DOI: 10.3727/096504017X14841698396900

EU-1006 provisionally accepted 1/2/2017 for publication in *Oncology Research*

Simultaneous, But Not Consecutive, Combination With Folinate Salts Potentiates 5-fluorouracil Antitumor Activity In Vitro and In Vivo

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Running Title: Folinate salts and 5-fluorouracil combination

ABSTRACT

The combination of folinate salts to 5-fluoruracil (5-FU)-based schedules is an established clinical routine in the landscape of the colorectal cancer treatment. The aim of this study was to investigate the pharmacological differences between the sequential administration of folinate salts (1 h before, as in clinical routine) followed by 5-FU and the simultaneous administration of both drugs. Proliferation and apoptotic assays were performed on human colon cancer cells exposed to 5-FU, calcium (CaLV) or disodium (NaLV) levofolinate or their simultaneous and sequential combination for 24 and 72h. *TYMS* and *SLC19A1* gene expression was performed with real time PCR. *In vivo* experiments were performed in xenografted nude mice, treated with 5-FU escalating doses and CaLV or NaLV alone or in simultaneous and sequential combination. The simultaneous combination of folinate salts and 5-FU was synergistic (NaLV) or additive (CaLV) in a 24h treatment in both cell lines. In contrast, the sequential combination of both folinate salts and 5-FU was antagonistic at 24 and 72h. The simultaneous combination of 5-FU and NaLV or CaLV inhibited *TYMS* gene expression at 24h, whereas the sequential combination reduced *SLC19A1* gene expression. *In vivo* experiments confirmed the enhanced antitumor activity of the 5-FU+NaLV simultaneous combination with a good toxicity profile, whereas the sequential combination with CaLV failed to potentiate 5-FU activity. In conclusion, only the simultaneous, but not the consecutive, *in vitro* and *in vivo* combination of 5-FU and both folinate salt formulations potentiated the antiproliferative effects of the drugs.

Key words: colon cancer; 5-fluorouracil; calcium levofolinate; disodium levofolinate; synergism; *TYMS; SLC19A1*

INTRODUCTION

5-Fluorouracil (5-FU) and other fluoropyrimidines (e.g. capecitabine, UFT, S-1, TAS-102), still represent essential drugs in combination regimens for the adjuvant or first/second line treatment of solid tumors such as colorectal neoplasms [1-3]. Their main mechanism of action involves the inhibition of thymidylate synthase (TS) by the active metabolite 5-fluoro-deoxyuridine-monophosphate (5-FdUMP) in the presence of folate (5,10-methylenetetrahydrofolate), a required cofactor for the reaction [1]. The formation of the ternary complex 5-FdUMP/TS/folate with covalent bonds leads to the stabilization of the complex itself, with prolonged inhibition of TS catalytic activity [1,4]. On the basis of these observations, the modulation of inhibitory effects of the fluoropyrimidines by folinate salts has been developed in preclinical and clinical settings, showing a preclinical enhancement [5-7] and a better clinical response to 5-FU/levofolinate combined treatment compared to the fluoropyrimidine alone [8].

Unfortunately, the simultaneous use of folinic acid salified with calcium, and mixed with the 5-FU, determined the precipitation of the formed calcium carbonate [9] and subsequent catheter obstruction [10-12]. Indeed, soon it became evident in patients that the simultaneous 5-FU and calcium levofolinate (CaLV) mix and infusion in a single catheter was impossible, and 5-FU and CaLV were infused with the aid of two separate pumps [10]. Thereafter, a sequential administration CaLV followed by 5-FU (administered first as bolus injection, then as continuous or chronomodulated infusions) was adopted in the clinical routine and now represents the standard therapeutic scheme to which new drugs have been added over the years, such as irinotecan, oxaliplatin, cetuximab and bevacizumab [13].

At the beginning of 2000s, the therapeutic armamentarium included both the CaLV and the newer formulation disodium levofolinate (NaLV) [14,15]. The latter showed comparable efficacy to the CaLV, with an objective response rate of 37.2 % in patients with advanced rectal cancer [14]. However, it was noted that in 51 evaluable patients, the

use of NaLV seemed favorably associated with a best time to progression [14]. More recently, a phase II randomized study of combined infusional NaLV and 5-FU *vs.* the CaLV followed by 5-FU, both in combination with irinotecan or oxaliplatin in patients with metastatic colorectal cancer, showed the same overall response rate in both the study arms with a comparable safety [16]. The favorable toxicity profile of the simultaneous clinical combination of 5-FU and NaLV was also recently confirmed in a 24h-infusion schedule [17]. The NaLV formulation can be easily administered simultaneously to 5-FU admixed in one ambulatory pump because it does not precipitate in the solution. This characteristic may have several clinical and nursing advantages, such as a shortening of the time necessary for the administration of drugs (with a consequent decrease in human resources) and a reduced discomfort for the patient [18], if compared to the sequential administration of CaLV followed by 5-FU.

The aims of the present study were i) to determine the effects of simultaneous administration of 5-FU and NaLV or CaLV on colon cancer cells compared to the sequential combination of the two drugs (CaLV or NaLV 1 h treatment before 5-FU administration), investigating both the antiproliferative and pro-apoptotic effects, and the changes in *Thymidylate Synthetase (TYMS)* and *Solute Carrier Family 19 Member 1* (*SLC19A1)* gene expression; ii) to evaluate the *in vivo* activity of simultaneous/consecutive administration of 5-FU and CaLV or NaLV on human colon cancer xenografts.

MATERIALS AND METHODS

Drugs, Cell Lines and Reagents

5-FU, NaLV and CaLV were generously provided by MEDAC (Wedel, Germany). The drugs were dissolved in sterile water at a concentration of 1 mM before their in vitro and in vivo use.

The human colon cancer cell lines HT-29 (*B-raf* mutated) and Caco-2 (wild type) were used between passage 2 and 5 from the original vial obtained by the American Type Culture Collection (Rockville, USA). Cells were cultured in sterile flasks of 75 cm^2 at 37 °C and 5% CO_2. Cells were maintained in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy), supplemented with penicillin 50 IU/ml, streptomycin 50 µl/ml, and with 10% fetal bovine serum (FBS) for the entire duration of *in vitro* experiments.

Each cell line was characterized for polymorphisms in the promoter region of the TS gene (*thymidylate synthase enhancer region*, TSER) by PCR amplifying a fragment containing 2 (TSER*2, 2R) or three repeats (TSER*3, 3R) of 28 bp in a thermocycler ABI PRISM 9700 (Life Technologies, Thermo Fisher Scientific Corporation, Carlsbad, USA) using the following primers [19]:

forward 5'- **GTGGCTCCTGCGTTTCCCCC** -3';

reverse 5'- **TCCGAGCCGGCCACAGGCAT** -3'.

The PCR analysis was performed in a total volume of 25 µl containing 200 ng DNA, 2 µl of 10 mM each primer, 0.5 µl of 10 mM deoxynucleotide Mix (Sigma-Aldrich), 0.15 U of 5U/µl GoTaq® DNA Polymerase (Promega, Milan, Italy), 2.5 µl of 5x Buffer with MgCl2 (Promega, Milan, Italy) and water DNase/RNase free. Cycling conditions were 1 cycle at 94°C for 5 min with hot start; 30 cycles of 40 sec at 94°C, 1 min at 62°C and 40 sec at 72°C; and 1 cycle elongation for 5 min at 72°C [20]. PCR products were maintained at 4 °C until electrophoresis on 3% agarose gel labeled with ethidium bromide.

Antiproliferative Effects of Drugs

Cells were seeded into sterile 24-well plates ($3x10^6$ cells/well). After 24h cells were treated with 5-FU, CaLV and NaLV (from 0.01 to 100 µM) for 24, 48, and 72h, whereas a group of control cells were exposed to vehicle alone (saline). After exposure, the cells were detached and viable cells were counted by the ADAM-MC cell

counter (Digital Bio, NanoEnTek Inc., Seoul, South Korea) as previously described [21]. Results were expressed as a percentage of the vehicle-treated control. The concentration level of the drugs which induced 50% of cell proliferation inhibition (IC_{50}) was calculated using the software GraphPad Prism v. 5.0 (GraphPad Software Inc., San Diego, CA, USA) with a non-linear regression method. Each experiment was conducted in triplicate, considering 9 wells for each drug concentration tested.

In Vitro Combination Studies

To evaluate the type of interaction (i.e. synergistic, additive, and antagonistic) between the drugs, 5-FU combined with NaLV or CaLV was explored with two different treatment schedules for 24h and 72h at a fixed molar concentration ratio of 1:1 in HT-29 and Caco-2 cells, as follows: (**A**) a simultaneous exposure with 5-FU (0.1-100 µM) plus NaLV or CaLV (0.1-100 µM) for 24h or 72h; (**B**) a sequential exposure with NaLV or CaLV (0.1-100 µM) given alone for 1 h, then 5-FU (0.1-100 µM) plus NaLV or CaLV (0.1-100 µM) were added for 24h or 72h. After drug exposure, the media of cell cultures were discarded and cells counted. Each experiment was conducted in triplicate, considering 9 wells for each drug concentration tested.

The effect of combination treatments was then assessed by the software CalcuSyn v. 2.0 (Biosoft, Cambridge, UK) using the method of Chou [22]. Briefly, the synergistic, additive or the antagonistic effect for 5-FU plus NaLV or CaLV was calculated on the basis of the multiple drug-effect equation, and quantified by the combination index (CI), where CI<1, CI=1 and CI>1 indicate synergism, additive effect and antagonism, respectively. Based on the classic isobologram, the CI value was calculated according to the following formula:

$CI = [(D)_1/(Dx)_1] + [(D)_2/(Dx)_2]$

where $(Dx)_1$ and $(Dx)_2$ are the IC_{50} values of the two drugs used alone, whereas $(D)_1$ and $(D)_2$ are the concentrations of the two drugs in combination to inhibit 50% cell growth.

Evaluation of Apoptosis

To quantify the apoptosis induced by the aforementioned pharmacological treatments, HT-29 and Caco-2 cell lines were treated for 24h alone and in combination 5-FU 2 µM, NaLV 2 µM and CaLV 2 µM (at the fixed molar concentration ratio of 1:1). After incubation with drugs, cells were collected and analyzed using the Cell Death Detection ELISA Plus Kit (Roche, Basel, Switzerland) as *per* manufacturer's instructions. The optical density was measured using a Multiskan Spectrum microplate reader (Thermo Labsystems, Milan, Italy) set to a wavelength of 405 nm (with a wavelength of 490 nm correction). All experiments were repeated three times with at least three replicates *per* sample.

Evaluation of TYMS and SLC19A1 Gene Expression

The assessment of *TYMS* and *SLC19A1* gene expression in tumor cells - exposed for 24h alone and in combination with 5-FU 2 µM, NaLV 2 µM and CaLV 2 µM (at the fixed molar concentration ratio of 1:1) - was conducted measuring mRNA concentrations by using the polymerase chain reaction after reverse transcription (RT-PCR). The mRNA concentration (µg/µL) was determined by measuring the absorbance at a wavelength of 260 nm. The reverse transcription reaction was carried out in a volume of 20 µl of a mixture containing the extracted RNA (2 µg), dNTP (0.8 mM), oligo (DT6) primers (50 µg/µL), MLV-RT (10 U/µL), 5x RT buffer, DTT (5 mM) and RNase inhibitor (2.5 U/µL). Reaction mixture was incubated at 37 °C for 1 hour in a ABI PRISM 9700 thermocycler (Life Technologies), and the cDNA was stored at -20 °C.

The quantification of gene expression (RT-PCR) of *TYMS* (Assay ID Hs00426586_m1), *SLC19A1* (Assay ID Hs00953344_m1) and the endogenous control gene GAPDH (Assay ID 4326317E) was performed by using TaqMan primers and probes (Life Technologies). Amplification reactions were performed in triplicate in 96-well plates in a total volume of 25 µl, containing cDNA equivalent to 20 ng of total RNA (5 µL), primers at optimal concentrations, 100 nM probe, and 12.5 µl of TaqMan Universal PCR master mix (Life Technologies). PCR was performed according the following steps: 50 °C/2 min, 95 °C/10 min, then 40 cycles at 95 °C/15 sec and 60 °C/1 min. Data were analyzed by PCR using the ABI PRISM program 7900HT Sequence Detector Software (Life Technologies). All experiments were repeated three times with at least three replicates *per* sample.

The PCR thermal cycling conditions and the optimization of primer concentrations were as per the manufacturer's instructions. Amplifications were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the quantification of gene expression was performed using the ΔΔCt calculation, where Ct is the threshold cycle. The amount of target, normalized to the endogenous control and relative to the calibrator (i.e., vehicle-treated control cells), was calculated using the formula $2^{-\Delta\Delta}$Ct.

Animals

Athymic Nude-Foxn1^{nu} male mice, weighing 25 g, were supplied by Envigo (Milan, Italy) and were allowed unrestricted access to sterile food and water. Housing and all procedures involving animals were performed according to the protocol firstly approved by the Academic Organization Responsible for Animal Welfare (OPBA, Organismo Preposto per il Benessere Animale) of the University of Pisa, in accordance with the Italian law D.lgs. 26/2014, and by the Italian Ministry of Health (authorization

number 557/2015-PR). Each experiment employed the minimum number of mice needed to obtain statistically meaningful results.

Subcutaneous HT-29 Xenografts

Animals were anesthetized with a combination (1:1) of tiletamine and zolazepam (Zoletil®) 50 mg/kg i.p. before inoculation procedure. HT-29 cell viability was assessed by trypan blue dye exclusion, and, on day 0, $1.3\times10^6\pm5\%$ cells *per* mouse were inoculated subcutaneously between the scapulae in 0.2 ml of culture medium without FBS, using an insulin syringe with a 0.5×16 mm needle. Animal weights were monitored and upon the appearance of a subcutaneous mass, tumor dimensions were measured using calipers (by two trained and expert technicians, independently). Caliper measurements were carried out every two days to determine tumor growth and tumor volume. Tumor volume (mm^3) was measured as follows: [(w_1 x w_1 x w_2) x ($\pi/6$)], where w_1 and w_2 were the smallest and the largest tumor diameter (mm), respectively. The mice were randomized into groups of six animals (n=6) and were administered with the experimental schedules when the mean of tumor volumes was around 100 mm^3. Data analysis was conducted by an investigator blinded to which group of animals represented treatments or controls.

Three different experiments were performed using escalating doses of 5-FU. In the first experiment, animals were randomized in four groups: group 1 (control group) was administered with vehicle alone (saline); group 2 received 5-FU 50 mg/kg i.p. once a week; group 3 received the CaLV 50 mg/kg i.p. once a week (slow infusion), followed 1h later by 5-FU 50 mg/kg i.p.; group 4 was treated with 5-FU 50 mg/kg i.p and NaLV 50 mg/kg, simultaneously in the same syringe. In the second experiment, animals were randomized in five groups: group 1 (control group) was administered with vehicle alone (saline); group 2 received 5-FU 100 mg/kg i.p. once a week; the group 3 received the CaLV 50 mg/kg i.p. once a week (slow infusion), followed 1h later by 5-FU 100 mg/kg

i.p.; group 4 and group 5 were treated with the simultaneous combination of 5-FU 100 mg/kg i.p plus NaLV 50 mg/kg (in the same injection) or CaLV 50 mg/kg i.p. (in separate injections), respectively. In the last experiment, animals were randomized in five groups: group 1 (control group) was administered with vehicle alone (saline); group 2 received 5-FU 150 mg/kg i.p. once a week; group 3 received the CaLV 50 mg/kg i.p. once a week (slow infusion), followed 1h later by 5-FU 150 mg/kg i.p.; group 4 and group 5 were treated with the simultaneous combination of 5-FU 150 mg/kg i.p plus NaLV 50 mg/kg (in the same injection) or CaLV 50 mg/kg i.p. (in separate injections), respectively. At the end of the study, animals were sacrificed with an overdose of anesthetic.

Statistical Analysis

Analysis of variance (ANOVA), followed by Student-Newman-Keuls test was used to compare data among different groups in *in vitro* and *in vivo* experiments. *P* values less than 0.05 were considered significant. Statistical analyses were performed using the GraphPad Prism software package version 5.0.

RESULTS

5-FU Inhibited Tumor Cell Proliferation in a Time- and Concentration-Dependent Manner

The analysis of DNA extracted from both HT-29 and Caco-2 cells revealed a TS promoter genotype of TSER 2/2.

5-FU has shown a significant time- and concentration-dependent inhibitory activity on cell proliferation. Indeed, the maximum effect of 5-FU was obtained in HT-29 cells at 72h (IC_{50}s 0.45±0.01 µM), whereas the Caco-2 cell line was slightly less sensitive to 5-FU concentrations at all the time points (e.g., 1.32±0.50 µM at 72h). **Table 1** reports all the calculated IC_{50}s at 24, 48 and 72h for the 5-FU drug in both the

colon cancer cell lines. The *in vitro* NaLV and CaLV IC_{50}s were experimentally achieved in order to calculate the synergistic, additive or antagonistic interaction between drugs, but resulted extremely high (>50 µM) in both cancer cell lines at all the time points (data not shown).

In Vitro Studies of Simultaneous or Consecutive Combination of 5-FU and NaLV or CaLV After 24 or 72 h Exposure

In order to mimic the clinical settings of the simultaneous infusion of NaLV plus 5-FU and the consecutive administration of CaLV 1h before of 5-FU injection, both the *in vitro* schedules were studied at 24h and 72h.

Synergistic and Additive Effects of Simultaneous Combination of 5-FU and NaLV or CaLV After 24 h Exposure

Simultaneous exposure of HT-29 and CaCo-2 cells to different concentrations of 5-FU and NaLV for 24h showed a strong synergism for 50% fraction of affected cells (CI<1, **table 2**). Interestingly, also the simultaneous treatments of 5-FU and CaLV for 24h in both CaCo-2 and HT-29 cells had additive effects for 50% fraction of affected cells (CI equal or around 1, **table 2**).

Antagonistic Effects of the Sequential Treatment of NaLV or CaLV (1 h) Followed by 5-FU After 24 h Exposure

Sequential exposure of HT-29 cells to different concentrations of NaLV (1 h before) and then 5-FU for 24 h showed an unexpected antagonism for all the percentages of fraction affected of cells (CI>1, **table 2**). Furthermore, also the sequential treatments of CaLV (1 hour) followed by 5-FU for 24h in HT-29 cells were strongly antagonist for 50% fraction of affected cells (CI>1, **table 2**).

Different Effects of the Simultaneous Treatments of 5-FU and NaLV or CaLV After 72 h Exposure in the Two Cell Lines

Simultaneous exposure of Caco-2 and HT-29 cells to different concentrations of 5-FU and NaLV for 72h showed a synergistic effect for 50% fraction of affected cells (CI<1, **table 2**). In contrast, the simultaneous treatments of 5-FU and CaLV for 72h in Caco-2 cells resulted frankly antagonistic (CI>1, **table 2**), whereas a moderate synergistic effect was maintained in the more sensitive HT-29 cell line (**table 2**).

Antagonistic Effects of the Sequential Treatment of NaLV or CaLV (1 h) Followed by 5-FU After 72 h Exposure

Sequential exposure of Caco-2 and HT-29 cells to different concentrations of NaLV (1 h before) and then 5-FU for 72h showed a marked antagonism for 50% fraction of affected cells (CI>1, **table 2**). Moreover, also the sequential treatments of CaLV (1 hour) followed by 5-FU for 72h in both cell lines were highly antagonist for half of affected cells (CI>1, **table 2**).

Induction of Apoptosis by the Simultaneous Combination of 5-FU and NaLV or CaLV in Colon Cancer Cells

The extent of DNA fragmentation was significantly higher after 24h in HT-29 cells treated with the simultaneous combination of 5-FU and NaLV (**figure 1A**; **P*<0.05 *vs*. vehicle-treated cells) and, although in a minor extent, with the simultaneous combination of 5-FU and CaLV (**figure 1B**; **P*<0.05 *vs*. vehicle-treated cells). Both NaLV and CaLV alone did not modulate the apoptotic process after 24h. Interestingly, both the sequential combinations (CaLV or NaLV followed by 5-FU) slightly enhanced the presence of chromatin fragments but they did not reach a statistical significance if compared to Caco-2 and HT-29 vehicle-treated cells (**figure 1A** and **B**).

5-FU and NaLV or CaLV Simultaneous Combination Inhibited TYMS Gene Expression in Colon Cancer Cells

To study the effect of the treatment with 5-FU, NaLV and CaLV alone and in simultaneous or sequential combination on the variation of *TYMS* expression, the gene expression of the 5-FU target was quantified in HT-29 colon cancer cell line after 24h. **Figure 2** shows the significant inhibition (**P*<0.05 *vs.* vehicle-treated cells) of the *TYMS* expression of cancer cells by 5-FU treatment and, above all, by the simultaneous combination of 5-FU and NaLV or CaLV (although in a lesser extent). Both NaLV and CaLV alone seem not to change significantly the *TYMS* gene expression. The sequential combination of NaLV or CaLV (1 h) and then 5-FU did not significantly change the *TYMS* expression after 24h if compared to vehicle-treated cancer cells (**figure 2**).

NaLV or CaLV and 5-FU Sequential Combination Inhibited SLC19A1 Gene Expression in HT-29 Colon Cancer Cells

To investigate the effect of the treatment with 5-FU, NaLV and CaLV alone and in simultaneous or sequential combination on the variation of *SLC19A1* expression was quantified in HT-29 colon cancer cell line exposed for 24 hours. **Figure 3** shows the significant inhibition (**P*<0.05 *vs.* vehicle-treated cells) of the *SCL19A1* expression of cancer cells by the sequential NaLV (1h before) plus 5-FU treatment and, above all, by the sequential CaLV (1h before) plus 5-FU treatment. On the contrary, the simultaneous combination of NaLV or CaLV plus 5-FU did not significantly change the *SCL19A1* expression after 24h if compared to vehicle-treated cancer cells (**figure 3**), as well as the drugs administered alone.

HT-29 Colon Cancer Tumor Xenografts In Vivo Studies

To evaluate the in vivo effect of the treatments on subcutaneous HT-29 tumors, three different experiments were performed using escalating doses of 5-FU (50, 100 and 150 mg/kg) combined with NaLV (50 mg/kg) or CaLV (50 mg/kg) simultaneously, and in the case of CaLV also in a sequential manner.

Experiment With 5-FU Dose of 50 mg/kg

HT-29 cells injected s.c. in CD nu/nu mice grew quite rapidly and tumor masses became detectable 6-10 days after xenotransplantation. Tumors in control animals showed a progressive enlargement in their dimensions, and a mean volume of 3,774 mm^3 was reached at the end of the experimental period at day 25 (**Figure 4A**). Both 5-FU alone and the simultaneous combination of 5-FU+NaLV were able to significantly inhibit tumour growth, and their therapeutic effect was significant starting on the 4th day after the beginning of the therapeutic schedule as compared to controls (**Figure 4A**). However, the simultaneous combination maintained a significant inhibitory effect during all the experimental period. Indeed, the tumor growth curve of NaLV+5-FU showed a divergent profile from that of 5-FU alone starting at days 18. On the contrary, in the group of animals receiving the combined, but sequential, treatment with CaLV followed 1 hour later by 5-FU, the reduction in tumor growth was not present, and the profile of the curve was superimposable to the one of controls (**Figure 4A**). Animals of both controls and CaLV+5-FU combination groups were sacrificed at day 25 for the high tumor volumes. Interestingly, all the drug schedules showed a constant tumor increase in all the treated groups, but with a significant delay in the case of the 5-FU+NaLV combination treatment. The toxicity profile was favourable at this dose level and acceptable for all the treatment groups, with a small loss of weight throughout the course of the experiment (**Figure 4B**).

Experiment With 5-FU Dose of 100 mg/kg

Injected HT-29 cells grew rapidly, and tumor masses became detectable 6 days after xenotransplantation. A mean volume of 3,973 mm^3 was reached at day 34 when the animals of the control group were sacrificed (**Figure 5A**). Both 5-FU and the simultaneous association of 5-FU+NaLV were able to significantly inhibit the tumor growth, and their efficacy became significant starting on the seventh and the fifth day, respectively after the beginning of treatments as compared to controls (**Figure 5A**). On average, the 5-FU+NaLV schedule was more effective, especially from day 23 until the end of the experiment. It is noteworthy that in the groups of animals receiving the simultaneous and sequential combination of 5-FU+CaLV the initial response was superimposable to the 5-FU alone group and significantly different from controls until day 14 (**Figure 5A**). However, at that day the experimental period finished due to their toxicity profiles as described below.

Figure 5B shows the toxicity profiles of the four different treatment schedules. Both 5-FU and 5FU+NaLV treatments were favorable and acceptable with a minor loss of weight throughout the course of the treatment (**Figure 5B**), whereas both the simultaneous 5-FU+CaLV combination and the sequential CaLV and 5-FU association caused a severe toxicity and a loss of weight that necessitated veterinary assistance at day 12 with an immediate fluid therapy (0.9% saline) and then at day 14, due to the nadir of body weight loss, the ethical sacrifice of all the animals, as suggested by guidelines (arrow, **Figure 5B**). It is noteworthy that the animals belonging to the simultaneous 5FU+NaLV combination group had a similar body weight profile to those treated with 5-FU alone (**Figure 5B**).

Experiment With 5-FU Dose of 150 mg/kg

Tumors in control animals showed a rapid enlargement in their dimensions; a mean volume of 392 mm^3 was reached at day 8 when the experiment was closed (**Figure 6A**). Both the simultaneous 5-FU+NaLV and 5-FU+CaLV were able to

significantly inhibit the tumor, and their efficacy became significant starting on the third day, after the single injection of high dose of 5-FU as compared to controls (**Figure 6A**). On average, the simultaneous combination schedules were more effective if compared to 5-FU alone and to the sequential administration of CaLV followed by 5-FU. Interestingly after day 6 the initial response of this schedule was lost and the tumor volumes of this group of mice started to grow similarly to the controls (**Figure 6A**). However, at that day the experimental period finished due to their toxicity profiles as described below.

Figure 6B shows the toxicity profiles of the four different treatment schedules. At this high 5-FU dose, the lack of weight is immediate for all the treated groups. Since the primary objective of this experiment was to establish the rapid antitumor effects of the schedules, the mice were sacrificed before the development of severe toxicity and weigh loss expected by the administered 5-FU dose.

DISCUSSION

The association of folinate salts to 5-FU-based schedules is an established clinical routine in the landscape of the colorectal cancer treatment because it has been experimentally proven to be active in past observations both in preclinical [5-7] and clinical settings [8,23].

In vitro experiments of the early nineties [7,24] suggested that prolonged simultaneous exposure to both 5-FU and folinic acid would increase cytotoxicity in human cancer cell lines in a schedule-dependent manner. Moreover, it was also demonstrated that simultaneous prolonged exposure to folinic acid during 5-FU administration might be optimal for the intracellular formation of the stable ternary complex with thymidilate synthase, 5-FU metabolites and folinic acid derivatives [25]. Ardalan and colleagues firstly translated these preclinical data in the clinical setting, treating patients with the simultaneous administration of 5-FU and CaLV [10]. However,

the crystallization of calcium salts with the consequent blockage of the catheters led the authors to use two separate pumps [10] in order to finish the clinical study. Based on these evidences, a 1- or 2-hour infusion of CaLV preceding 5-FU administration was introduced into the clinic for its better practicability [26]. This schedule become widely used, especially in European countries. However, as well pointed out by Luftner and colleagues in an editorial appeared in 2003, "this mode of application represented a compromise between the need for one i.v. device only and the pharmacological rationale of parallel toxification of 5-FU by folinic acid. However, keeping in mind the half lives of folinic acid with approximately 7 hours and 5-FU with a few minutes, this compromise a priori renounces optimal toxification of 5-FU, especially towards the end of the 24-h infusion" [27].

The aim of the present study was to better investigate if there were relevant pharmacological differences between the sequential administration of folate salts (1 h before) followed by 5-FU (a schedule adopted in the clinical routine mainly for practical reasons rather than on the basis of experimental results) and the simultaneous administration of both drugs *in vitro* and *in vivo* settings. Indeed, our *in vitro* data seem to suggest some important differences on the activity of the combination of 5-FU and NaLV or CaLV in both colon cancer cell lines with the same TSER genetic profile but with different mutation status (i.e. HT-29 *b-raf* mutated, Caco-2 wild type). As expected by the previous published scientific literature, the simultaneous combination of both folinate salts with 5-FU enhanced the antiproliferative activity of the chemotherapeutic drug; in particular, the association of NaLV and 5-FU was synergistic at 24 and 72h, whereas the combination with CaLV was simply additive in the 24 h schedule. Instead, and surprisingly, the sequential combination of NaLV or CaLV (1 h before) and then 5-FU resulted not additive but even antagonistic in a 24 and 72h treatment. Moreover, the simultaneous combination (but not the sequential one) of 5-FU and NaLV or CaLV

(although in a lesser extent) significantly increased also the percentages of apoptotic cells after 24 h treatments.

A possible explanation of this counterintuitive antagonistic effect may come from the gene expression analysis of treated cancer cells. Indeed, the sequential combination (but not the simultaneous one) of 5-FU and CaLV or NaLV (although in a much lesser extent) significantly inhibited the folate transporter *SLC19A1* (solute carrier family 19, member 1) gene expression after 24 h exposure. *SLC19A1/RFC*-1 is ubiquitously expressed and is the major folate transporter in mammalian cells and tissues [28,29]. The *SLC19A1/RFC-1* gene encodes the reduced folate carrier which functions optimally at physiological pH, transferring reduced folates, including leucovorin, into the cells [30]. Interestingly, Odin and colleagues [31] significantly associated *SLC19A1/RFC-1* expression with the disease-free survival of colorectal cancer patients treated with a 5-FU-based therapy using leucovorin. The authors suggested that the poor response to 5-FU plus leucovorin therapy in some patients was linked to low expression of this gene [31]. The low expression of the folate transport gene *SLC19A1/RFC-1* in tumor cells after the sequential administration of the two drugs, would result in lower levels of intracellular folates, and a reduced stabilization of the ternary complex comprising the active cofactor 5,10-methylenetetrahydrofolate, the thymidylate synthase and fluorinated dUMP, and may correlate with a decreased effect of the combination, measured by antiproliferative and proapoptotic activity. On the contrary, the simultaneous combination of folinate salts and 5-FU did not alter the transporter expression, making available a higher amount of stable ternary complexes with a strong synergistic antiproliferative effect. This result is unlikely related to 5-FU, considering that this drug enter the cells by transport mechanisms unrelated to *SLC19A1/RFC-1* [32].

However, other folate pathway genes than the analyzed *SLC19A1/RFC-1* are likely to be of importance. For instance, the expression of the gene *TYMS* which

encodes the 5-FU target thymidylate synthase, is known to affect response to treatment [33]. In our experiments, the simultaneous combination (but not the sequential one) of 5-FU and NaLV or CaLV (although in a lesser extent) significantly inhibited the *TYMS* gene expression after 24 h exposure. Of note, *TYMS* is known to play a central role in 5-FU response to therapy because the higher expression of *TYMS* has been previously reported to be associated with the resistance to 5-FU [34,35]. Among resistance mechanisms reported so far, tumors with elevated *TYMS* have been shown to have highly proliferative and metastatic characteristics [36]; moreover 5-FU sensitivity and patients' survival have been inversely related to the level of TYMS protein and enzymatic activity in cancer cells, and 5-FU-resistant tumors commonly express high levels of TYMS protein [37]. Thus, it is conceivable, at least *in vitro*, that the sequential administration, differently from the simultaneous one, of folinate salts and 5-FU may cause an antagonistic effect through the significant inhibition of the transport mechanism of folate, whereas, the simultaneous combination of the drugs may synergize its effects through the significant decrease of *TYMS* gene expression and an unchanged *SLC19A1/RFC-1* expression.

The in vivo experiments confirmed the enhanced antitumor activity of the simultaneous 5-FU plus NaLV combination at different 5-FU doses. These positive effects could be explained on the basis of our *in vitro* findings but also with the possible pharmacokinetic advantage of the simultaneous administration of the two drugs. Indeed, the NaLV maximum concentration, and thus the availability of LV in plasma and tissues, is contextual with the maximum concentration reached by 5-FU, enhancing the possibility of stabilization of the ternary complex that can be achieved by high levels of 5,10-methylenetetrahydrofolate [31]. Moreover, this association obtained also a good toxicity profile in nude mice, similarly to the published clinical studies of Hartung et al. [14] and Kuhfahl et al. [15] on the simultaneous administration of NaLV and 5-FU where no haematological toxicity grade III/IV was observed. On the contrary, the

sequential association of CaLV (1h before) [38] followed by 5-FU at the lowest and at the highest dose (50 and 150 mg/kg) did not exert a significant therapeutic effect, whereas at the 5-FU dose of 100 mg/kg, the combination showed an antitumor activity similar to 5-FU alone but with the appearance of a severe toxicity that led to a precocious mice suppression. Unfortunately, combined administration of CaLV and 5-FU resulted in high grade toxicity. However, it cannot be excluded that this important toxicity may depend on the calcium salt formulation of the drug with the possible crystallization and precipitation of the formed calcium carbonate [9] during the simultaneous persistence in peritoneal cavity of the two drugs, although this event was not recorded at the autopsy of the mice.

In conclusion, the simultaneous *in vitro* administration of folate salts with 5-FU leads to an enhanced antiproliferative effect. Unfortunately, this increased activity is lost in the sequential combination probably due to the inhibition of *SLC19A1/RFC-1* gene expression. The *in vivo* experiments confirmed the enhanced antitumor activity of the simultaneous combination of NaLV+5-FU with a good toxicity profile of this schedule. The present preclinical findings seem to suggest that folinate salts may be better administered with 5-FU simultaneously in a single pump (i.e. NaLV) or with the aid of two separate ones (i.e. CaLV), rather than sequentially, as currently applied in routine oncology practice. However, a confirmation of this preclinical data in a clinical setting could be achieved only through a properly designed phase III, randomized clinical trial.

Acknowledgements

The authors thank Dr. Giada Salvia for her technical assistance with *in vivo* experiments. This study was supported by unconditional funds provided by MEDAC (Wedel, Germany). The authors declare that they have no conflict of interest.

REFERENCES

1. Wilson PM, Danenberg PV, Johnston PG, Lenz HJ, Ladner RD. Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. Nat Rev Clin Oncol 2014;11(5):282-98.
2. Kwakman JJ, Punt CJ. Oral drugs in the treatment of metastatic colorectal cancer. Expert Opin Pharmacother 2016;17(10):1351-61.
3. Salvatore L, Rossini D, Moretto R, Cremolini C, Schirripa M, Antoniotti C, Marmorino F, Loupakis F, Falcone A, Masi G. TAS-102 for the treatment of metastatic colorectal cancer. Expert Rev Anticancer Ther 2015;15(11):1283-92.
4. Rustum YM. Modulation of fluoropyrimidines by leucovorin: rationale and status. J Surg Oncol Suppl 1991;2:116-23.
5. Lonn U, Lonn S. Increased levels of DNA lesions induced by leucovorin-5-fluoropyrimidine in human colon adenocarcinoma. Cancer Res 1988;48(15):4153-7.
6. Moran RG, Keyomarsi K. Biochemical rationale for the synergism of 5-fluorouracil and folinic acid. NCI Monogr 1987(5):159-63.
7. Moran RG, Scanlon KL. Schedule-dependent enhancement of the cytotoxicity of fluoropyrimidines to human carcinoma cells in the presence of folinic acid. Cancer Res 1991;51(17):4618-23.
8. Grem JL. Biochemical modulation of 5-FU in systemic treatment of advanced colorectal cancer. Oncology (Williston Park) 2001;15(1 Suppl 2):13-9.
9. Trissel LA, Martinez JF, Xu QA. Incompatibility of fluorouracil with leucovorin calcium or levoleucovorin calcium. Am J Health Syst Pharm 1995;52(7):710-5.
10. Ardalan B, Chua L, Tian EM, Reddy R, Sridhar K, Benedetto P, Richman S, Legaspi A, Waldman S, Morrell L and others. A phase II study of weekly 24-hour infusion with high-dose fluorouracil with leucovorin in colorectal carcinoma. J Clin Oncol 1991;9(4):625-30.

11. Bruch HR, Esser M. Catheter occlusion by calcium carbonate during simultaneous infusion of 5-FU and calcium folinate. Onkologie 2003;26(5):469-72.

12. Ardalan B, Flores MR. A new complication of chemotherapy administered via permanent indwelling central venous catheter. Cancer 1995;75(8):2165-8.

13. Loupakis F, Cremolini C, Schirripa M, Masi G, Falcone A. Cytotoxic triplets plus a biologic: state-of-the-art in maximizing the potential of up-front medical treatment of metastatic colorectal cancer. Expert Opin Biol Ther 2011;11(4):519-31.

14. Hartung G, Hofheinz RD, Wein A, Riedel C, Rost A, Fritze D, Kreuser ED, Drees M, Kuhnel J, Hehlmann R and others. Phase II study of a weekly 24-hour infusion with 5-fluorouracil and simultaneous sodium-folinic acid in the first-line treatment of metastatic colorectal cancer. Onkologie 2001;24(5):457-62.

15. Kuhfahl J, Steinbrecher C, Wagner T, Wagner H, Fritze D, Link H, Schulte F, Pichlmeier U, Kreuser ED. Second-line treatment of advanced colorectal cancer with a weekly simultaneous 24-hour infusion of 5-fluorouracil and sodium-folinate: a multicentre phase II trial. Onkologie 2004;27(5):449-54.

16. Bleiberg H, Vandebroek A, Deleu I, Vergauwe P, Rezaei Kalantari H, D'Haens G, Paesmans M, Peeters M, Efira A, Humblet Y. A phase II randomized study of combined infusional leucovorin sodium and 5- FU versus the leucovorin calcium followed by 5-FU both in combination with irinotecan or oxaliplatin in patients with metastatic colorectal cancer. Acta Gastroenterol Belg 2012;75(1):14-21.

17. Terjung A, Kummer S, Friedrich M. Simultaneous 24 h-infusion of high-dose 5-fluorouracil and sodium-folinate as alternative to capecitabine in advanced breast cancer. Anticancer Res 2014;34(12):7233-8.

18. Bourget P, Moriceau A, Amin A, Vidal F, Cassard B, Clement R. Stability of irinotecan and sodium levofolinate admixtures in polyolefin bags: clinical and nursing considerations. European Journal of Hospital Pharmacy Practice 2011;17(6):66-69.

19. Kawakami K, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. Anticancer Res 1999;19(4B):3249-52.

20. Adleff V, Hitre E, Koves I, Orosz Z, Hajnal A, Kralovanszky J. Heterozygote deficiency in thymidylate synthase enhancer region polymorphism genotype distribution in Hungarian colorectal cancer patients. Int J Cancer 2004;108(6):852-6.

21. Bocci G, Fioravanti A, Orlandi P, Di Desidero T, Natale G, Fanelli G, Viacava P, Naccarato AG, Francia G, Danesi R. Metronomic ceramide analogs inhibit angiogenesis in pancreatic cancer through up-regulation of caveolin-1 and thrombospondin-1 and down-regulation of cyclin D1. Neoplasia 2012;14(9):833-45.

22. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58(3):621-81.

23. Machover D, Goldschmidt E, Chollet P, Metzger G, Zittoun J, Marquet J, Vandenbulcke JM, Misset JL, Schwarzenberg L, Fourtillan JB and others. Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil and high-dose folinic acid. J Clin Oncol 1986;4(5):685-96.

24. Keyomarsi K, Moran RG. Mechanism of the cytotoxic synergism of fluoropyrimidines and folinic acid in mouse leukemic cells. J Biol Chem 1988;263(28):14402-9.

25. Boarman DM, Allegra CJ. Intracellular metabolism of 5-formyl tetrahydrofolate in human breast and colon cell lines. Cancer Res 1992;52(1):36-44.

26. Weh HJ, Wilke HJ, Dierlamm J, Klaassen U, Siegmund R, Illiger HJ, Schalhorn A, Kreuser ED, Hilgenfeld U, Steinke B and others. Weekly therapy with folinic acid (FA) and high-dose 5-fluorouracil (5-FU) 24-hour infusion in pretreated patients with metastatic colorectal carcinoma. A multicenter study by the Association of Medical Oncology of the German Cancer Society (AIO). Ann Oncol 1994;5(3):233-7.

27. Luftner D, Jozereau D, Possinger K. Catheter occlusion by calcium carbonate: a well-known problem persists in spite of better knowledge. Onkologie 2003;26(5):425-6.

28. Hou Z, Matherly LH. Biology of the major facilitative folate transporters SLC19A1 and SLC46A1. Curr Top Membr 2014;73:175-204.

29. Matherly LH, Wilson MR, Hou Z. The major facilitative folate transporters solute carrier 19A1 and solute carrier 46A1: biology and role in antifolate chemotherapy of cancer. Drug Metab Dispos 2014;42(4):632-49.

30. Zhao R, Goldman ID. Folate and thiamine transporters mediated by facilitative carriers (SLC19A1-3 and SLC46A1) and folate receptors. Mol Aspects Med 2013;34(2-3):373-85.

31. Odin E, Sonden A, Gustavsson B, Carlsson G, Wettergren Y. Expression of Folate Pathway Genes in Stage III Colorectal Cancer Correlates with Recurrence Status Following Adjuvant Bolus 5-FU-Based Chemotherapy. Mol Med 2015;21:597-604.

32. Li H, Chung SJ, Shim CK. Characterization of the transport of uracil across Caco-2 and LLC-PK1 cell monolayers. Pharm Res 2002;19(10):1495-501.

33. Kaiyawet N, Rungrotmongkol T, Hannongbua S. Effect of halogen substitutions on dUMP to stability of thymidylate synthase/dUMP/mTHF ternary complex using molecular dynamics simulation. J Chem Inf Model 2013;53(6):1315-23.

34. Sinicrope FA, Rego RL, Halling KC, Foster NR, Sargent DJ, La Plant B, French AJ, Allegra CJ, Laurie JA, Goldberg RM and others. Thymidylate synthase expression in colon carcinomas with microsatellite instability. Clin Cancer Res 2006;12(9):2738-44.

35. Nomura T, Nakagawa M, Fujita Y, Hanada T, Mimata H, Nomura Y. Clinical significance of thymidylate synthase expression in bladder cancer. Int J Urol 2002;9(7):368-76.

36. Ahn JY, Lee JS, Min HY, Lee HY. Acquired resistance to 5-fluorouracil via HSP90/Src-mediated increase in thymidylate synthase expression in colon cancer. Oncotarget 2015;6(32):32622-33.

37. Peters GJ, Backus HH, Freemantle S, van Triest B, Codacci-Pisanelli G, van der Wilt CL, Smid K, Lunec J, Calvert AH, Marsh S and others. Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta 2002;1587(2-3):194-205.

38. Ishihara Y, Matsunaga K, Iijima H, Hasegawa G, Suzuki T, Sato A, Kobayashi T, Yang M, Hoffman RM. The combination of 5-FU, leucovorin and CPT-11 (FOLFIRI) prolongs survival through inhibition of metastasis in an orthotopic model of colon cancer. Anticancer Res 2010;30(2):403-8.

Table 1. IC_{50} values of single drugs after 24, 48 and 72 hours of *in vitro* exposure		
drugs	**HT-29**	**CaCo-2**
	IC_{50} ± SD [µM]	*IC_{50} ± SD [µM]*
5-FU 24 h	12.27±0.93	14.56±4.84
5-FU 48 h	7.57±2.58	11.20±3.63
5-FU 72 h	0.45±0.01	1.324±0.50

Table 2. Combination index (CI) values at 50% of affected cell fraction in human HT-29 and Caco-2 colon cancer cells after 24 and 72h of simultaneous and consecutive combination treatment of 5-fluorouracile (5-FU) and sodium levofolinate (NaLV) or calcium levofolinate (CaLV)

	Simultaneous treatment 5-FU + NaLV or CaLV				**Consecutive treatment NaLV or CaLV (1h) → 5-FU**			
	24h		72h		24h		72h	
Cell lines	NaLV	CaLV	NaLV	CaLV	NaLV	CaLV	NaLV	CaLV
HT-29	0.427	1.340	0.811	0.906	4.136	32.462	9.297	2.584
CaCo-2	0.793	1.063	0.962	27.974	4.923	7.778	12.622	23.076

FIGURE LEGENDS

Figure 1. Apoptotic effects of sequential and simultaneous combinations of 5-FU (2 µM), NaLV (2 µM) and CaLV (2 µM) on HT-29 (A) and Caco-2 (B) colon cancer cell lines treated for 24 hours. Columns and bars indicate mean values ± SD, respectively. **P*<0.05 *versus* vehicle-treated controls.

Figure 2. *TYMS* gene expression in HT-29 cells exposed for 24 hours to 5-FU alone and to the sequential and simultaneous combinations of 5-FU (2 µM), NaLV (2 µM) and CaLV (2 µM). Columns and bars indicate mean values ± SD, respectively. **P*<0 .05 *versus* vehicle-treated controls.

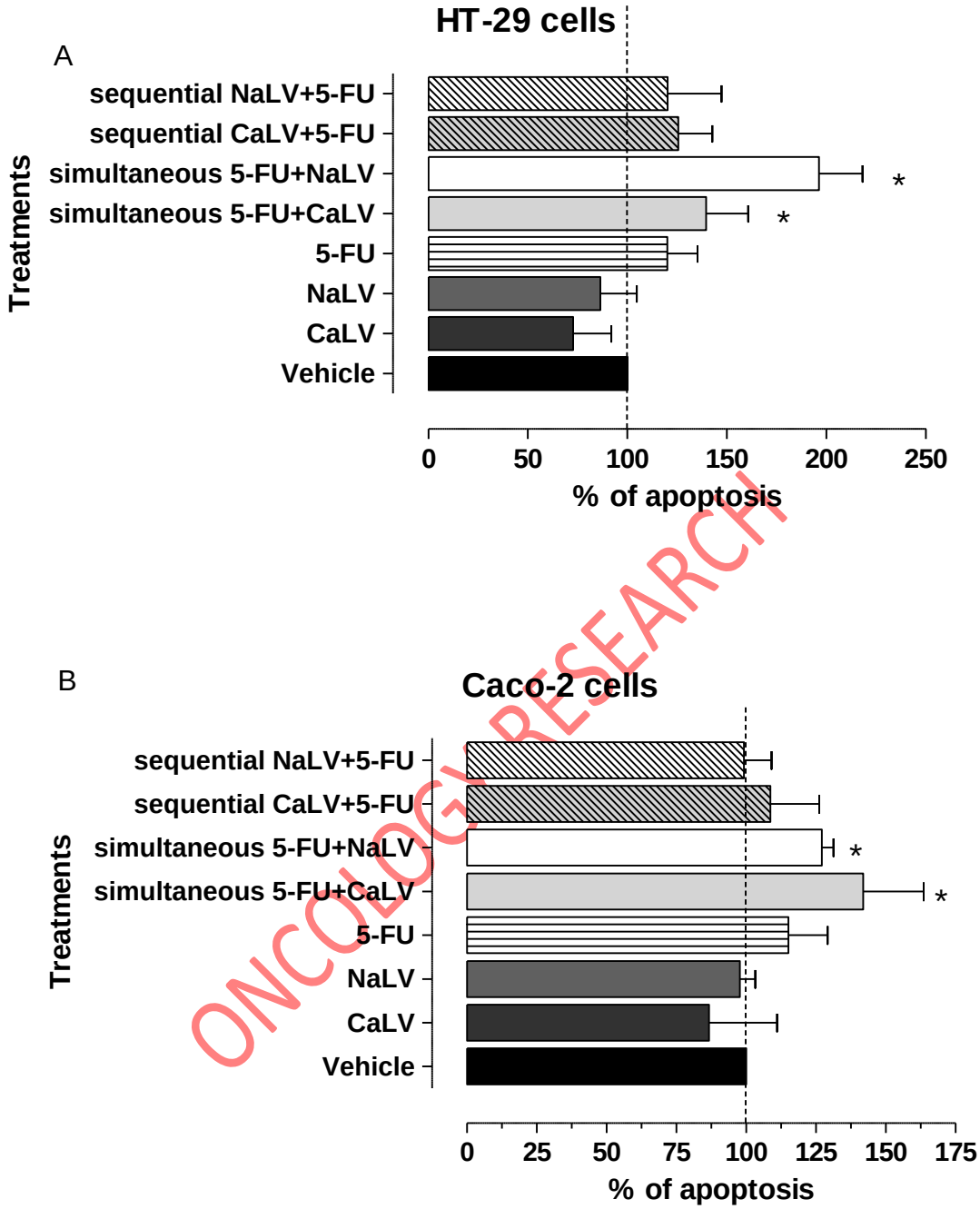
Figure 3. *SCL19A1* gene expression in HT-29 cells exposed for 24 hours to sequential and simultaneous combinations of 5-FU (2 µM), NaLV (2 µM) and CaLV (2 µM). Columns and bars indicate mean values ± SD, respectively. **P*<0 .05 *versus* vehicle-treated controls.

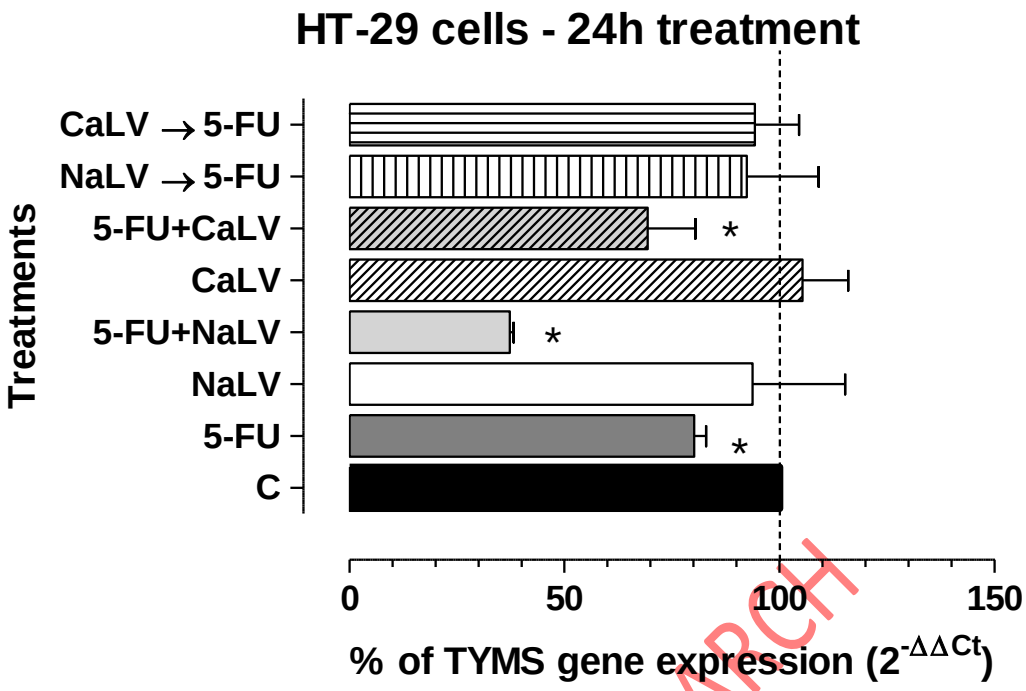
Figure 4. A) Chemotherapeutic effect of 5-FU 50 mg/kg i.p. alone and in simultaneous or sequential combination with NaLV or CaLV 50 mg/kg i.p. on HT-29 tumors xenotransplanted in CD *nu/nu* mice (*n*=6 for each group). **P*<0.05 with respect to controls. Symbols and bars, mean ± SD. (**B**) Body weight of HT-29 tumor-bearing control mice and mice treated with 5-FU alone or in simultaneous or sequential combination with folinate salts. Symbols and bars, mean ± SD.

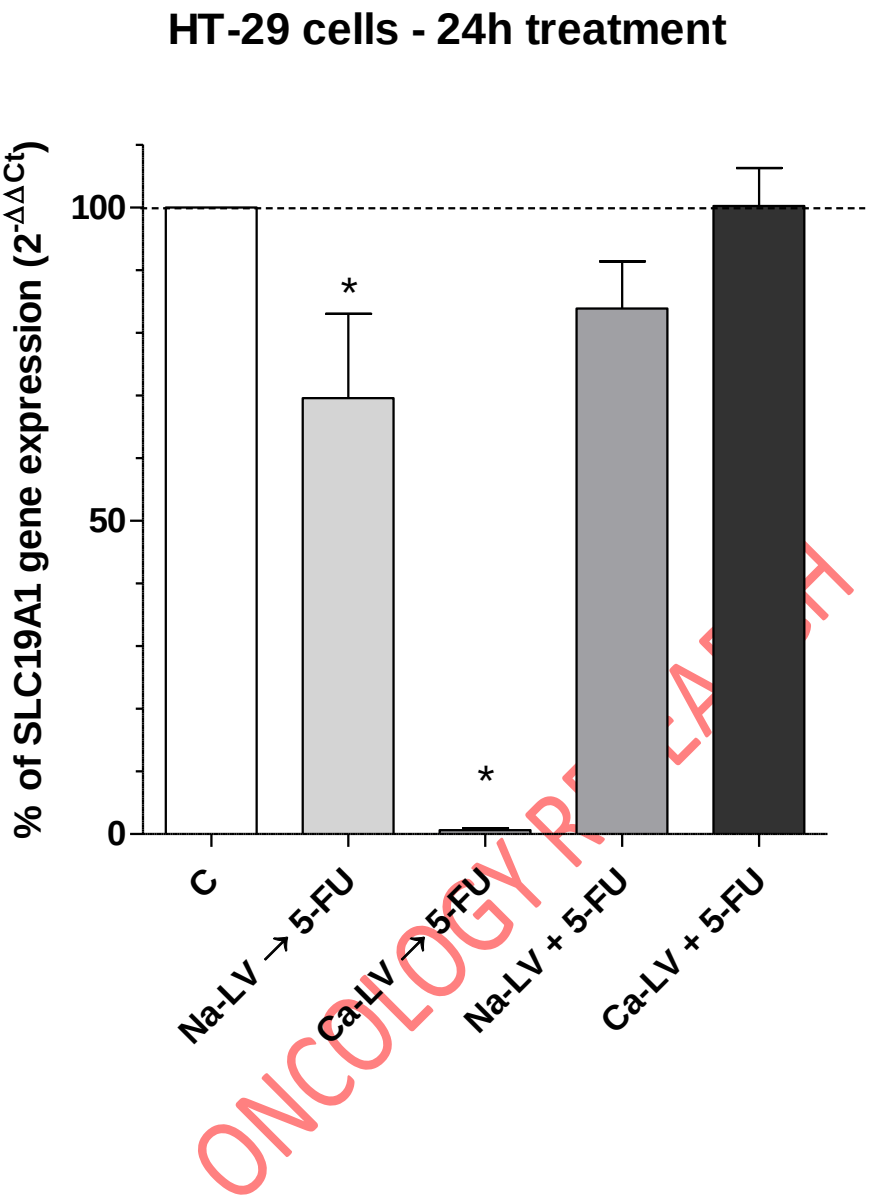
Figure 5. A) Chemotherapeutic effect of 5-FU 100 mg/kg i.p. alone and in simultaneous or sequential combination with NaLV or CaLV 50 mg/kg i.p. on HT-29 tumors xenotransplanted in CD *nu/nu* mice (*n*=6 for each group). **P*<0.05 with respect to controls. Symbols and bars, mean ± SD. (**B**) Body weight of HT-29 tumor-bearing

control mice and mice treated with 5-FU alone or in simultaneous or sequential combination with folinate salts. Both the simultaneous 5-FU+CaLV combination and the sequential CaLV and 5-FU association caused a severe loss of mouse weight requiring fluid therapy (0.9% saline) at day 12, and then at day 14 the ethical sacrifice of all the animals (*arrow*). Symbols and bars, mean ± SD.

Figure 6. A) Chemotherapeutic effect of the single injection of 5-FU 150 mg/kg i.p. dose alone and in simultaneous or sequential combination with NaLV or CaLV 50 mg/kg i.p. on HT-29 tumors xenotransplanted in CD *nu/nu* mice (*n*=6 for each group). **P*<0.05 with respect to controls. Symbols and bars, mean ± SD. (**B**) Body weight of HT-29 tumor-bearing control mice and mice treated with 5-FU alone or in simultaneous or sequential combination with folinate salts. Symbols and bars, mean ± SD.







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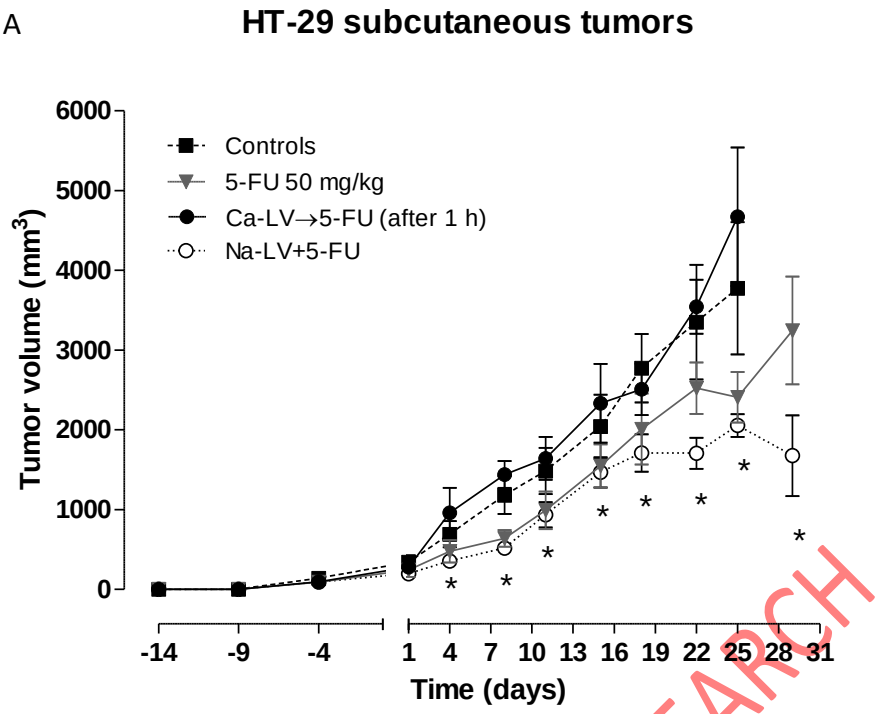

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