5 mM aspirin for 5 min (5′), 10 min (10′), 15 min (15′), or 30 min (30′). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 protein. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

To further evaluate the relative involvement of ERK1/2 and PI3-kinase pathways in Caco-2 cells survival, we analyzed also the effects, during serum deprivation, of PD98059 or LY294002 inhibitor treatments alone and in combination with 2 and 5 mM aspirin (Fig. 10, A and B; Table 1).

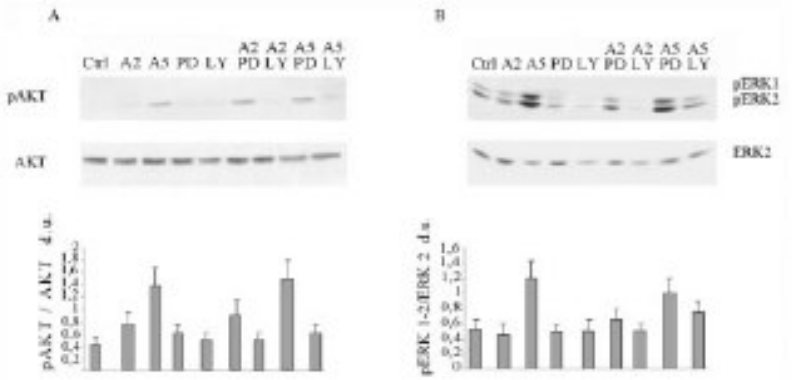

Fig. 10 Effect of PD98059 and LY294002 treatments alone and in combination with aspirin on (Ser 437) phosphorylated AKT and total AKT (A) and phosphorylated ERK1/2 and total ERK2 (B) expression in Caco-2 cells. Western immunoblot analysis was performed on protein lysates from control cells cultured in the absence of serum for 96 h (Ctrl), from cells treated with 2 mM aspirin (A2), 5 mM aspirin (A5), 40 μM PD98059 (PD), 50 μM LY294002 (LY), or from cells cotreated with 2 mM aspirin and 40 μM PD98059 (A2PD), 2 mM aspirin and 50 μM LY294002 (A2LY), 5 mM aspirin and 40 μM PD98059 (A5PD), or 5 mM aspirin and 50 μM LY294002 (A5LY). The immunoblots were stripped and reblotted with antibodies against total AKT or ERK2 proteins. The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

TABLE 3

Effect of PD98059 and LY294002 treatments, alone or in combination with aspirin, for 96 h on apoptosis during Caco-2 cell starvation

Apoptosis was calculated as the percentage of cells showing a subdiploid DNA peak as described under *Materials and Methods*. Data are expressed as mean ± S.D.

Treatment	Aspirin		
	0	2 mM	5 mM
		%	
No Serum	19±4	8±2	11±2
PD98059	8±2	7±3	10±3
LY294002	35±5	40±5	45±5

Treatment with the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 alone did not cause any relevant change in activation status of AKT protein, slightly decreased ERK1/2 phosphorylation, but reduced apoptosis with respect to control cells (Fig. 10 Table 1). The aspirin induced activation of AKT persisted in the presence of PD98059 (Fig. 10 A) but PD98059 did not counteract 5 mM aspirin-induced activation of ERK1/2 kinases (Fig. 10 B), thus suggesting that ERK1/2 kinases were activated by aspirin independently from MAP kinases. On the contrary, the PI3- kinase inhibitor LY294002 alone reduced basal activation of AKT (Fig. 10A) and further increased apoptosis from 19 to 35% with respect to control cells (Table 3). Furthermore, LY294002 in cotreatment with aspirin completely reduced AKT activation (Fig. 10 A) and partially reduced ERK1/2 activation induced by aspirin (Fig. 10 B), whereas increased apoptosis levels up to 40 and 45% in the presence of aspirin at 2 and 5 mM, respectively (Table 3). These data suggest that in Caco-2 cells ERK1/2 kinase pathway could be, at least in part, under PI3-kinase pathway control. Similar data were obtained in aspirin-treated cells for 168h (data not shown). Because treatments with aspirin, through the activation of AKT protein via PI3-kinase pathway stimulation, and with PD98059, by decreasing MAP kinase activity, both reduce apoptosis, the above-mentioned findings support the hypothesis that PI3-kinase inhibition and MAP kinase activation are involved in apoptotic

response to serum deprivation in Caco-2 cells. I have previously shown that in Caco-2 cells cotreated with aspirin and topoisomerase inhibitors, apoptosis and overall toxicity induced by topoisomerase inhibitors were reduced (Ricchi et al., 2002). To further correlate this cytoprotective effect of aspirin with the activation status of AKT, I assayed AKT activation status after 17 M Vp-16 and 5 mM aspirin cotreatment, an experimental condition where maximal effect on overall viability have been observed (Ricchi et al., 2002). As shown in Fig. 11, phosphorylation status o AKT protein was 2-fold increased both in cells treated with 5 mM aspirin and cells cotreated with aspirin at 5 mM and Vp-16, compared with untreated cells, whereas phosphorylation status of AKT protein was unmodified after Vp-16 treatment compared with untreated cells.

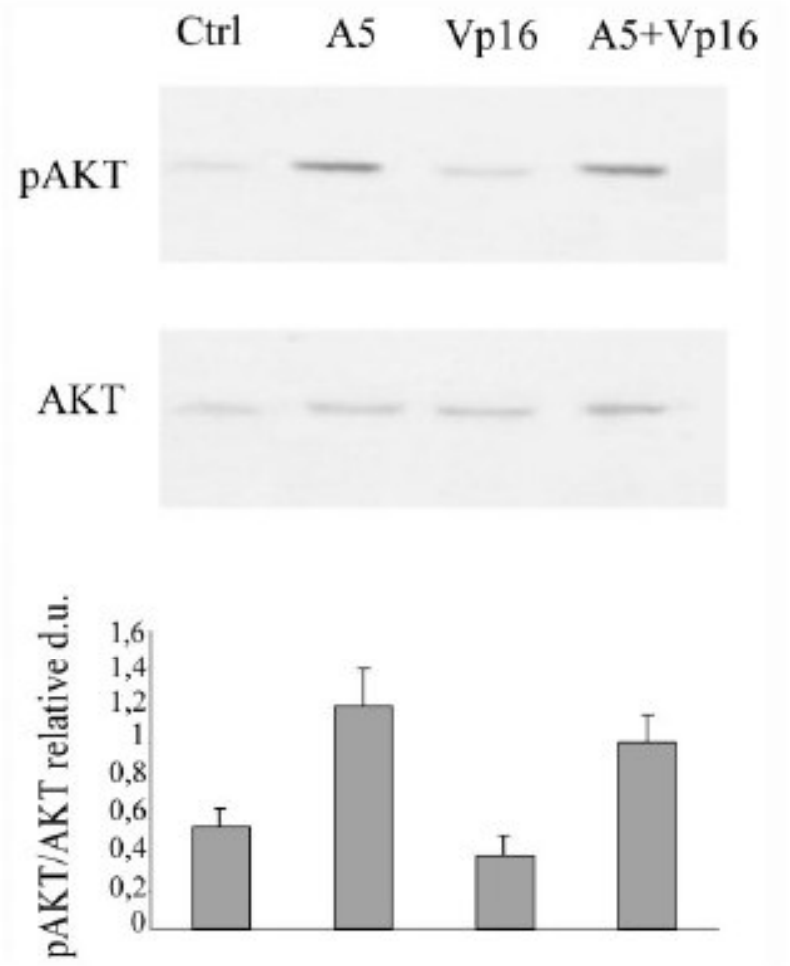

Fig.11 Effect of aspirin and Vp-16 treatments on the phosphorylation status of AKT protein in Caco-2 cells. Experiments were performed in complete medium. Vp-16 at the concentration of 17 μM or aspirin at the concentration of 5 mM were added alone or in combination for 48 h at day 4 of culture. Western immunoblot analysis of (Ser 437) phosphorylated AKT (top) and total AKT (bottom). The immunoblots were stripped and reblotted with antibody against total AKT protein. Protein lysates were from untreated cells (Ctrl), or cells treated with aspirin at 5 mM (A5), Vp-16 for 48 h at 17 μM (Vp 16), or 17 μM Vp-16 and 5 mM aspirin (A5 + Vp16). The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

Finally, in the search for substrates of Akt that could be relevant to the survival-promoting effects of Akt in Caco-2 cells, I also evaluated the effects of aspirin treatment on phosphorylation of (Ser 136) Bad in the same experimental system. No effects were detected on phosphorylation status of Bad at Ser 136 both after acute (5–30 min) and 96-h aspirin treatments (data not shown). On the contrary, according to our previous data (Ricchi et al., 2002), I found that aspirin long-term treatment caused a dose-dependent increase in the bcl-2 levels (data not shown). Together, these results strongly suggest that the aspirin cytoprotective effect resides in the ability to activate AKT protein and to deliver antiapoptotic signals in the presence of different apoptotic stimuli. As reported previously, treatment with aspirin dose dependently inhibited growth (Ricchi et al., 1997). Furthermore, aspirin (2–5 mM) did not significantly affect Caco-2 cells viability and counteracted G2/M phase transition induced by topoisomerase inhibitors (Ricchi et al., 2002). My data mentioned above on cell cycle further reinforced the idea that aspirin treatment could also regulate some of the proteins involved in control of cell cycle checkpoints. Furthermore, it was previously shown that p21*Cip*/*WAF*1, but not p27 levels, were elevated in terminal differentiated and nondividing Caco-2 cells (Evers et al., 1996; Gartel et al., 1996) and were almost undetectable in Caco-2 proliferating cells (Zarrilli et al., 1999). I therefore evaluated the effect of aspirin treatment on p21*Cip*/*WAF*1 expression. Because it has been reported that induction of p21*Cip*/*WAF*1 could be a downstream event of the activation of the AKT/PKB and ERK1/2 kinase pathways (Olson et al., 1998; Lawlor and Rotwein, 2000a,b), I also studied whether PD98059 or LY294002 treatment, alone or in combination with 2 and 5 mM aspirin for 96 h could modify p21*Cip*/*WAF*1 expression in Caco-2 serum-starved cells.

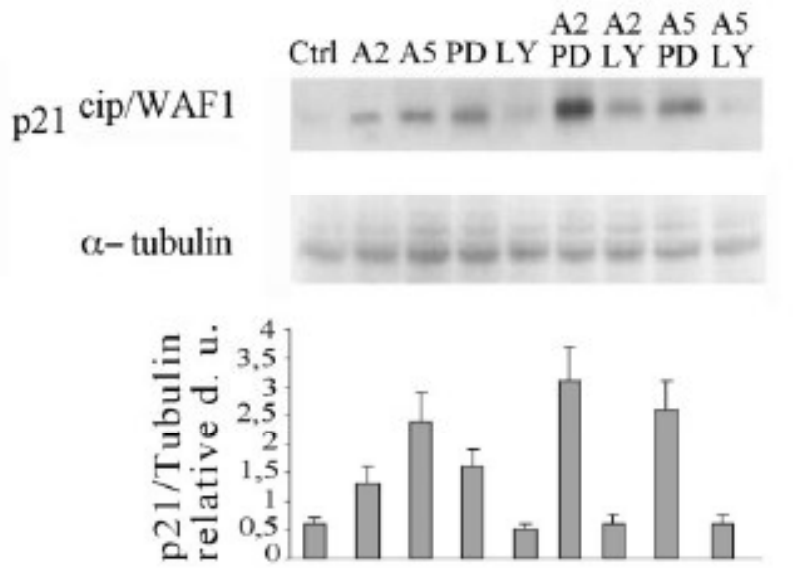

Fig.12 Effect of 40 μM PD98059 or 50 μM LY294002 treatments, alone or in combination with 2 and 5 mM aspirin on $p21^{Cip/WAF1}$ (top) and α-tubulin (bottom) expression in serum-starved cells. Western immunoblot analysis was performed on protein lysates from control cells (Ctrl), cell treated with 2 mM aspirin (A2), 5 mM aspirin (A5), 40 μM PD98059 (PD), 50 μM LY294002 (LY), 2 mM aspirin and 40 μM PD98059 (A2PD), 5 mM aspirin and 50 μM LY294002 (A2LY), 5 mM aspirin and 40 μM PD98059 (A5PD), or 5 mM aspirin and 50 μM LY294002 (A5LY). The autoradiographs shown are representative of three separate experiments. Histograms represent the mean densitometric analysis of three experiments plus S.D. bars.

As shown in Fig.12, p21*Cip*/*WAF*1 levels increased by approximately 2-fold and 4-fold in cells treated with aspirin at 2 and 5 mM, compared with untreated cells. PD98059 alone, or in combination with 2 and 5 mM aspirin, increased p21*Cip*/*WAF*1 levels by 2.7-, 5-, and 4.4-

fold, respectively, compared with untreated cells. On the contrary, LY294002 alone did not affect basal level of p21*Cip*/*WAF*1, whereas in combination with aspirin completely suppressed the increase of p21*Cip*/*WAF*1 expression induced by aspirin. The above-mentioned data therefore demonstrated that the induction of p21*Cip*/*WAF*1 in Caco-2 serum-starved cells was a downstream event of the activation of the PI3-kinase survival pathway. Then, I examined whether these treatments had any impact on cell cycle parameters under the same experimental conditions. Table 4 shows the effect of PD98059 and LY294002, alone or in combination with aspirin, on cell cycle distribution of Caco-2 cells. PD98059 alone and in the presence of aspirin increased the percentage of cells in G1 and S phases and almost suppressed the percentage of cells in G2/M phase of cell cycle compared with control cells. Interestingly, LY294002 alone increased the percentage of cells in G1 phase and reduced the percentage of cells in G2/M phase compared with control cells; on the contrary, LY294002 in cotreatment with aspirin at 2 and 5 mM increased the percentage of cells in G2/M phase from 7 to 15%(p 0.05) and from 4 to 32% (p 0.02) with respect to aspirin-treated cells.

TABLE 4

Effect of PD98059 and LY294002 treatments, alone or in combination for 96 h with aspirin, on cell cycle distribution during Caco-2 cell starvation

DNA cell cycle analysis was performed by propidium iodide labeling as described under *Materials and Methods*. Data are expressed as mean ± S.D.

	% of Cell Cycle Distribution		
Treatment	G_0/G_1	S	G_2/M
Control	56 ± 5	32 ± 5	12 ± 3
2 mM aspirin	53 ± 8	41 ± 5	7 ± 3
5 mM aspirin	61 ± 3	35 ± 3	4 ± 1
PD	58 ± 6	41 ± 8	1 ± 0
PD + 2 mM aspirin	59 ± 7	40 ± 7	1 ± 0
PD + 5 mM aspirin	56 ± 5	39 ± 8	5 ± 3
LY	72 ± 8	25 ± 7	3 ± 2
LY + 2 mM aspirin	60 ± 7	23 ± 7	15 ± 3
LY + 5 mM aspirin	54 ± 9	14 ± 5	32 ± 6

The occurrence of this molecular mechanism and this effect of aspirin also in vivo system.

In this study, I aimed to test the ability of aspirin to activate PI3K/AKT pathway in vitro and in vivo in methylcholanthrene induced fibrosarcoma cells (Meth A) transplanted into BALB/c nude mice.

Meth A cells were exposed for 72 hours at different concentrations of VP-16 and aspirin alone and in combination.Aspirin was used at 1, 2, and 3 mmol/L and VP-16 was used at 34 and 68 μmol/L. Apoptosis was evaluated through the analysis of cells positive for Annexin V staining at flow cytometry. Spontaneous apoptosis of Meth A cells in culture medium was in the average 10%. Treatment with aspirin up to 3 mmol/L concentration did not increase significantly apoptosis in Meth A cells with respect to basal level (Fig. 13, top).

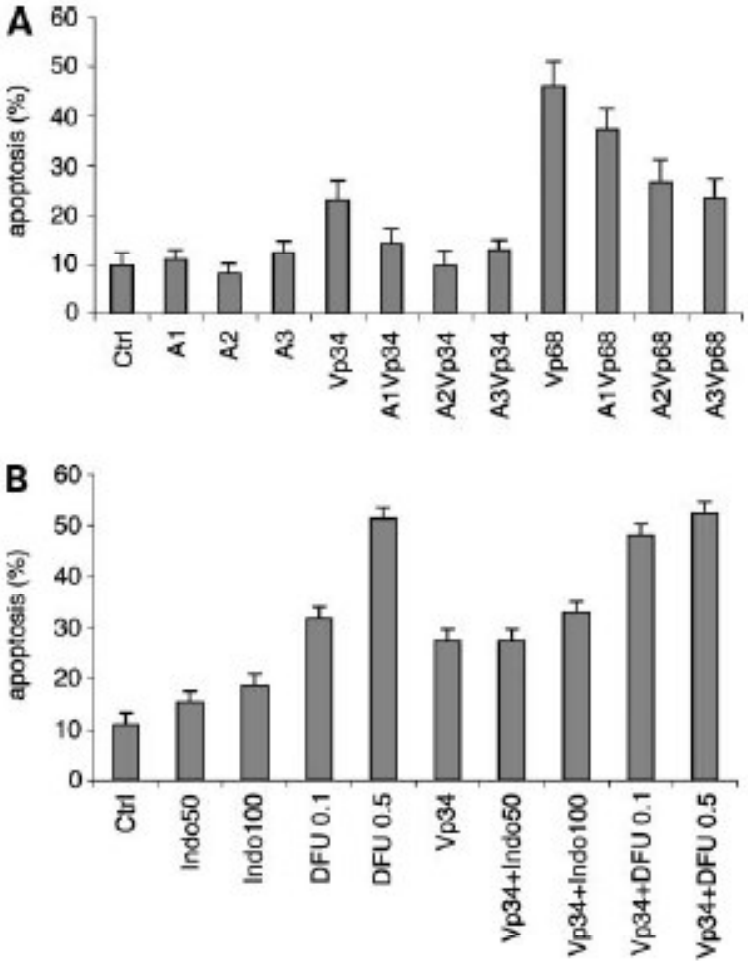

Figure 13 **A**, effect of 1 mmol/L (*A1*), 2 mmol/L (*A2*), and 3 mmol/L (*A3*) aspirin and 34 μmol/L (*Vp34*) and 68 μmol/L (*Vp68*) VP-16 treatments on apoptosis in Meth A cells. Cells were incubated with aspirin and VP-16 alone or in combination at the indicated concentrations for 72 h. **B**, effect of Indomethacin (50 and 100 μmol/L) and DFU (0,1, and 0.5 μmol/L) and VP (34 μmol/L). Cells were incubated with Indomethacin and DFU and VP34 alone and in combination for 72 h. Apoptosis was calculated as the percentage of cells positive at Annexin V-FITC as described in Materials and Methods. *Columns*, mean of triplicate experiments; *bars*, SD.

The percentage of apoptosis increased upto 11% following treatment with 5 mmol/L aspirin (data not shown). Conversely, VP-16 at 34 and 68 μmol/L dose dependently increased apoptosis from 26% to 46% (Fig. 14, top). In addition, the levels of apoptosis were significantly reduced ($P < 0.05$) for each concentration of VP-16 when given in the presence of all concentration of aspirin compared with single-agent treatment; Meth A cells cotreated with aspirin at 3 mmol/L were particularly resistant to VP-16-induced apoptosis ($P < 0.002$). Similar results were obtained when levels of apoptosis were evaluated with propidium iodide staining analysis (data not shown). These results indicated that VP-16 was an active anticancer agent in Meth A cells and that aspirin treatment reduced the ability of the drug to induce apoptosis. To evaluate whether the effects of aspirin cotreatment on anticancer drug responsiveness were also obtainable toward others anticancer agents, we tested the effect of

aspirin treatment on cisplatin-induced apoptosis. Meth A cells were cotreated with aspirin at 1, 2, and 3 mmol/L and cisplatin at 2, 5, 10, and 20 μmol/L under the above mentioned experimental conditions. Aspirin cotreatment dose-dependently reduced cisplatin-induced apoptosis for each concentration of cisplatin tested. The effect of aspirin on apoptosis was of the same range of magnitude of that observed in VP-16 and aspirin cotreated Meth A cells (data not shown).

To evaluate whether the cytoprotective effects of aspirin treatment on Meth A cells were dependent on the inhibition of COXs activity, I analyzed the effect of VP-16 cotreatments with DFU, a COX-2selective inhibitor, and with indomethacin, a nonsteroidal antiinflammatory drug that shows a COX-1/COX-2 ratio called the inhibition of COX isoform similar to that of aspirin. I used two different concentration of indomethacin and DFU both at doses well recognized to inhibit COX-1 and/or COX-2. Neither indomethacin at 50 and 100 mol/L nor DFU at 0.1 and 0.5 mol/L, respectively, reduced VP-16-induced apoptosis in Meth A cells (Fig. 13, bottom). These results, therefore, indicated that the cytoprotective action was unique to aspirin and was not shared by indomethacin.

To show that aspirin-induced cytoprotective effect correlated with the activation of AKT in Meth A cells, I assayed AKT activation status following VP-16 treatment in the presence or absence of aspirin. Lysates from cells treated with 1, 2, and 3 mmol/L aspirin alone or in the presence of VP-16 at 34 Amol/L for 72hours were probed for phosphorylated AKT at Ser473. The bands were quantified and normalized to total AKT protein kinases, respectively. As shown in Fig. 14, aspirin treatment, but not VP-16 treatment, induced activation of AKT protein with respect to control cells.

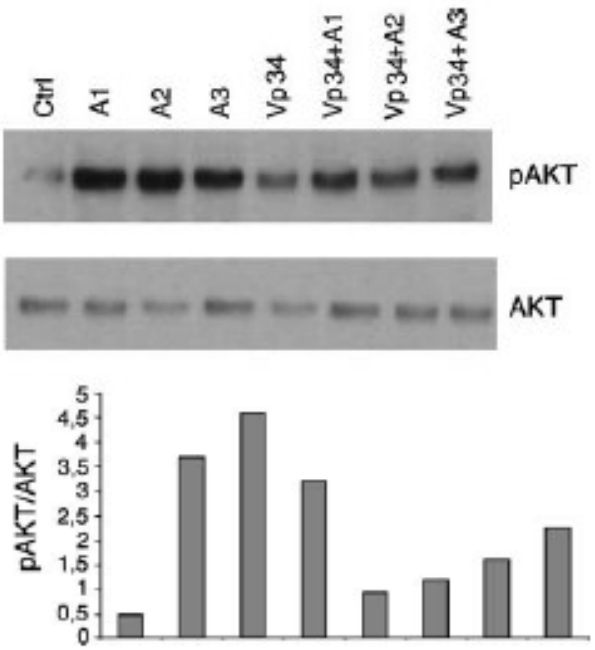

Figure 14 Effect of 1, 2, and 3 mmol/L aspirin and 34 μmol/L VP-16 treatment alone and in combination on (Ser^{437}) phosphorylated AKT and total AKT expression in Meth A cells. Western blot analysis was done on protein lysates from cells cultured as described in Fig. 1. The immunoblots were stripped and reblotted with antibodies against total AKT. One of three separate experiments yielded similar results.

The relative densitometric analysis showed 7.4-, 9.5-, and 6.9-fold increase for AKT protein phosphorylation status following aspirin treatment at 1, 2, and 3 mmol/L, respectively (Fig. 14). Moreover, phosphorylation status of AKT protein were 2-, 3.5-, and 4.2-fold increased

also in cells cotreated with 1, 2, and 3 mmol/L aspirin andVP-16, respectively, compared with untreated cells.To further confirm the involvement of PI3K/AKTsurvival pathway, I evaluated the effect of the PI3K inhibitor LY294002 on the activation of AKT in aspirin treated and cotreated Meth A cells. The use of LY294002 suppressed the activation induced by aspirin, restoring the basal level of AKT activation status (data not shown).
To investigate whether our in vitro finding occurred also in vivo, I evaluated the effect of aspirin and VP-16 treatments in nude mice injected i.p. with Meth A tumor cells.
BALB/c mice average starting weight was 24.75 g. Because the rapid growth of Meth A tumor cells induced ascites in control group, weight gain was used to monitor tumor burden in the animals. Figure 15 shows the percentage of increase in body weight with respect to initial weight in control group; after 5 days, when Meth A cells induced visible ascites, and after 14 days from the inoculum, an average 10% and 53% increase (38.2g mean weight; data not shown) in weight was reached, respectively (Fig. 15).

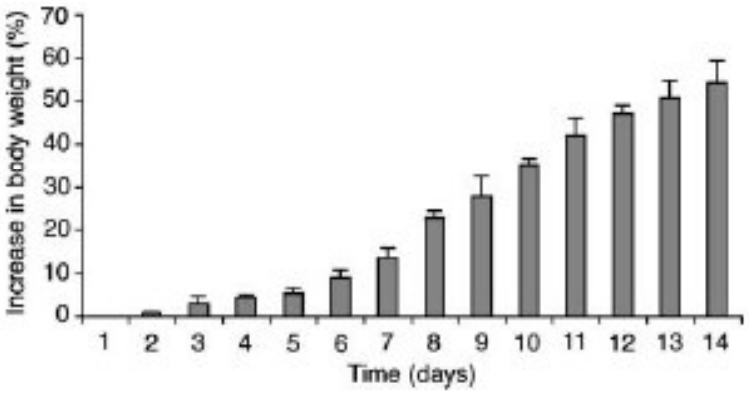

Figure 15 Effect of Meth A cells injection on body weight gain of BALB/c mice. *Y axis,* percentage of weight increase with respect to animal weight recorded at day 1 of inoculum. *Columns,* mean of triplicate experiments; *bars,* SD.

Later, surviving animals reaching a size not compatible with normal life and suffering for the great ascites developed were killed for bioethical reasons. Treatments started after 5 days from inoculum; animals (six for each group) were treated i.p. with either aspirin (15 and 45 mg/kg) and VP-16 (4 mg/kg) alone or in combination with aspirin (at the dose of 15, 30, and 45 mg/ kg) or with vehicle (control group). In case of coadministration, to mimic experimental condition obtained in vitro, aspirin and VP-16 were simultaneously injected at different sides of peritoneum.
During the first 7 days of treatment (days 1–7), when animal death was not yet observed, follow-up was based on the calculation of the percentage of increase in animal weight with respect to first day of treatment (Fig. 16); during the subsequent days (days 8–32), the effects of different treatments were evaluated recording animal survival and were displayed with Kaplan-Meier curves (Fig. 17).

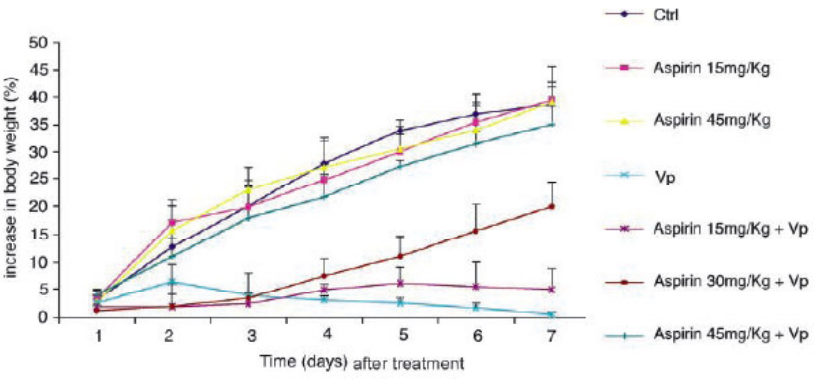

Figure 16 Effect of aspirin treatment at 15, 30, and 45 mg/kg and VP-16 (*Vp*) treatment (4 mg/kg) alone and in combination on percentage of increase in body weight. The percentage was calculated with respect to animal body weight recorded at day 1 of treatment. Treatments started after 5 d from i.p. injection of 2×10^6 Meth A sarcoma cells and body weight gain was monitored for 7 d. *Points,* mean of triplicate experiments; *bars,* SD. Representative experiment. Two separate experiments yielded similar results. Mann-Whitney *U* test was used to compare increase in body weight (%) at day 7 among different treatment groups.

Treatment with VP-16 completely arrested tumor growth and ascites generation, and the suppression of Meth A tumor cell growth was associated with an 2 % of reduction in body weight at days 5 to 7 (Fig. 16). After 8 days from VP-16 treatment, 33.3% of animals died for causes not directly linked with weight increase (bleeding ascites and cachexia). In other 50% of animals, after a variable lag time from treatment, Meth A cells resumed a growth rate comparable with control, and animals were harvested for bioethical reason when they reached an average weight of 35 g (data not shown). In remaining 16.6% of animals, no tumor growth was detected until 25 days of observation (Fig. 17) and they remained tumor free for a period of observation of 40 days after treatment (data not shown). Therefore, although VP-16 treatment was not able to definitively cure almost all animals, tumor growth was arrested and mouse survival duration was significantly increased with respect to control ($P < 0.001$) for all the duration of follow-up. Aspirin treatment as single agent at 15 and 45 mg/kg had no effect on animal body weight and survival with respect to the control group (Figs. 16 and 17); no precocious mortality for gastric toxicity was observed in the aspirin-treated groups. The combination of aspirin at 45 mg/kg and VP-16 did not significantly inhibit xenograft ascitic growth and did not produce any effects on animal survival compared with VP-16-treated group ($P < 0.001$), displaying a trend similar to control group (Figs. 16 and 17). These data suggest that the highest dose of aspirin abolished the activity and the efficacy of VP-16 in tumor-bearing mice.

The combination of aspirin at 30 mg/kg and VP-16 produced an increase in body weight of a lesser extent compared with that observed in control group. However, a more rapid increase in weight gain after 5 days of treatment was observed in cotreated animal when compared with animal treated with VP-16 alone ($P < 0.002$at day 7; Fig. 16).

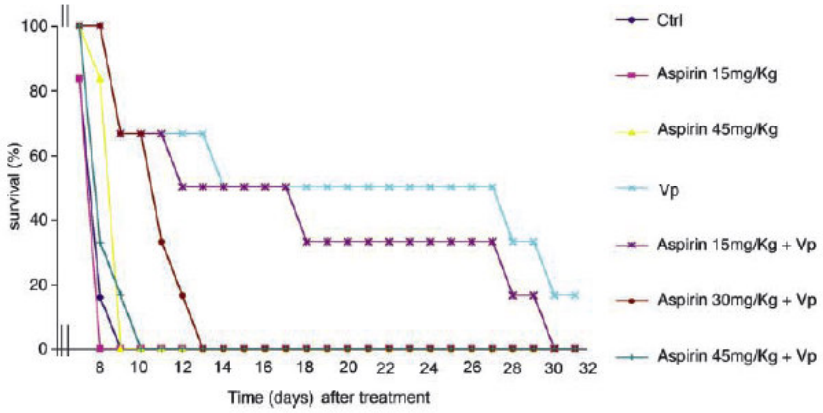

Figure 17 Effect of VP-16 and aspirin treatment on survival of Meth A tumor-bearing mice. Five days following i.p. injection of 2×10^6 Meth A sarcoma cells, groups of mice were treated with aspirin at 15, 30, and 45 mg/kg or VP-16 at 4 mg/kg alone and in combination. Proportion of surviving animals in each group after 7 d from treatment as a function of days post-tumor challenge. The decrease in survival of aspirin at 30 and 45 mg/kg and VP-16 cotreated mice over VP-16-treated group was statistically significant at days 12 and 15, respectively, as determined by the log-rank test. Representative experiment. Two separate experiments yielded similar results.

After this lag of time, a statistically significant difference in survival was observed between these two groups of animals (Fig. 17); in fact, in group of cotreated animals, 33% of the

animals died for causes not directly linked with weight increase, and after day 15 from treatment, no animal survived (Fig. 17). These results indicate that aspirin cotreatment at 30 mg/kg attenuates the activity and the efficacy of VP-16 although at a lesser extent than cotreatment at 45 mg/kg. Finally, in animals treated with the combination of aspirin at 15 mg/kg and VP-16, the body weight reduction was slightly minor ($P < 0.02$) compared with that observed in animal treated with VP-16 alone (Fig. 16). Similarly, a minimal but not statistically significant difference in survival probability was observed (Fig. 17). These results suggest that 15 mg/kg aspirin is able to reduce the activity but not the efficacy of VP-16-based therapy.

To analyze the molecular pathways associated with aspirin treatment in Meth A–bearing mice, one animal was harvested 4 hours after completing treatment and ascitic fluid containing tumor cells from previously considered experimental points was collected for biochemical evaluation. Consistently with our in vitro finding, treatment with aspirin at 15 and 45 mg/kg alone determined a 3.8- and 6.5-fold increase in activation status of AKT compared with control group, respectively (Fig. 18).

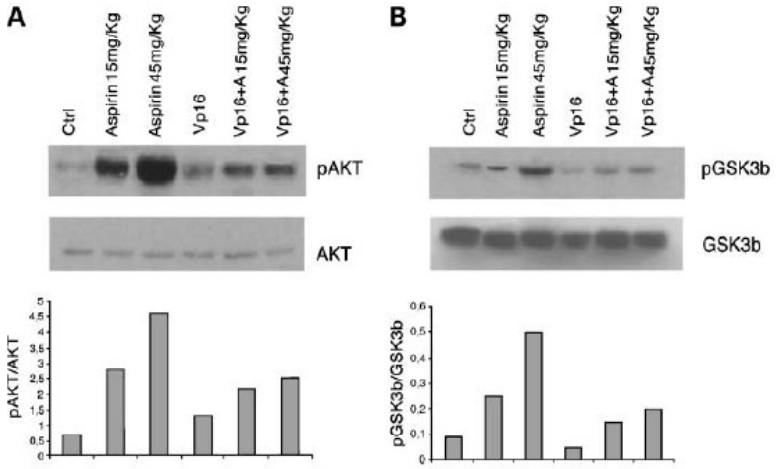

Figure 18 **A,** effect of aspirin treatment (15 and 45 mg/kg) and VP-16 (4 mg/kg) alone and in combination on phosphorylated AKT and total AKT expression. Western blot analysis was done on protein lysates from ascitic cells collected from BALB/c mice 4 h after the end of each treatment. Immunoblots were stripped and reblotted with antibody against total AKT protein. **B,** effect of aspirin treatment (15 and 45 mg/kg) and VP-16 (4 mg/kg) alone and in combination on phosphorylated Ser^9 GSK-3β. The immunoblots were stripped and reblotted with antibody against total GSK-3 protein. Representative experiment. Three separate experiments yielded similar results.

In addition, aspirin cotreated groups showed higher levels of activation of AKT with respect to vehicle-treated group (Fig. 18). Between several downstream targets of AKT, GSK-3 represents a convergence site of multiple signaling pathways involved in cell fate (Li et al. 2000, Loberg et al. 2002). Phosphorylation at Ser21 (-isoform) or Ser9 (-isoform) of GSK-3h by AKT leads to inhibition of its activity and reduces apoptosis. Inactivation of GSK-3 by AKT may thus contribute to antiapoptotic effects of PI3K/AKT signaling (Loberg et al., 2002). Therefore, I evaluated the effect of different treatments on activation status of GSK-3.

As shown in Fig. 18B, phosphorylation status of GSK-3 in Ser9 was increased in aspirin-treated mice. The effect was of lesser extent with respect to that observed for AKT phosphorylation and persisted, at 45 mg/kg aspirin treatment, also in the presence of VP-16. These results indicate that aspirin activates the AKT-dependent survival pathway leading to increased phosphorylation of GSK-3 at Ser9.

DISCUSSION

Mounting evidence suggest that aspirin and other NSAID are able to interfere with mitogenic signalling causing arrest of cells in the G0/G1 phase of the cell cycle and, depending on the doses and the drug, to induce apoptosis as conventional cytotoxic drugs (Lu et al, 1995; Shiff et al, 1995, 1996; Piazza et al, 1995; Arber et al, 1997).
For these properties NSAIDs have been sporadically employed in clinical cancer therapy; more frequently, they are utilised as analgesic, occasionally concurrently chemotherapy administration without a profound knowledge of the potential interaction with anticancer drugs and their efficacy. To date, very little information has been reported on this subject; a paper describes the first extensive screen of commercially available NSAIDs with anticancer drugs and discusses the potential clinical benefits of such combinations (Duffy et al, 1998). More recently, several studies have investigated the possibility of Cox-2 selective inhibitor-mediated enhancement of chemotherapeutic drug toxicity (Soriano et al, 1999; Hida et al, 2000). There are not reports comparing the effects of Cox-2 selective inhibition on responsiveness of colon cancer cells to chemotherapy with that obtained with aspirin, the most frequently used medicinal drug also for its anti-aggregating properties and in the clinical treatment of inflammatory diseases. In particular, the use of aspirin for analgesia and antipyresis (650 mg of oral administration, six times more on average than the anti-platelet dose) results in peak plasma concentration of 25 mg ml (which corresponds approximately to 0.15 mM). On the other hand, the high dose of aspirin required for the treatment of arthritis (4 – 6 g of oral administration) determines aspirin plasma level up to 300 mg ml, which corresponds to 2 mM (Insel, 2001). The main purpose of the first part of my study is to evaluate if combined treatment in vitro would produce interactive effects that could be relevant in the clinical use. We have previously demonstrated that aspirin and NS-398 treatments are both associated with the inhibition of proliferation and induction of apoptosis of Caco-2 cells (Ricchi et al, 1997; Di Popolo et al, 2000). Numerous studies suggest that any interference with mitogenic signalling and cell cycle cause a modification in the responsiveness to conventional cytotoxic drugs. In this study , I investigate whether aspirin and NS-398 interference with cell proliferation has any effect on Caco-2 cells responsiveness to the topoisomerase poisons irinotecan and etoposide, anticancer agents that specifically require DNA synthesis to exert their toxicity.The data reported herein demonstrate that aspirin and NS-398 act as anticancer agents in Caco-2 colon cancer cells with cytostatic properties and with different ability to induce apoptosis at high dosages. Also, I show that they differently modify anticancer responsiveness to chemotherapy of Caco-2 cells. I have evaluated this effect mainly towards the induction of apoptosis. In fact, there is emerging evidence that resistance to antitumour treatments relies on reduced sensitivity to apoptosis induction (Fisher, 1994; Nagata, 1997); in this regard, my results demonstrate that aspirin treatment is able to counteract the proapoptotic effect of anticancer drugs in vitro. Aspirin co-treatment produces, at all concentrations investigated, a reduction of anticancer drug-induced apoptosis in Caco- 2 cells and, at 5 mM, a reduction of S/G2 phase cell cycle accumulation.
I hypothesise that the effects of high doses of aspirin cotreatment on anticancer drug-induced toxicity could be mainly explained by the almost complete inhibition of DNA synthesis and G1 cell cycle arrest obtained at this dose of aspirin that have been shown also to alter cell cycle-related proteins (Law et al, 2000; Marra et al, 2000); the reduced ability of such anticancer drugs to induce apoptosis in quiescent cells have been already observed (Nitiss and Wang, 1996: Lin et al, 1998). Furthermore, it is a common observation that agents able to interfere with cell cycle usually prevent the action of drugs active in the next phase of cell cycle (Lin et al, 1998).

Moreover, I clearly demonstrate by PI- and annexin V-staining that the reduced levels of apoptosis obtained in aspirin co-treatments are associated with an increase in cell viability of Caco-2 cells; this effect is also evident by the analysis of plating efficiency in aspirin-Vp 16 co-treated cells; thus, these data indicate that aspirin co-treatment interferes not only with apoptotic death but also with overall toxicity induced by these anticancer drugs. In this regard, aspirin and its metabolite sodium salicylate have been found to be protective against neurotoxicity elicited by the excitatory amino acid glutamate in rat primary neuronal cultures (Grilli et al, 1996); thus, my data may represent a further evidence of aspirin cytoprotective property against a cellular damage.
In addition, PI- and annexin V-staining assay indicated that the cytoptotective effect of aspirin co-treatment is present not only in Caco-2 cells but also in the Cox-2 negative, Cox-1 positive Sw 480 colon cancer cell line. These data may suggest that aspirin interferes with topoisomerase poison-induced toxicity through a Cox-independent mechanism. On the other hand, my data show that NS-398 co-treatment may alternatively reduce or increase anticancer drug induced apopotosis, depending on the concentration. These effects are less powerful compared with those observed in the presence of aspirin.
NS-398 at 1mm concentration completely inhibits the biosynthesis of prostaglandin E2 in Caco-2 cells (Acquaviva et al, unpublished data) without affecting anticancer drug responsiveness; NS 398, at 10 mm concentration, causes minimal enhancement of anticancer drug-induced apoptosis without modifying cell cycle parameters. On the contrary, my data show that NS-398, at dose of 50 mM, is cytoprotective and counteracts anticancer drugs induced cell cycle perturbation. Based on these findings, I postulated that the described effects of increasing dose of NS-398, as in the case of aspirin, might be dependent on regulation of cell cycle regulatory protein (Hung et al, 2000) and not related on the inhibition of Cox-2 activity; these data could also reinforce the hypothesis of the concurrent presence of Cox-2-dependent and independent mechanisms of action in the antiproliferative effect of individual selective Cox-2 inhibitors (Grosch et al, 2001; Smith et al, 2000).
The second aim of my study, was tried to better elucidate the molecular mechanisms that link the aspirin-dependent effect on cell cycle to the resistance to other apoptotic stimuli. I used serum deprivation-induced apoptosis in Caco-2 cells as a model system to evaluate aspirin interference with death/ survival pathways. My data show that in Caco-2 cells apoptosis induced by serum deprivation is a late event accompanied with increase in G2/M cell cycle phase. These findings are in agreement with those obtained in different experimental systems in which PI3-kinase activity was found to be required for growth factor-dependent survival and differentiation (Kennedy et al., 1997; Eves et al., 1998) and in which MAP kinase cascade was responsible for cell cycle progression (Aliaga et al., 1999; Abbott and Holt, 1999). My data show that aspirin treatment in Caco-2 cells interferes with serum deprivation-induced apoptosis through PI3-kinase and ERK kinase pathways. I also show that the effect of aspirin on survival is mediated through the activation of the PI3-kinase. In fact, aspirin treatment in the presence of the specific PI3-kinase inhibitor LY294002 is able to revert the effect of aspirin on Caco-2 cells survival being even more toxic than LY294002 alone. On the other hand, the inhibition of MEK activity by PD98059 alone or in cotreatment with aspirin does not have the same effect of LY294002 and maintain the cytoprotective effect of aspirin. Further studies are needed to clarify at molecular level the role of PI3-kinase pathway in the observed aspirin-dependent activation of ERK kinase. That aspirin treatment has a direct effect on PI3-kinase and ERK kinase pathways is demonstrated by my data showing a transient activation of these pathways by acute aspirin treatment. However, because the majority of my data are obtained with aspirin long-term treatment, I cannot exclude that aspirin might have also an indirect effect through the IGFII/IGF-Ir autocrine loop that sustains Caco-2 cells survival (Zarrilli et al., 1994, 1996). Therefore, further studies are needed also to elucidate at molecular levels the site(s) of aspirin interference with the IGFII/IGF-Ir-

dependent PI3-kinase survival pathway and the mechanism(s) responsible for PI3-kinase activation.
My data show that aspirin not only prevents apoptosis but also the increase in G2 cell cycle after Caco-2 cells serum deprivation and dose dependently induces p21*Cip*/*WAF*1 expression.
Mounting evidence indicates that p21*Cip*/*WAF*1 is a downstream target of the PI3-kinase/AKT pathway. In fact, it has been shown in muscle cells that IGFI/IGF-I receptor activation regulates survival and differentiation through the stimulation of PI3-kinase that in turn activates AKT, which stimulates the expression of p21*Cip*/*WAF*1 (Lawlor and Rotwein, 2000a,b).
However, although it has been shown that AKT stimulates p21*Cip*/*WAF*1 expression, other reports indicate that the activated AKT phosphorylates p21*Cip*/*WAF*1 to prevent p21*Cip*/*WAF*1 nuclear localization and the p21*Cip*/*WAF*1-dependent cell cycle arrest, as well as Mdm2, to prevent Mdm2 nuclear localization and Mdm2-dependent degradation of p53, thus determining cell cycle progression (Zhou et al., 2001a,b). The data presented herein indicate that aspirin activates a survival pathway that is responsible for the induction of p21*Cip*/*WAF*1 also in Caco-2 cells and that there is a strict correlation between p21*Cip*/*WAF*1 expression and its inhibitory function toward cell cycle progression. In fact, as well as restoring apoptosis induced by serum deprivation, treatment with LY294002 in the presence of aspirin leads to p21*Cip*/ *WAF*1 suppression and G2/M accumulation. Thus, the aspirin dependent activation of AKT/PKB protein and the induction of p21*Cip*/*WAF*1expression provides a mechanism for its ability to prevent apoptosis and G2/M phase accumulation induced by prolonged serum deprivation and Etoposide treatment and indicates a novel tool by which aspirin may control cell cycle without resulting in a toxic treatment in Caco-2 cells.
In accordance with our data, the activation of a similar pattern has been recently shown by Yu and collaborators, who demonstrated that the overexpression of the receptor tyrosine kinase p185ERbB2 increased p21*Cip*/*WAF*1 expression, conferred resistance to taxol-induced apoptosis, and prevented cell entrance to G2/M phase (Yu et al., 1998). On the other hand, we found also that inhibition of the MEK pathway led to induction of p21*Cip*/*WAF*1 expression and G2/M cell cycle phase reduction; similarly, the highest increase of G2/M phase was observed during 5 mM aspirin and LY294002 cotreatment in presence of activation of ERK1/2 kinases and p21*Cip*/*WAF*1 suppression. Although the mechanism by which PD98059 treatment increases p21*Cip*/*WAF*1 level remains unclear, the above-mentioned data both confirme the protective effect of PD98059 against apoptotic stimuli (Cho et al., 2002; Cuda et al., 2002) and strongly suggest that the apoptotic response to serum deprivation in Caco-2 cells involves also the activation of MAP kinase pathway and p21*Cip*/*WAF*1 suppression both responsible for G2/M accumulation.
These finding are in agreement with other reports showing that p21*Cip*/*WAF*1 expression could play a role in enhancing cell survival (Cheng et al., 2000), protecting various cell types from apoptosis after anticancer drug treatment (Waldman et al., 1997). Further studies are needed to clarify the effect of aspirin treatment on p21*Cip*/*WAF*1 expression and its phosphorylation status on the control of cell cycle check points in Caco-2 cells and the mechanism by which the coordinated activity of PI3-kinase and MAP kinase pathways regulates its expression and the survival signaling. I found previously the involvement of PI3K/AKT pathway in cytoprotective property of aspirin in cultured colon cancer Caco-2cells (19).To verify that aspirin is able to activate this pathway in other cancer cells and ,also in vivo, I used the fibrosarcoma Meth A cells as a system to evaluate aspirin interference with anticancer drug-induced apoptosis in vitro and in vivo. My data showed for the first time that the antiapoptotic effects of aspirin are not cell type specific and operated in vivo through the activation of PI3K/AKT pathway. My data showed that aspirin exposure, but not other COX-2selective and nonselective inhibitors, prevented apoptosis induced by VP-16 and cisplatin in Meth A cells. The in vitro data were consistent with previous results showing that aspirin protected cells from apoptosis through activation of PI3K/AKT. Because the AKT protein is

the downstream effector of PI3K, further studies are needed to clarify at molecular levels the site(s) of aspirin interference with the PI3K survival pathway and the mechanism(s) responsible for PI3K activation. Preliminary data from our group indicate that aspirin is able to induce acetylation status of p85 regulative subunit of PI3K. It is possible to postulate that the structural change by acetylation increase the level of PIP3, as shown previously (19), and induce activation of PDK1 kinase.

Furthermore, the in vitro data reinforce the evidence coming from our and other previous experiments (6, 18, 19, 24) that high doses of aspirin slightly affected or may increase basal level of apoptosis but were protective from apoptosis induced by external toxic stimuli. This at the molecular level confirms the aspirin pleiotropic activity and suggests that the aspirin interference with different survival/death pathways determines a global prosurvival promoting effect.

One of the major purposes of this study was to evaluate whether combined treatment in vivo would produce effects that could be relevant in the clinical use.My data indicated that aspirin as single agent did not affect Meth A cell growth while provided evidence that aspirin administration reduced in vivo the activity and the efficacy of VP-16 treatment in Meth A–bearing mice. My data indicated also that in vivo aspirin operated in a dose-response fashion to reduce the outcome of anticancer treatment. This trend was evident behind the lowest dose and indicated that coadministration of minimum 30 mg/kg aspirin was required to produce a significant effect on animal survival.

Furthermore, aspirin-induced increase of animal body weight and decrease of survival strictly correlates with the activation of AKT. My results agree with previous data showing that AKT activation in vivo is a major factor in the resistance of tumor cells to apoptosis induced by chemotherapeutic drugs (29).

In addition, my in vivo data indicated that aspirin inhibited the activity of GSK-3, a key factor not only for survival and chemoresistance (30) but also for the attenuation of the cellular action of insulin (31). Because it has been shown that high-dose aspirin could improve glucose metabolism in humans (16) and that GSK-3 activity inhibitors may therapeutically benefit in treating insulin resistance and type 2diabetes (32), the data of this report should encourage further evaluation of the in vivo mechanism for aspirin hypoglycemic effect.

Although an expanding literature is showing the role of nonsteroidal anti-inflammatory drugs used alone or in combination with conventional anticancer drugs and their ability to alter toxicity profile of anticancer drugs (33, 34), the use of aspirin in combination with anticancer drugs has never been investigated in vivo. In this study, I used doses of aspirin in vitro and in vivo approximately corresponding to that required for the treatment of arthritis and rheumatic fever. In fact, the clinical treatment of inflammation requires 2 to 5 g aspirin oral administration, which determines aspirin plasma level up to 1 to 3 mmol/L (35). In my experiments, the antiapoptotic effect of aspirin was observed starting at dose at least 10 times higher than those used for antiplatelet therapy (75 mg/d). Therefore, our finding could be of relevance in the clinical condition where treatment schedules involving both cytotoxic agents and high doses of aspirin are required, suggesting that cotreatment with aspirin could limit the outcome of anticancer therapy.

In conclusion, my data reinforce the emerging and wide observation coming from neuronal models (17) that aspirin operates also in vivo as cytoprotective agent. I also show that aspirin cytoprotective effect is mediated through the activation of AKT pathway. Because AKT pathway mediates survival against a wide variety apoptotic stimuli, it should be also verified the possible involvement of this pathway as mechanism by which aspirin exerts neuroprotection.

MATERIALS AND METHODS

Cell growth and culture. Caco-2 cells were routinely grown in 100 mm plastic dishes at 37°C in a humidified incubator 5% CO2-95% air atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, glutamine (2 mM), penicillin (100 u ml71), and streptomycin (100 mg ml71) and buffered with N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) (20 mM). The culture medium was changed every other day. Confluence was reached 6 – 8 days.
Etoposide and irinotecan were a gift from Bristol-Myers- Squibb (Rome, Italy) and Rhone Poulen, respectively; anticancer drugs were diluted in DMEM to prepare 500x concentrated solutions. Aspirin (Sigma, Milan, Italy) was dissolved in a 0.1 M Tris-HCl pH 7.8 solution. The solutions were buffered with Tris base to obtain the final pH equal to that of control DMEM medium and prepared every two weeks. Experiments were performed in the absence or in the presence of aspirin. In aspirin untreated cells (control) anticancer drugs were added for 48 h at day 4 of culture. In treated cells aspirin was added at day 3 of culture for 24 h. Anticancer drugs were added for 48 h in presence of aspirin or NS 398 (co-treatment) without changing media. LY294002 (Calbiochem, Darmstadt, Germany) was dissolved in dimethyl sulfoxide and prepared as a 16.2 mM stock solution and added at a final concentration of 50 M. PD98059 (Calbiochem) was dissolved in dimethyl sulfoxide and prepared as an 8 mM stock solution and added at final concentration of 40μM.
The transplantable sarcoma Meth A cells were kindly provided from Dr. Pramod Srivastava.Original tumor cell lines were induced by a single s.c. injection of 0.1 mg 3-methylcholantrene dissolved in 0.1 mL sesame oil. Meth A cells have a welldefined immunogenicity and typically produce either solid (20, 21). The cells were maintained by continuous propagation in the peritoneal cavity of BALB/c mice (6–7 weeks old).
In addition, Meth A cells can routinely grown in 100-mm plastic dishes at 37°C in a humidified atmosphere of 5% CO2 in air in DMEM supplemented with 10% FCS, glutamine (2mmol/L), penicillin (100 units/mL), and streptomycin (100 Ag/mL) and buffered with HEPES (20 mmol/L). All experiments in vitro were done after one passage from peritoneal cavity.
Indomethacin (Sigma, Milan, Italy) was dissolved in DMSO and prepared every week, 5,5-Dimethyl-3-(3-fluorophenyl)-4-(4-methylsulfonyl)-phenyl-2(5H)-furanone (DFU) was kindly provided by Merck Frosst (Kirkland, Quebec, Canada), dissolved in DMSO, and prepared as 10 mmol/L stock solutio

Cell cycle analysis and apoptosis detection.In order to define the cell cycle distribution and apoptosis rate, Caco-2cells were trypsinised, pelletted, fixed and Propidium Iodide (PI) stained as previously described (Nicoletti et al, 1991). PI staining fluorescence of individual cells was analysed by using a FACS Calibur flow cytometer apparatus (Becton & Dickinson, Mountain View, CA, USA) and the MODFIT analysis software. For each sample, at least 20 000 events were stored. Apoptosis/necrosis ratio was additionally evaluated by using annexin V-FITC/PI double staining technique. Briefly, trypsinized Caco-2 and Meth-A were collected, including floating apoptotic cells and the cells spontaneously detached during washing procedure, and annexin V-FITC and PI co-stained by using a detection kit from Medical & Biological Laboratories Co, Ltd, Naka-ku Nagoya Japan, according to the manufacturer's instructions. Fluorescence analysis was performed by a flow cytometer apparatus (Becton & Dickinson, Mountain View, CA, USA) and the Cell Quest analysis software. For each sample, at least 30 000 events were stored. Quadrant settings were based on the negative control. Each experiment was repeated at least three times.

Plating efficiency assay. To determine anticancer drug responsiveness of Caco-2 cells a plating efficiency assay was performed following drug removal. After each treatment, cells

were trypsinised, washed and seeded (15 000 cells 6 well) in triplicate in 24 multiwell cluster dishes and counted at days 1, 4, 6, 8 and 11 of culture.

Western blot analysis. Mouse monoclonal antibody anti- Bcl-2, rabbit polyclonal anti-human p21*Cip*/*WAF*1 antibody, mouse monoclonal antibodies against phosphorylated ERK1/2, and rabbit polyclonal against total ERK2 protein were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). (Ser 473) Phosphorylated AKT and total AKT, phosphorylated glycogen synthase kinase (GSK)-3α/β (Ser21/Ser9) and GSK-3β were detected by using rabbit polyclonal antibody and mouse monoclonal antibody, from Cell Signaling Technology Inc. (Beverly, MA).Lysates from adherent cells collected by scraping were centrifuged at 12 000 g for 15 min at 4°C. The protein concentration in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad, Richmond CA, USA) and 70μg of total protein from each sample was analysed. Proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis and transferred on nitrocellulose membrane (Hybond-ECL Nitrocellulose, Amsherman, Rainham, UK). Membranes were blocked in 5% non-fat dry milk, and after three washes, were incubated for 1 h at 4°C with 0.5 mg ml71 of mouse monoclonal primary antibody in PBS. After five washes, filters were
incubated for 1 h at 4°C with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Bio Rad) diluted 1 : 2000 in PBS, 0.1% Tween. The membranes were then washed and protein bands were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Control for loading and transfer was obtained by probing with anti-a-tubulin (Sigma) at1 : 4000 dilution. For quantitation of immunoblots, relative intensities of bands were quantified by densitometry with a desk scanner (Pharmacia Discovery system) and RFLPrint software (PDI, New York, USA). Cells were washed in cold PBS and lysed for 10 min at 48C with 1 ml of lysis buffer (50 mM Tris, pH 7.4, 0.5% NP40, 0.01% SDS) containing protease inhibitors.

PI3-Kinase Assay. Cells lysates (300 μg) were immunoprecipitated with a pTyr antibody (Santa Cruz Biotechnology, Inc.). Pellets were washed in cold PBS and then in 100 mM Tris-HCl, pH 7.4, supplemented with 500 mM LiCl, 1 mM EDTA, and 0.2 mM NaVO4.
Pellets were further resuspended in 30mMHEPES, pH 7.5, and 6.25mM MgCl2 and 125 M cold ATP; the kinase reaction was initiated by addition of 2 g/ l phosphatidylinositol (Sigma) and 10 Ci/ l [32P]ATP (3000 Ci/mmol) and performed for 15 min at 37°; the reaction was stopped by addition of 5 M HCl and 0.5 M EDTA and methanol/chloroform (1:1). After mixing vigorously and centrifuging to separate the phases, the organic layer was collected and separated by thin layer chromatography (TLC). [32P]phosphoinositides were visualized by autoradiographs, scraped from TLC, and counts per minute were quantified by a -counter (Beckman Coulter, Inc., Fullerton,CA).

Statistical Analysis. Statistical comparisons were performed using the Mann-Whitney *U* test. A probability value 0.05 was considered a significant difference.

In vivo Studies. Female BALB/c mice (6–7 weeks old) were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved and mice were maintained in accordance to institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized at the University of Naples Medical School Animal Facility for 1 week before injection with cancer cells. All these animals received 1 million Meth A tumor cells in 0.2mL saline on day 0. VP-16 was used at 4 mg/kg, and aspirin was used at 15,30, and 45 mg/kg. After 5 days, when established ascites were evident and animal weight started to increase, seven mice per group were treated i.p. at the indicated doses of aspirin alone or in combination with VP-16. Drugs were given (on days 1–3) for 3 days every 2 4hours. Control group was injected with vehicle. All animals were injected i.p. with an

equal volume of solvent as a control (300 AL). Then, mice were randomly divided into seven groups [1 control group, 1 VP-16-treated group, 2aspirin-treated groups (15 and 45 mg/kg), and 3 aspirin and VP-16 cotreated groups], with each group containing seven animals. Of each group, six animals were observed daily to monitor their survival and their weight increase and one animal was killed 4 hours after completing treatment; ascitic fluid containing tumor cells was collected in PBS with proteases and phosphatases inhibitors and processed as described in Western Blot Analysis.

Statistical Analysis. Statistical comparisons were done using the Mann-Whitney U test and log-rank test. $P < 0.05$ was considered a significant difference.

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