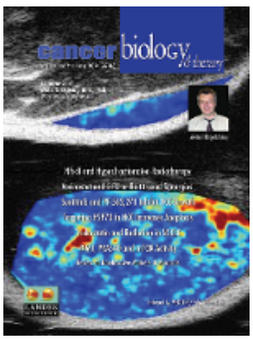

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Research Paper

Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5FU or raltitrexed

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Key words: HDAC inhibitor, vorinostat, thimidilate synthase, p53, colorectal cancer, 5-fluorouracil, raltitrexed

Despite the introduction of several novel anticancer agents almost 50% of colorectal cancer (CRC) patients die for cancer suggesting the necessity of new therapeutical approaches. In this study we demonstrated that the HDAC inhibitor vorinostat exerted potent antiproliferative effect in a panel of mut- and wt-p53 human CRC cell lines. Moreover, in combination with 5-fluorouracil modulated by folinic acid (5FU-FA) or with Raltitrexed (RTX), both commonly used in the treatment of this disease, it showed a clear schedule-dependent synergistic antiproliferative interaction as demonstrated by calculating combination indexes. Only simultaneous, or 24 h pretreatment with vorinostat followed by either agent, produced synergistic effect paralleled by evident cell cycle perturbations with major S-phase arrest. Moreover, we provided for the first time evidences that vorinostat can overcome resistance to both 5FU and RTX. Downmodulation of Thymidilate synthase (TS) protein induced by vorinostat within 24 h, represented a key factor in enhancing the effects of both drugs in sensitive as well as resistant tumor cells. Furthermore, p53, whose wild-type expression is critical for sensitivity to 5FU and RTX, was upregulated by vorinostat in wt- and downregulated in mut-p53 cells, suggesting an additional mechanism of the antiproliferative synergistic interactions observed. Overall these data add new insights in the mechanism of vorinostat antitumor effect and suggested that the association of vorinostat plus 5FU-FA and/or RTX should be clinically explored.

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Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the western countries. Although higher response rates have been achieved using the latest poly-chemotherapy regimens, including agents such as 5-fluorouracil (5FU), raltitrexed (RTX), oxaliplatin, irinotecan and the introduction of new molecular targeted drugs such as cetuximab and bevacizumab, almost 50% of the patients die for cancer with an average overall survival of 24 mo in metastatic stage.[1] Intrinsic or acquired resistance to chemotherapy as well as the genetic flexibility of cancer cell genome resulting in multiple and often compensatory survival and proliferative signals, are the cause of the limited activity of anti-cancer strategies.

Histone deacetylase inhibitors (HDAC-Is) represent a new class of anticancer agents that by enhancing histone acetylation modulate the expression of cell cycle regulation, survival and differentiation genes, thus affecting multiple pathways.[2-4] Several HDAC-Is exhibit antitumor effects in preclinical animal models at amounts that have little or no toxicity[5] and some of them are in advanced clinical studies either as single agents or in combination with conventional chemotherapy or biologicals.[3,4,6-9] These compounds act very selectively altering the transcription of fewer than 2–5% of expressed genes[4,10] and by mechanisms that cannot be solely attributed to the level of histone acetylation.[11] Acetylation of other proteins such as p53 and other transcription factors has been also described.[12] Among the most promising HDAC-Is, vorinostat (SAHA, suberoylanilide hydroxamic acid) has shown significant preclinical and clinical antitumor activity in both haematologic malignancies and solid tumors, including CRC, and represents the first HDAC-I to be recently approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma who has progressive, persistent or recurrent disease during or following two systemic therapies.[4,9,13]

Several reports, including microarray studies, have shown that the expression of the enzyme thymidilate synthase (TS) can be regulated by HDAC-Is.[14-16] In details, Glaser et al. have shown

that different HDAC-Is may control the expression of a "core" set of genes, eight upregulated and five downregulated, including TS, in several cell lines.[15] TS is an essential enzyme for the de novo synthesis of thymidilate (dTMP) and subsequently DNA synthesis, and is a critical target for 5FU and RTX.[17] Moreover, high levels of TS expression have been correlated with patients' poorer overall survival in several tumors, including CRC.[18] The active metabolite of 5FU, 5-fluorodeoxyuridine monophosphate (5FdUMP), inhibits the dTMP synthesis by forming a stable ternary complex with TS and methyl donor 5,10-methylene tetrahydrofolate (CH2THF).[17,19,20] Folinic Acid (FA), a precursor for CH2THF, increases the formation of the stabilized ternary complex when associated with 5FU, resulting in enhanced cytotoxicity in preclinical models[17] as well as in a significant improvement in response rate and overall survival in advanced CRC.[21] RTX is a direct and selective quinazoline antifolate TS inhibitor with a similar response rates to 5Fu modulated by FA (5FU-FA) and has been licensed in many countries for the treatment of metastatic colorectal cancer.[22-24] Preclinical and clinical studies have demonstrated a strong association between increased TS expression and development of resistance to both 5FU and RTX.[20,25-27] Moreover, exposure of cancer cells to 5FU or other TS inhibitors acutely upregulates TS synthesis. The latter effect is likely due to the inhibition of the negative-feedback mechanism in which TS binds its own mRNA and inhibits translation.[20,28,29] In addition, TS protein, recently reported to have an oncogene-like activity,[30] decreases the expression of genes involved in the regulation of proliferative and survival pathways such as c-myc and p53 through binding theirs mRNAs.[31,32] On the other hand, expression of wild-type p53 has been shown to be required for 5FU- and RTX-induced antitumor effects.[28,29,33-35] Therefore new strategies overcoming these molecular mechanisms of chemo-resistance are required.

In this study, we demonstrated in CRC cells a potent antitumor activity of vorinostat paralleled by a downregulation of TS protein expression which was independent from the status of p53. Furthermore, we demonstrated that vorinostat decreased mutant, but not wild-type, p53 protein. On the basis of these observations, we have investigated if the combination of vorinostat plus 5FU-FA or RTX, enhanced cell growth inhibition. Results showed that simultaneous exposure or sequential treatment of vorinostat followed by 5FU-FA or RTX produced an evident synergistic antiproliferative effect, as demonstrated by median drug effect analysis calculating a combination index, independently from p53 status. Finally, taking advantage of either 5FU or RTX resistant cells, we have shown, for the first time, that vorinostat can overcome resistance to both drugs. Additional investigations on biochemical modulation of TS and p53 expression as well as on cell cycle analysis have also been performed in order to explain the molecular mechanism of the described synergistic interaction, in both sensitive and resistant cells.

Results

Vorinostat induces growth inhibition and modulates p53 and TS protein expression in CRC cells. We demonstrated that vorinostat induced antiproliferative effect on a wide variety of cell lines derived from human CRCs with different p53 protein status (Fig. 1A). Moreover, vorinostat induced similar antiproliferative effect in doxorubicin- and 5FU-resistant cells, LoVoDX and HT29FU, compared to their parental LoVo and HT29 cell lines, demonstrating no cross-resistance with other cytotoxic drugs (Fig. 1A).

Moreover, in agreement with previous reports,[14,15] we demonstrated that TS protein expression was downmodulated in time-dependent manner by vorinostat in wild-type p53 (wt-p53) LoVo and LS174T as well as in mut-p53 HT29 and SW620, cell lines. In details, a clear downregulation of TS expression was evident between 6–12 h in all cell lines with almost undetectable levels of the corresponding band observed thereafter in some cell lines (Fig. 1B). This effect was also dose-dependent and was evident even at doses below the $IC_{50}{}^{72\,h}$ (Fig. 1C).

Interestingly, we demonstrated in wt-p53 LoVo and LS174T that vorinostat induces a time-dependent increase of p53 protein expression, which was evident after 6 h of treatment (Fig. 1B). The expression of wt p53 increases in all cell lines with the doses of vorinostat up to $IC_{50}{}^{72h}$ values, decreasing at higher doses (Fig. 1C). On the contrary, in mutant p53 (mut-p53) HT29 and SW620 cell lines, we observed a dose- and time-dependent reduction of p53 protein expression, which appears evident after 12 h of treatment (Fig. 1B and C). Finally, taking advantage of isogenic-paired HCT116 colon cancer cells that were either wt (+/+) or null (-/-) for p53, we showed in both cell lines a downregulation of TS expression after 12 h of treatment. In wt HCT116 $p53^{+/+}$ we demonstrated, as shown before for wt-p53 cells, that TS downregulation was paralleled by a time-dependent increase in p53 protein expression (Fig. 1B).

All these data suggest that the decrease of TS protein expression induced by vorinostat is independent from p53 status and is paralleled by a opposite modulation of wild-type and mutant p53 protein expression.

Sequence-dependent synergistic antiproliferative effect of vorinostat in combination with 5FU-FA or RTX. On the basis of the latter results and considering that alterations in p53 function and TS expression were associated with resistance to both 5FU and RTX,[20,26-28,33,34] two antitumor agents currently used for the treatment of colon cancer patients, we investigated the potential cooperative antitumor effect of vorinostat in combination with each drug. IC_{50} values for 5FU, 5FU-FA and RTX, varied slightly among the different cell lines examined and notably, in all cells FA induced a reduction in the IC_{50} value of 5FU (Fig. 1D). Thus we evaluated, by median drug effect analysis calculating combination index values, the interaction of vorinostat either with 5FU modulated by FA (5FU-FA), or with RTX, on cell proliferation in LoVo, LS174T, HT29 and SW620 cell lines. Since the modulation of both TS and p53 protein expression induced by vorinostat was observed within 24 h, and in order to define the best treatment schedule, simultaneous or sequential (24 h previous exposure to either agent) treatments were performed. Combination indexes calculated at 50% of cell lethality (CI_{50s}) reported in Table 1 showed that simultaneous (*sequence I*) or 24 h previous exposure to vorinostat (*sequence II*) produced, in all four cell lines tested, synergistic effect, while 24 h previous exposure to 5FU-FA (*sequence III*) resulted only in additive effect. Interestingly, similar results were shown also

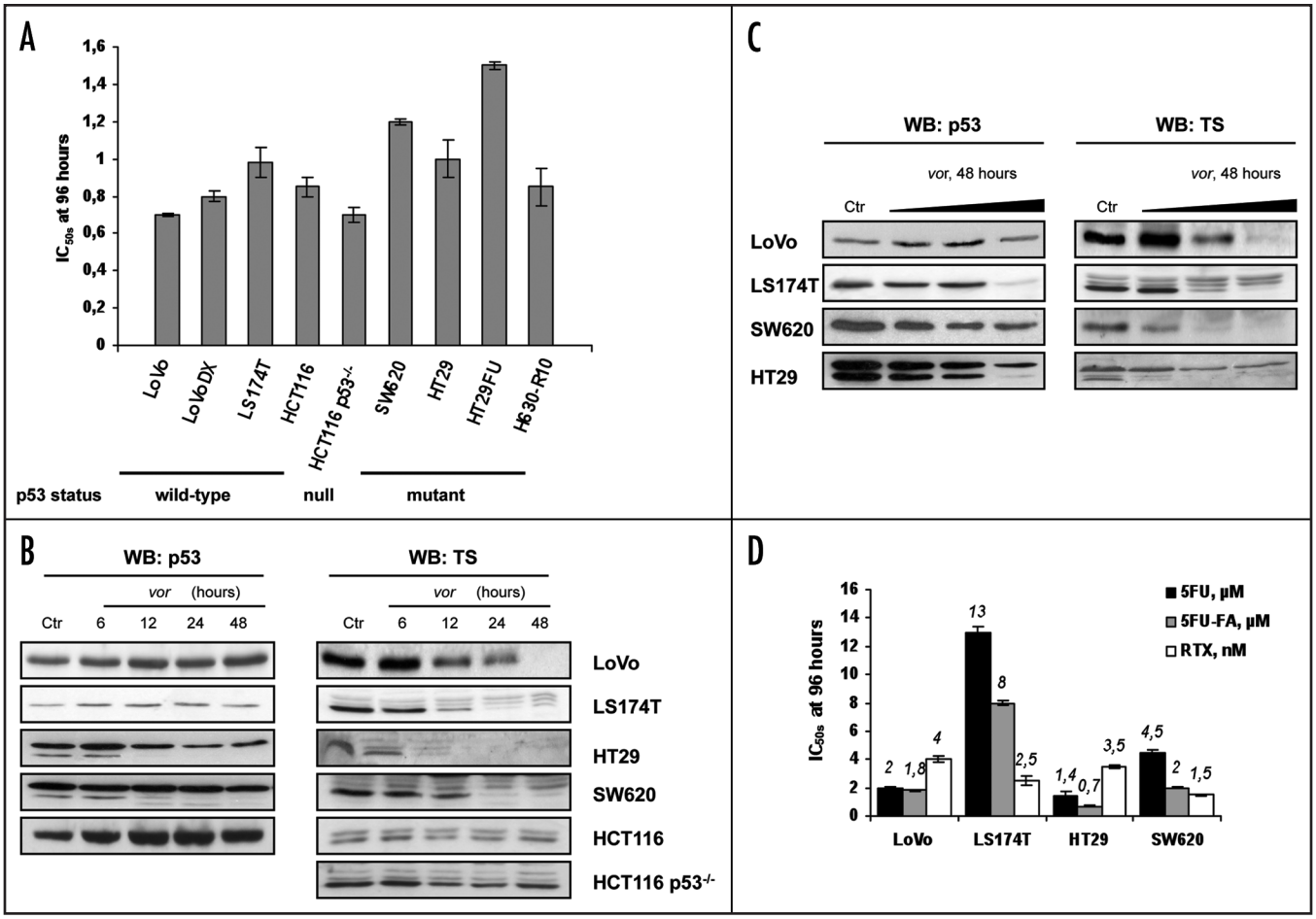

Figure 1. Effects of vorinostat on cell growth and expression of p53 and on TS. (A) IC_{50} values computed at 96 h of treatment (IC_{50}^{96h}) with vorinostat on CRC cell lines with different p53 status. Data represent the means from at least three different experiments performed in quadruplicates, *bars*, SD. Cell growth assessment was performed by SRB colorimetric assay as described in "Materials and Methods." p53 and TS proteins expression analyzed by western blotting in LoVo, LS174T, HT29, SW620, HCT116 and HCT116 $p53^{-/-}$ cells treated with vorinostat at concentration corresponding to IC_{50}^{72h} and harvested at indicated times (B) or treated with increasing doses of vorinostat for 48 h (in lane 3 cells were treated at dose corresponding to IC_{50}^{72h}) (C). 50 µg of whole cell lysates were resolved by 10%-SDS-PAGE and detected with specific antibody. Ponceau staining ensured the equal loading of samples in each lane. *Ctr*, untreated cells; *vor*, vorinostat-treated cells. (D) IC_{50}^{96h} values for 5FU, 5FU-FA and RTX in LoVo, HT29, SW620 and LS174T CRC cell lines. Data represent the means from at least three different experiments performed in quadruplicates, *bars*, SD.

for vorinostat/RTX combination treatments, however, synergistic antiproliferative effect and schedule-dependency was less evident compared to vorinostat/5FU-FA combination. Furthermore, in all cell lines, we observed from 0.95 to 15 magnitude (fold) of dose reduction in the IC_{50} values (DRI_{50}) of either vorinostat, or 5FU-FA, or RTX, in combination setting compared with the concentrations of the drugs used alone. Again, best values of DRIs were observed for simultaneous treatments or when vorinostat preceded either 5FU-FA or RTX (Table 1). Taken together these results showed that scheduling appears to be critical for the potentiating antitumor effect of vorinostat on TS targeting agents such as 5FU-FA or the pure inhibitor RTX.

Effects of vorinostat plus 5FU-FA combination treatment on TS and p53 proteins expression. To explore the mechanism involved in the synergistic interaction between vorinostat and 5FU-FA, we analyzed the effect of each agent, alone or in combination, and for all three different sequences of treatment described before, on TS and p53 proteins expression, in both LoVo and HT29 cell lines. In order to appreciate differences between the effects of combinations versus each drug alone, we have used low doses (IC_{30}) of both vorinostat and 5FU-FA. The inhibition of TS upon the formation of a ternary complex between FdUMP, TS and the methyl donor, as well as the increase of TS level, are common findings after 5FU treatment.[17] In both LoVo and HT29 cell lines we demonstrated that 5FU-FA induced within 24 h a clear ternary complex, as shown by the appearance of the upper 38 Kd band[17] (Fig. 2A and B). Vorinostat as single agent downregulates TS expression and in combination treatment antagonized 5FU-FA-induced TS upregulation. This effect is visibly evident when the two drugs were given simultaneously (Fig. 2A) and when vorinostat preceded 5FU-FA exposure (*Sequence II*, Fig. 2B), but it is less evident when 5FU-FA preceded vorinostat exposure (*Sequence III*, Fig. 2B). However, it should be pointed out that the formation of the ternary complex is always achieved in combination treatments,

Table 1 **CI and dose reduction index (DRI) values for vorinostat (vor) and 5FU-FA or RTX sequence combination treatments**

Cell lines	p53 status	Treatment	Sequence	$CI_{50} \pm SD$[a]	DRI ± SD[b]	
					vor	5FU-FA or RTX
LoVo	**wt**	vor + 5FU-FA	I	0.70 ± 0.15	2.85 ± 0.49	2.80 ± 1.69
		vor -> 5FU-FA	II	0.76 ± 0.13	2.10 ± 0.56	3.74 ± 3.16
		5FU-FA -> vor	III	0.83 ± 0.01	1.75 ± 1.76	2.90 ± 1.40
		vor + RTX	I	0.79 ± 0.09	2.36 ± 1.60	4.70 ± 3.20
		vor -> RTX	II	0.83 ± 0.09	1.50 ± 0.14	6.33 ± 6.03
		RTX -> vor	III	0.87 ± 0.27	1.55 ± 0.07	6.40 ± 3.39
LS174T	**wt**	vor + 5FU-FA	I	0.55 ± 0.21	2 ± 1.27	3.05 ± 0.49
		vor -> 5FU-FA	II	0.47 ± 0.13	2.23 ± 1.65	4.35 ± 0.35
		5FU-FA -> vor	III	0.99 ± 0.23	1.94 ± 1.08	3.4 ± 0.28
		vor + RTX	I	0.49 ± 0.22	1.49 ± 0.15	15.34 ± 10.06
		vor -> RTX	II	0.69 ± 0.12	1.53 ± 0.03	10.85 ± 6.26
		RTX -> vor	III	0.76 ± 0.11	1.36 ± 0.27	10.55 ± 6.88
HT29	**mut**	vor + 5FU-FA	I	0.48 ± 0.09	1.93 ± 0.02	6.6 ± 3.8
		vor -> 5FU-FA	II	0.73 ± 0.02	1.25 ± 0.41	9.2 ± 5.7
		5FU-FA -> vor	III	0.98 ± 0.10	1.1 ± 0.33	6.7 ± 2.6
		vor + RTX	I	0.63 ± 0.23	3.25 ± 2.6	6.23 ± 3.53
		vor -> RTX	II	0.7 ± 0.06	1.83 ± 0.70	5.65 ± 0.63
		RTX -> vor	III	1.05 ± 0.07	1.31 ± 0.35	9.03 ± 4.7
SW620	**mut**	vor + 5FU-FA	I	0.52 ± 0.17	2.45 ± 1.76	4.5 ± 0
		vor -> 5FU-FA	II	0.45 ± 0.21	3.67 ± 2.56	9 ± 5.09
		5FU-FA -> vor	III	0.82 ± 0.14	2.25 ± 1.76	3.95 ± 2.33
		vor + RTX	I	0.86 ± 0.35	1.75 ± 0.91	7.83 ± 6.73
		vor -> RTX	II	0.74 ± 0.28	1.5 ± 1.69	5.25 ± 0.21
		RTX -> vor	III	1.07 ± 0.32	0.95 ± 0.42	3.4 ± 1.27

[a]CIs were calculated for 50% of cell lethality (CI_{50}) by the Chou-Talalay equation. Means ± SD of at least three different experiments performed in quadruplicates. [b]DRI_{50} represents the order of magnitude (fold) of dose reduction obtained for 50% of cell lethality effect in combination setting compared with each drug alone. Means ± SD of at least three different experiments performed in quadruplicates.

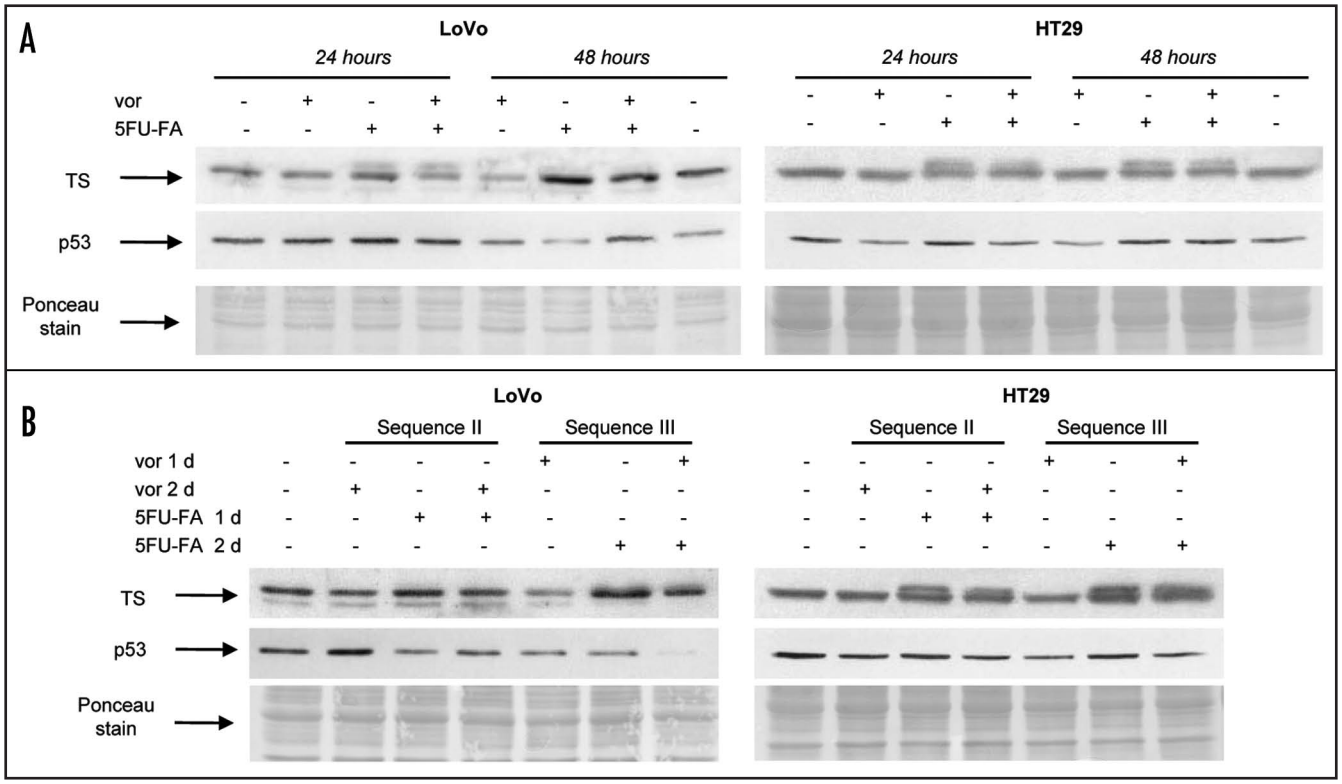

Figure 2. For figure legend, see page 786.

Figure 2. Effect of vorinostat (vor) and/or 5FU-FA on TS and p53 proteins expression in LoVo and HT29 cells. TS and p53 proteins expression were determined by western blotting in LoVo and HT29 cells untreated or treated with each drug alone (using IC_{30}^{72h}) or in combination. (A) Cells treated simultaneously were harvested after 24 and 48 h. (B) In sequential treatment cells were harvested after 48 h from the exposure to the first agent, consequently cells were exposed to either agent for 1 d or 2 d. 50 μg of whole cell lysates were resolved by 10%-SDS-PAGE, and then immuoblotted. Ponceau staining ensured the equal loading of samples in each lane.

indicating that vorinostat does not affect the biochemical inhibition of TS induced by 5FU-FA.

On the other hand, the regulation of p53 expression by vorinostat/5FU-FA combination demonstrated a different behaviour on the basis of p53 status. In wt-p53 LoVo cells vorinostat induced a slight upregulation of p53 expression and antagonized the 5FU-FA-induced downregulation of the protein when given simultaneously or when precede 5FU-FA exposure (Fig. 2A and B), while when vorinostat followed 5FU-FA we observed an evident downregulation of p53 with almost undetectable protein expression (Fig. 2B). On the contrary, in HT29 cells, we demonstrated that vorinostat downregulates the expression of mut-p53, as single agent or in combination with 5FU-FA, regardless of the schedule (Fig. 2A and B).

Taken together these results implies that vorinostat downregulates TS expression even in combination treatment but does not influence the biochemical inhibition of TS by 5FU-FA. Moreover, differential modulation of wt- and mut-p53 proteins by vorinostat, also in combination treatments, suggest potential additional mechanisms of the observed synergistic antitumor effect.

Cell cycle perturbations induced by vorinostat plus 5FU-FA combination treatment. To investigate whether the mechanism of the observed synergistic interaction might involve cell cycle perturbations, the effects of low doses (IC_{30}) of vorinostat and 5FU-FA, either alone or in combination, were assessed on cell cycle kinetic in LoVo and HT29 cell lines for all three different sequences of treatment described before. In both LoVo and HT29 cell lines and for all the three sequence of treatment tested, vorinostat did not induce major modifications while, as expected, 5FU-FA treatment induced a late S-phase arrest in LoVo and a clear early s-phase arrest in HT29 cells (Fig. 3A and B). However, when we combined 5FU-FA with vorinostat, we observed different patterns of cell cycle perturbation, depending on the sequence of treatment. In details, in LoVo cell line, we demonstrated that combination treatment *sequence I* determined 67% of cells arrested in middle- late-s phase, while in *sequence II* and *III* we observed a similar or faint increase of s-phase arrested cells, compared to 5FU-FA single agent treatment, respectively (Fig. 3A). In HT29 cell line we observed a strong perturbation of cell cycle in *sequence I*, with 65% of cells arrested in s-phase and undetectable cells in G_2/M phase, and in *sequence II,* with 80% of cells arrested in early s-phase; on the contrary in *sequence III* we observed a decrease of s-phase arrested cells compared with 5FU-FA single agent treatment, 58% vs. 71%, respectively (Fig. 3B).

In summary these data suggest that sequencing is also important for the type and the magnitude of cell cycle perturbations induced by vorinostat and 5FU-FA combination and that, at least in part, p53 status can influence these results.

Vorinostat reverted chemo-resistance to either 5FU or RTX in HT29FU and H630-R10 resistant cell lines. Intrinsic or acquired resistance to 5FU or to RTX is often related to TS protein overexpression. In order to verify if vorinostat can overcome resistance to either agent, we used two resistant cell models: HT29FU and H630-R10 cell lines. In HT29FU, a sub-line selected by adaptation to 5FU, showing basal high levels of TS,[36] we demonstrated eleven and five-fold higher IC_{50} values for 5FU-FA and for RTX, respectively, than parental HT29 cells (Figs. 4A vs. 1D). In H630-R10, a mut-p53 cell line carrying an amplified TS gene[29] we showed strong resistance to RTX, with mean IC_{50} value between 50- to 100-fold higher than those observed for the other CRC cell lines, while the IC_{50} value for 5FU-FA was comparable to those of the other cell lines (Figs. 4A vs. 1D). In both HT29FU and H630-R10 cells IC_{50} values for vorinostat were similar to those observed for the parental HT29 or the other cell lines examined (Fig. 1A). When we evaluated the simultaneous treatment of vorinostat plus 5FU-FA, or RTX, on cell proliferation, we demonstrated a synergistic antiproliferative effect in both cell lines, as shown by CI_{50} and DRI_{50} values reported in Figure 4B and C, respectively. In details, as shown in Figure 4C we observed in HT29FU cells more than 8-fold of reduction in the IC_{50} value (DRI_{50}) for 5FU-FA and of 30-fold for RTX, when combined with vorinostat. On the other hand, in H630-R10 we observed almost 25-fold of reduction in the IC_{50} value for RTX.

Notably, also in HT29FU cells vorinostat induced downregulation of TS (Fig. 4D) as well as of mutated p53 protein expression within 24 h. Furthermore, the marked upregulation of TS ternary complex and p53 expression induced by 5FU-FA treatment in HT29FU cells was strongly antagonized by vorinostat (Fig. 4E).

Taken together these results showed that simultaneous treatment with vorinostat can revert chemo-resistance to both 5FU-FA and RTX.

Discussion

In this study we have demonstrated that the HDAC-I vorinostat exerted potent antiproliferative effect in a panel of mut- and wt-p53 human CRC cell lines. Moreover, in combination with 5-FU modulated by FA (5FU-FA), or with RTX, both commonly used in the treatment of this disease, it showed a clear schedule-dependent synergistic antiproliferative interaction. In details, we showed that simultaneous exposure, as well as 24 h pretreatment with vorinostat followed by 5FU-FA or RTX, produced a synergistic antiproliferative effect independent from p53 status. Furthermore, we provided for the first time evidences that vorinostat can overcome resistance to both 5FU and RTX.

We also presented data demonstrating that the downmodulation of TS protein expression induced by vorinostat, in a

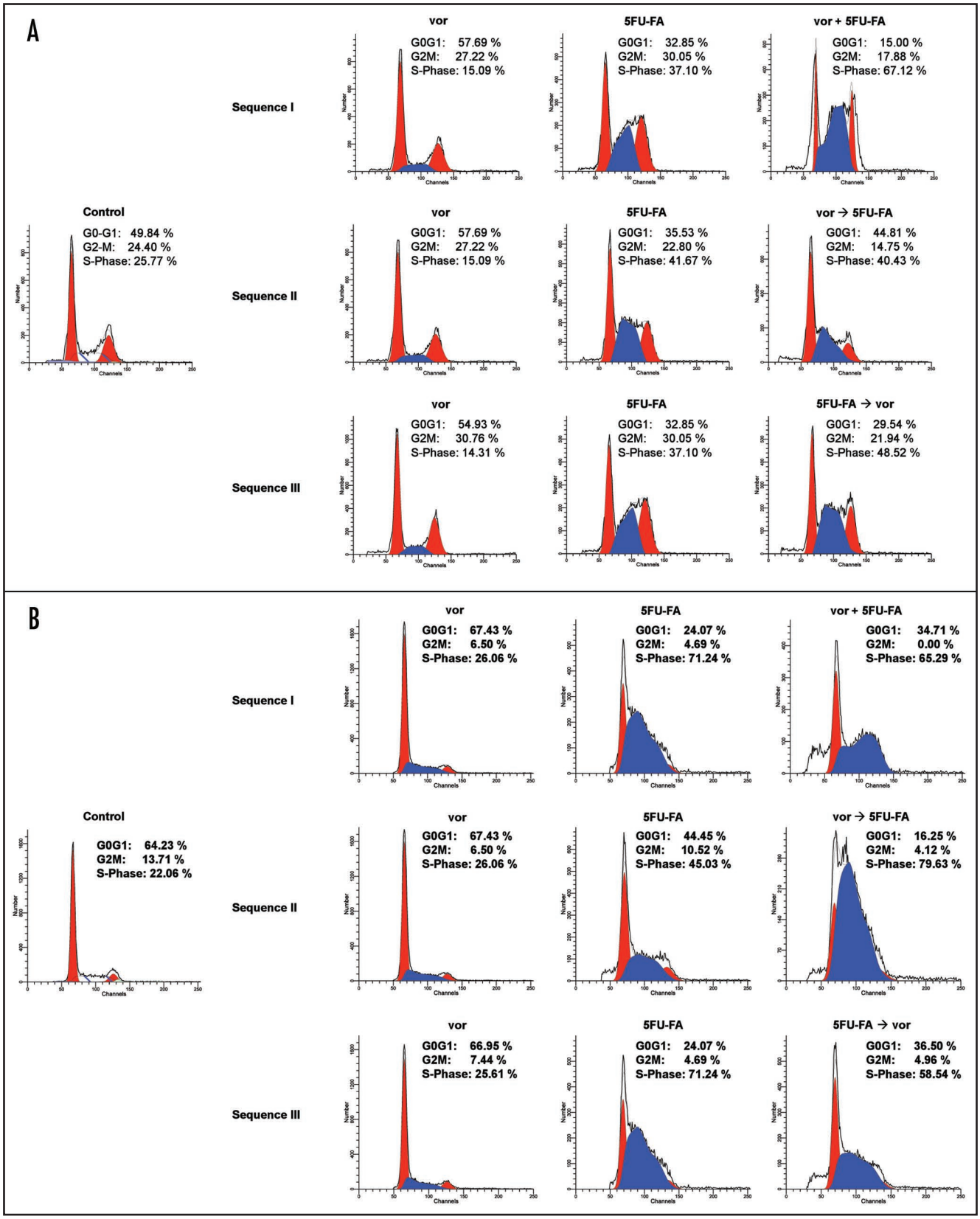

Figure 3. For figure legend, see page 788.

Figure 3. Effect of vorinostat and/or 5FU-FA on cell cycle kinetic of LoVo and HT29 cells. Cell cycle analysis was performed after PI staining on LoVo (A) and HT29 (B) cells exposed to vorinostat and 5FU-FA combination treatment for all three different sequences of treatment. Cells, treated with each drug alone, or in combination for all three schedules (*Sequence I* = simultaneous treatment; *Sequence II* = vorinostat preceded 5FU-FA exposure; *Sequence III* = 5FU-FA preceded vorinostat exposure) as described in Materials and Methods, were harvested after 72 h from the first treatment. Cells were treated with concentrations of each drug corresponding to IC_{30}^{72h} (vorinostat 0.5 μM and/or 5FU-FA 1 μM in LoVo cells; vorinostat 0.5 μM and/or 5FU-FA 0.5 μM in HT29 cells).

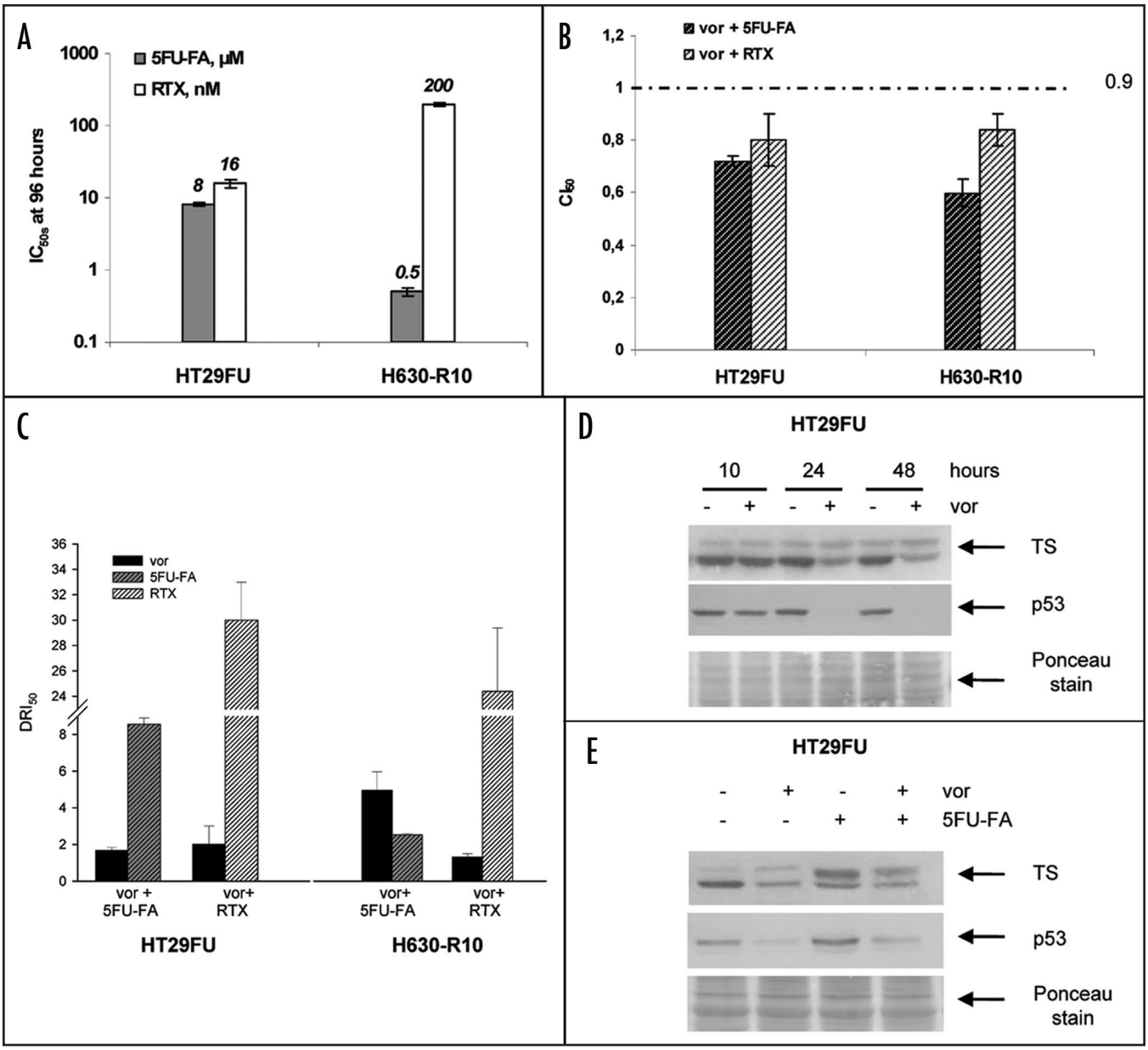

Figure 4. Effects of vorinostat in combination with 5FU-FA or RTX on resistant HT29FU and on H630-R10 cells. (A) IC_{50}^{96h} values for 5FU-FA and RTX in 5FU-resistant HT29FU cells and in H630-R10 cells. (B) CI values calculated at 50% of cell lethality (CI_{50s}) for vorinostat plus 5FU-FA or plus RTX combination treatments, in HT29FU and H630-R10 cell lines. (C) Dose reduction index values calculated at 50% of cell lethality (DRI_{50s}) for vorinostat plus 5FU-FA or plus RTX combination treatments, in HT29FU and H630-R10 cell lines. Data in A, B and C represent the means of at least three different experiments performed in quadruplicate; *bars*, SD. Western blot analysis of TS and p53 proteins expression in HT29FU cells, treated with vorinostat at IC_{50}^{72h} for the indicated time points (D), or simultaneously with vorinostat and 5FU-FA at IC_{30}^{72h} for 48 h (E). 50 μg of whole cell lysates were resolved by 10%-SDS-PAGE and detected with specific antibody. Ponceau staining ensured the equal loading of sample in each lane.

dose-dependent manner and within 24 h from beginning of treatment, represents a key factor in enhancing the effect of 5FU-FA and of a pure TS inhibitor such as RTX, in both sensitive and resistant tumor cells. Investigations on biochemical modulation of TS expression showed that vorinostat downregulated TS protein expression independently of p53 status, and in combination treatment inhibits TS induction by 5FU-FA in both sensitive and resistant cells. However, vorinostat did not affect the formation of the stable and inactive ternary complex between the 5FU-metabolite FdUMP, TS and the methyl donor CH2THF, indicating that it does not influence the biochemical inhibition of TS. Previous studies, demonstrated that TS mRNA and protein are both downregulated by HDAC-I through two independent mechanisms: at the transcriptional level and through modulation of protein degradation by a mechanism involving acetylation of the chaperone protein Hsp90.[14,15]

Moreover, in our study we demonstrated that p53 protein, whose functional wild-type expression is critical for drug sensitivity to TS inhibitors such as 5FU and RTX,[35] is upregulated by vorinostat in wt-p53 cells but downregulated in mut-p53 cells, as single agent or in combination treatment. It as been previously reported that p53 protein acetylation, upon HDAC inhibition, is essential for preventing the degradation and for leading to an open conformation that allowed the protein to bind DNA.[40] On the other hand, degradation of mutant p53 protein induced by vorinostat is in agreement with data from two other reports also showing depletion of mut-p53 by HDAC-Is.[41,42] In addition, it was demonstrated that a novel gain of function conferred by certain p53 mutants was linked to fluoropyrimidine chemoresistance,[34,43] and several clinical studies have revealed higher resistance to fluoropyrimidine therapy of tumors expressing p53 mutants.[33] More prominently, it was recently reported, in colon cancer cells transfected with mut-p53, an increased TS mRNA and protein expression as well as activity, associated with decreased sensitivity to 5-FU and antifolates, compared to the wild-type parental cells.[35]

On these basis we proposed that the opposite modulation of wt- and mut-p53 protein by vorinostat could be an additional mechanism explaining the synergistic interaction with TS inhibitors.

We have clearly shown that the downregulation of TS by vorinostat is evident in wild-type, mutant as well as null (-/-) p53 cells, suggesting that it is independent from p53 status. However, reciprocal regulation between TS and p53 has been demonstrated by different studies showing that TS can regulate p53 at the translational level[32] and that p53 can inhibit transcription from mouse TS promoter.[44,45] On this regard we can not exclude that the modulation of wt- and mut-p53 protein by HDAC-I can also affect TS expression and additional investigations are needed to clarify this aspect.

Intrinsic or acquired resistance to 5FU or RTX is often related to TS protein overexpression.[17] We demonstrated synergistic antiproliferative effect of vorinostat in combination with either 5FU-FA or RTX in HT29FU cells, selected for resistance to 5-FU and cross-resistant to RTX, and in mut-p53 H630-R10 cells carrying amplification of TS gene and strongly resistant to RTX. Notably, we have also showed that vorinostat strongly downregulated basal as well as 5FU-FA-induced mutant p53 and TS protein, confirming data observed in sensitive cells and suggesting potential mechanisms by which vorinostat may overcome the resistance to TS inhibitors.

Finally, we suggested that cell cycle perturbation could explain, at least in part, the schedule dependent-synergistic antiproliferative effect between vorinostat and 5FU-FA. In fact, if vorinostat/5FU-FA combination did not affect cell cycle perturbation typical of TS inhibition such as S-phase arrest, simultaneous treatment (*sequence I*), or vorinostat followed by 5FU-FA (*sequence II*), determined an increase and/or a different shape of S-phase cell cycle arrest, in both wt- and mut-p53 cells, compared to 5FU-FA treated cells. On the other hand, when 5FU-FA was applied first in combination treatment, we observed cell cycle kinetics similar to those seen in 5FU-FA treated cells, consistently with the additive antiproliferative effect observed.

Overall these results demonstrated that vorinostat had antiproliferative activity in cells derived from human CRC and can be combined with cytotoxic drugs currently used for this disease. Recent studies demonstrated that HDAC-Is can potentiate 5FU-induced inhibition of cell growth in colon and gastric cancer cells.[14,16] However, at least to our knowledge, our study is the first to demonstrate a synergistic effect of an HDAC-I such as vorinostat in combination with the pure inhibitor of TS RTX and with 5FU bio-modulated by FA (5FU-FA), the latter being the cornerstone of chemotherapy regimens in the colorectal cancer. We also provided evidences demonstrating that the mechanism of the synergistic interaction observed lay in the modulation of both TS and p53 protein expression. Finally, our study was also the first to show that vorinostat can overcome resistance to 5FU or RTX. We have recently shown preclinically and clinically the feasibility of 5FU-FA and RTX combination treatment in colon cancer[38,46] and it appears intriguing to test both agents in combination with vorinostat. In conclusion, although a gap exists between bench and bed side, these data may be useful for the design of future clinical trials combining HDAC-I and 5FU-FA or/and RTX.

Materials and Methods

Materials. Clinical grade vorinostat was provided by Merck & Co. Inc., (Rahway, NJ USA). Stock solutions were prepared in DMSO and diluted to appropriate concentrations in culture medium before addition to the cells. 5FU was supplied by Teva Pharmaceutical Industries Ltd., (Netanya, Israel), FA was obtained from Wyeth Pharma GmbH (Münster, Germany), RTX was supplied by AstraZeneca (Macclesfield, UK). TS antibody was supplied by Rockand Immunochemicals, Inc., (Gilbertsville, PA USA); p53 antibody was supplied by Monosan (Uden, Netherlands). Sulforhodamine B (SRB) was obtained from ICN Biomedicals (Irvine, CA USA). Enhanced chemiluminescence (ECL) immunodetection reagents were from GE Healthcare (Milan, Italy). All media, serum, antibiotics and glutamine were from Cambrex Bio Science (Verviers, Belgium).

Cell culture and cell proliferation assay. LoVo, SW620, HT29 and LS174T cell lines were all from American Type Culture Collections (Rockville, MD USA), doxorubicine resistant LoVo cells (LoVo-DX) were kindly provided by Dr. G.

Zupi, (IFO, Rome, Italy), TS-overexpressing H630-R10 cell line were kindly provided by Dr. Peters (VU University Medical Center, Amsterdam, Netherlands), HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ were a gift from Dr. Vogelstein (Johns Hopkins University, Baltimore, MA). 5FU-resistant HT29 cell line (HT29FU) were selected by continuous exposure of sensitive HT29 cells to step wise increasing concentrations of 5FU; after several passages we selected the surviving cells that had similar doubling time of parental sensitive HT29 cells but showed an IC_{50} value for 5FU >11-fold higher.[36] LoVo, LoVo-DX, HCT116 $p53^{+/+}$ HCT116 $p53^{-/-}$ and H630-R10 cell lines were grown in RPMI 1640 medium, whereas HT29, HT29FU, SW620 and LS174T were grown in Dulbecco's Modified Eagle's medium (DMEM). Both media were supplemented with 10% heat-inactivated fetal bovine serum, 50 units/mL penicillin, 500 μg/mL streptomycin and 4 mmol/L glutamine in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell survival/proliferation was measured in 96-well plates by a spectrophotometric dye incorporation assay using SRB, as previously described.[37] Treatment schedule was performed throughout all the experiments as follows: 24 h after seeding (1 x 10^3 cells/well), cells were exposed to the indicated drugs and cell growth assessment was performed at indicated time points. Since FA was always tested at 10 μM, and it did not exhibit any effect on cell proliferation when given alone, 5FU-FA was considered as a single drug. Experimental conditions were tested in quadruplicates, and experiments were performed at least three times for each cell line.

Protein extraction and western blotting. Cells grown and treated as indicated, were collected, lysed, separated on SDS polyacrilamide gel electrophoresis (PAGE) as described elsewhere.[37] After electrophoresis, proteins were transferred to nitrocellulose paper, immunoblotted with specific antibodies and probed with the appropriate horseradish peroxidase-linked IgG. Immunoreactive bands were detected by ECL.

Drugs combination studies. Drug combination studies in vitro were based on concentration-effect curves generated as a plot of the fractions of unaffected (surviving) cells versus drug concentrations. Serial dilutions of the equipotent doses of the two agents in combination (vorinostat and 5FU-FA or RTX) were tested in three different sequences of treatment. *Sequence I*: vorinostat and 5FU-FA or RTX were given together 24 h after seeding and cell growth assessment was performed after 96 h; *sequence II*: vorinostat was added 24 h after seeding followed by 5FU-FA or RTX after 24 h and cell growth assessment was performed after 72 h (cells were exposed for 96 h to vorinostat and for 72 h to 5FU-FA or RTX); *sequence III*: 5FU-FA or RTX was added 24 h after seeding followed by vorinostat after 24 h (cells were exposed for 96 h to 5FU-FA or RTX and for 72 h to vorinostat).

Synergism, additivity, or antagonism was quantified by determining the combination index (CI) calculated by the Chou-Talalay equation as described elsewhere.[37-39] The CI < 0.9, CI = 0.9–1.2, and CI > 1.2 indicate synergistic, additive or antagonisic effect, respectively. Dose reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone. The linear correlation coefficient (*r*) of the median-effect plot is considered a measure of conformity of the data according to the mass-action law principle when the experimental measurement is assumed to be accurate. A *r* value equal to 1 indicates perfect conformity while a poor value may be the result of biological variability or experimental deviations. For all our experiments *r* values were between 0.91 and 0.98 indicating a good conformity of the data.

Analysis of cell cycle kinetic. Analysis of cell cycle kinetic was performed at indicated times on LoVo and HT29 cells treated with vorinostat and/or 5FU-FA or RTX in the three different sequences of treatment, as previously reported.[37] Briefly, adherent and floating cells were harvested, fixed in 70% ethanol and stored at -20°C until analysis. After nuclear DNA staining with propidium iodide, flow cytometry was done in duplicate by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). For each sample, 20,000 events were stored and cell cycle analysis was done by the ModFit LT software (Verity Software House, Inc., Topsham, ME). FL2 area versus FL2 width gating was done to exclude doublets from the G_2-M region.

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