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## Title page

# Effect of a novel vacuolar-$H^+$-ATPase inhibitor on cell and tumor response to camptothecins

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# Running title page

**Running title:** vacuolar-$H^+$-ATPase inhibitor and topotecan therapy

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**Abbreviations:** BCRP, breast cancer resistant protein.

## Abstract

The vacuolar-$H^+$-ATPase, functionally expressed in cell membranes, is known to play a relevant role in intracellular pH regulatory mechanisms, because it is implicated in pumping protons into extracellular environment or in sequestrating excess protons into acidic vacuolar compartments. Since tumor cells exist in hypoxic microenvironment and produce acidic metabolites, this regulatory mechanism is recognized as a protective function. The study was designed to investigate the effect of NiK-12192, an indole derivative identified as an effective inhibitor of vacuolar-$H^+$-ATPase, on the cytotoxic activity of two camptothecins, i.e. topotecan and SN38 (the active metabolite of irinotecan). The cellular studies performed in two pairs of human colon carcinoma cell lines, i.e. LoVo and LoVo/DX (overexpressing P-glycoprotein) and HT29 and HT29/Mit (overexpressing BCRP) indicated an enhancement of the antiproliferative effect of camptothecins by concomitant exposure to subtoxic concentrations of NiK-12192. Studies of subcellular distribution indicated that, while topotecan alone localized mainly in mitochondria and endoplasmic compartment, the simultaneous presence of NiK-12192 caused a cytoplasmatic redistribution. In HT29/Mit cells, NiK-12192 reverted the pattern of acidification induced by topotecan. The potentiation of topotecan efficacy by NiK-12192 was documented by an increased efficacy of the combination in both the HT29 tumor xenografts, being more evident in the topotecan-resistant HT29/Mit tumor. In conclusion, the vacuolar-$H^+$-ATPase inhibitor NiK-12192 was able to potentiate the cytotoxic/antitumor effects of camptothecins, either in *in vitro* or *in vivo* systems. Such finding supports a potential interest for the use of vacuolar-$H^+$-ATPase inhibitors in combination therapy to improve camptothecin efficacy.

## Introduction

Despite the recent development of target-specific innovative agents, cytotoxic agents still remain the milestone of antitumor therapy. Unfortunately, their toxicity and drug resistance are major clinical obstacles to effective treatment and the lack of tumor responsiveness to conventional agents is a common event in patients with solid tumors. Drug resistance is often a multifactorial phenomenon involving a number of defence mechanisms. The overexpression of efflux transport proteins, such as P-glycoprotein, multidrug resistance protein and BCRP, represents a frequent mechanism mediating the development of the multidrug resistant phenotype (Glavinas et al., 2004). In addition to the transporter-mediated efflux, the cellular uptake of drug may be impaired by the acidic extracellular microenvironment, produced by the high glycolytic metabolism of tumor cells and by the export of protons from the intracellular compartments, which is increased to prevent cellular acidosis (Vaupel et al., 1989). Extrusion of acidic metabolites produces an extracellular pH lower than that of surrounding normal cells and is essential to maintain a physiological intracellular pH. These homeostatic mechanisms cause a pH gradient between intracellular and extracellular compartments and between cytoplasm and intracellular organelles. The pH gradient has been implicated in drug sequestration in acidic vescicles and might contribute to the multidrug resistant phenotype (Raghunand and Gilles, 2000; Simon et al., 1994). Extracellular acidification could modulate several cell functions such as cell growth, differentiation, angiogenesis and metastasis.

Tumor cells often exist in a hypoxic microenvironment and develop high glycolytic activity which, in turn, produces acidic metabolites. Four major types of pH regulatory mechanisms have been identified in tumor cells, i.e. $Na^+$-$H^+$ exchangers, bicarbonate transporters, proton-lactate symporters, and proton pumps (Putnam, 2001). Among them, the vacuolar-$H^+$-

ATPase has been shown as essential (Nishi and Forgac, 2002). At the cell surface vacuolar-$H^+$-ATPase plays a role in maintaining an intracellular environment favorable for growth, while producing an acidic extracellular environment favorable for cell invasion (Montcourrier et al., 1994; Sennoune et al., 2004). In the intracellular organelles membranes, vacuolar-$H^+$-ATPase plays a role in drug compartmentation observed in resistant cells (Larsen et al., 2000). The vacuolar-$H^+$-ATPase has been shown to be overexpressed in multidrug resistant cells, and altered cytosolic pH has been implicated in drug resistance (Raghunand and Gilles, 2000; Simon et al., 1994). Thus, vacuolar-$H^+$-ATPase may be a potential target to increase the therapeutic efficacy of antitumor agents, by modulating pH-dependent cellular resistance mechanisms (Izumi et al., 2003). The role of vacuolar-$H^+$-ATPase inhibitors in tumor therapy is a matter of investigation, and recent studies have shown the ability of proton-pump inhibitors to sensitize tumor cells to various cytotoxic agents (Luciani et al., 2004; McSheehy et al., 2003; Ouar et al., 2003).

Vacuolar-$H^+$-ATPase inhibitors have been known for many years, with the natural compound bafilomycin A1 being the most extensively studied (Bowman et al., 1988). However, bafilomycin A1 is not able to distinguish among the various types of vacuolar-$H^+$-ATPase; therefore the *in vivo* treatment is toxic, due to an inhibition of all the essential vacuolar-$H^+$-ATPases and to systemic alteration of cellular physiology (Farina and Gagliardi, 2002). Starting from bafilomycin A1, extensive structure-activity relationship studies allowed to identify the key structural requirements for the vacuolar-$H^+$-ATPase inhibitory activity (Gagliardi et al., 1999). SB242784, a potent and selective inhibitor of the osteoclast vacuolar-$H^+$-ATPase, has been identified as a potent inhibitor of *in vitro* (Nadler et al., 1998) and *in vivo* bone resorption (Visentin et al., 2000). Further modification of the SB242784 structure, in which the dienic chain was replaced by substituted phenyl ring, led to the identification of new classes of vacuolar-$H^+$-ATPase inhibitors (Farina et al., 2001). Among the compounds of the series, NiK-12192, a novel

substituted indol-2yl-N-(2,2,6,6-tetramethylpiperidin-4-yl)benzamide (Fig. 1), was selected for development due to its capability to inhibit the enzyme in various biological systems.

In the present study we investigated the role of NiK-12192 in the antitumor therapy of camptothecins, which are well-established cytotoxic drugs that target DNA topoisomerase I (Zunino and Pratesi, 2004). Due to their chemical structure, pH modulation is expected to be a critical factor for their cytotoxic and antitumor effect (Flowers et al., 2003). The interaction of topotecan or SN38 with NiK-12192 was studied in LoVo and HT29 human colorectal carcinoma cell lines and in two drug-resistant sublines, overexpressing P-glycoprotein and BCRP, respectively. Due to the synergism observed in the cell lines between topotecan and NiK-12192, such a combination was tested *in vivo* using HT29, both parental and resistant, tumor xenografts. The combination of the two drugs resulted in a substantial increase in tumor response, more relevant in the HT29/Mit xenograft, the BCRP overexpressing subline.

## Materials and Methods

**Drugs.** Topotecan (Hycamtin; Glaxo SmithKline S.p.A., Verona, Italy), SN38 (provided by Prof. L. Merlini, University of Milan, Milan), bafilomycin A1 (Sigma-Aldrich, Milan, Italy) and NiK-12192 (Fig. 1, provided by Nikem Research, Milan, Italy) were used throughout the study.

For *in vitro* studies, stock solutions (1 mg/ml) were stored in dimethylsulfoxide for NiK-12192 and SN38 and in ethanol for topotecan, and diluted before the use in culture medium. Maximal final concentrations of dimethylsulfoxide and ethanol in culture medium were 0.6 and 0.1%, respectively, which have been verified to be non toxic on our cell systems.

For *in vivo* studies, topotecan was dissolved in sterile distilled water and NiK-12192 was dissolved by adding absolute ethanol and Cremophor ELP (both 5% of the final volume) and diluted just before use by adding 0.9% NaCl.

**Bafilomycin-Sensitive ATPase Assay.** Inhibition of bafilomycin-sensitive ATPase activity was assayed by measuring the release of inorganic phosphate, during 30 minutes of incubation, of the microsomal fraction in 96-well plates at 37°C, as described by Boyd et al. (Boyd et al., 2001). Procedures for the preparation of membranes have been already described (Boyd et al., 2001). All the ATPase assays were performed in the presence of oligomycin and vanadate as inhibitors of F- and P-ATPases, to exclude possible interactions.

**Cell and tumor lines.** The combination of camptothecins and NiK-12192 was investigated in two human colorectal carcinoma cell lines, HT29 and LoVo. The panel included the HT29/Mit subline, selected for resistance to mitoxantrone, overexpressing BCRP, and the

LoVo/DX subline, selected for resistance to doxorubicin, overexpressing P-glycoprotein (Grandi et al., 1986; Perego et al., 2001). HT29 and HT29/Mit cell lines were maintained in McCoy's medium (BioWhittaker, Verviers, Belgium) and LoVo and LoVo/DX cells in F12 medium (BioWhittaker), both added with 10% heat-inactivated fetal bovine serum (Life Technologies Inc., Gaithersburg, MD). All cell lines were maintained in a humidified atmosphere with 5% $CO_2$ at 37°C.

For *in vivo* studies, tumor lines were obtained by s.c. injection in nude athymic mice of exponentially growing tumor cells, and were maintained by successive passages of tumor fragments, as previously described (Pratesi et al., 1989).

**Antiproliferative activity studies in cell cultures**. Cells were seeded in 96 well plates. After 24 h, cells were exposed to NiK-12192, to the camptothecins or to both compounds for 72 h. After treatment, cells were fixed in TCA 50% and processed for sulforodamine B assay (Skehan et al., 1990). Dose-response curves were plotted, and analysis of drug interaction was done by a modified method of Drewinko et al. (Zanchi et al., 2005). Combination index is calculated as the ratio SF*a* x SF*b*/SF*ab* where SF*a*, SF*b* and SF*ab* are the surviving fraction of cells treated with the compound *a* or *b* or *a* and *b* together, respectively. Combination index >1 indicates greater than additive effects, i.e. synergism (the higher the value, the greater the degree of synergism), combination index = 1 indicates additivity, and combination index < 1 indicates antagonism.

**Subcellular localization of NiK-12192 and topotecan.** HT29 and HT29/Mit cells were seeded on coverlips and treated, 48 h later, with topotecan (22 μM) with or without NiK-12192 (1

μM) for 40 min. The fluorescence imaging analysis was performed by means of an Argus VIM 100 processor digital system (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany), using a high sensitive ISIT camera (Hamamatsu C2400-09), coupled to a Leica fluorescence microscope (Wetzlar, Germany). Excitation light was provided by a 100 W Hg lamp, and selected by a 366 nm (T%=40) interferential filter. The images were acquired through a 430 nm long pass filter, by means of a 95 X Leitz objective (iris diaphragm, N.A. 1.10-1.33), digitally stored on a magnetic mass memory support and processed by means of the Hamamatsu Argus 100 control program. The emission of the two drugs was collected contemporary in cells treated with the combination, because of the superimposition of the fluorescence of topotecan and NiK-12192.

**Determination of lysosomes acidity.** Cells were treated 24 h after seeding with NiK-12192 (1 μM), topotecan (0.1 μM for HT29 or 5 μM for HT29/Mit) or the combination of the two compounds. After 24 h of treatment, trypsinized cells were stained with acridine orange (Sigma Chemicals, St. Louis, MO) at the final concentration of 1 μg/ml in PBS for 17 min, washed and analyzed by FACScan or observed by a fluorescence microscope. In acridine orange-stained cells, the cytoplasm and nucleus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of acidity and/or the volume of the cellular acidic compartment (Traganos and Darzynkiewicz, 1994). Therefore, by comparing the mean red/green fluorescence ratio within different cell populations, it is possible to measure a change in the degree of acidity and/or the fractional volume of their cellular acidic compartment. Dot plots of FL1 (green fluorescence) and FL3 (red fluorescence) obtained by FACScan analysis were subdivided in 8 regions and the ratios of the mean values for red and for green fluorescence are reported. Slides were prepared with

the same samples and observed by DMRB (Leica) fluorescence microscope equipped with a 100-W lamp. Images were recorded with a Spot Insight digital camera (Delta Sistemi, Roma, Italy). No interference between acridine orange lysosomal staining and topotecan and/or NiK-12192 is expected, because of the different excitation emission wavelengths of acridine orange (excitation at 490 nm and emission in the range of 530-640 nm). In any case, a quenching effect would result in a decrease of the emission intensity of acridine orange, rather than in a change of the emission colour.

**Western blot analysis.** Adherent and floating cells were lysed and prepared for SDS-PAGE as described (Zuco et al., 2004). After separation on SDS-PAGE, proteins were transferred to nitrocellulose filters. Membranes were incubated with anti-BCRP primary antibody (Alexis Biochemicals, Lasen, Switzerland) overnight and with peroxidase-conjugated anti-mouse antibody to reveal immunoreactive bands using the enhanced chemiluminescence detection system from Amersham Bioscences (Amersham, United Kingdom). Anti-actin antibody, used as control of loading, was from Sigma.

**Antitumor activity *in vivo.*** Antitumor activity experiments were performed using 8-11 week-old female athymic nude CD-1 mice (Charles River, Calco, Italy). Mice were maintained in laminar flow rooms with constant temperature and humidity. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy), according to the United Kingdom Coordinating Committee on Cancer Research Guidelines (Workman et al., 1998).

Experimental groups included 5 mice bearing bilateral s.c. tumors (9-10 tumors/group). Tumor growth was followed by biweekly tumor diameter measurements using a Vernier caliper.

Tumor weight (TW) was calculated, considering tumor density =1, according to the formula: TW (mg) = tumor volume ($mm^3$) = $d^2xD/2$, where d and D are the shortest and the longest diameter, respectively. Drug treatments started when mean TW was about 100 mg. Drugs were delivered orally, by gavage in a volume of 10 ml/kg of body weight according to different treatment schedules and doses. Details are reported in the Results. In combination studies, NiK-12192 was delivered starting 1 h after each topotecan treatment. Control mice were treated orally with solvent, in parallel with drug treatments.

Drug efficacy was assessed as: a) tumor weight inhibition % (TWI%) = 100 – (mean TW treated mice/mean TW control mice x 100), evaluated during and after drug treatment; b) the $log_{10}$ cell kill (LCK) achieved by the drug treatment, according to the formula: (T-C)/DTx3.32, where T and C are the mean time (days) required for treated (T) and control (C) tumors, respectively, to reach a determined weight (1 g), and DT is the doubling time of control tumors. In order to define a synergistic efficacy, in the groups treated with the combination the "expected TWI% value" was calculated by summing TWI% induced by topotecan + TWI% induced by NiK-12192 on the surviving fraction of tumor. The results achieved represent the TWI% due to an additive effect of the two drugs. Synergism is defined when the "observed TWI%" of the combination was superior to the "expected" value (Berenbaum, 1989).

Drug tolerability was assessed in tumor-bearing mice as: a) lethal toxicity, i.e., any death in treated mice occurring before any death in control mice; b) body weight loss percentage (BWL%) = 100 – (body weight on day x/body weight on day 1 x 100), where x represents a day after or during the treatment period.

Experimental groups were sacrificed by cervical dislocation when mean TW was at a maximum of 1±0.5 g.

**Statistical analysis**. Student's t test (two-tailed) was used to compare treated and control samples throughout all the study. P values lower than 0.05 were considered statistically significant. Analyses were performed with Graph Pad Prism, version 4.0 (Graph Pad Software Inc., San Diego , CA).

## Results

**Inhibition of vacuolar-$H^+$-ATPase activity.** The results shown in Table 1 indicate that NiK-12192 was an effective inhibitor of vacuolar-$H^+$-ATPase activity in various systems including bovine chromaffin granules and membranes from human osteoclastoma, human kidney cortex and human liver.

**Antiproliferative activity.** The antiproliferative activity of the tested camptothecins (topotecan and SN38) or their combination with NiK-12192 was investigated in colon carcinoma cells, including sublines characterized by overexpression of transport systems. Specifically, HT29/Mit cells, selected for resistance to mitoxantrone and characterized by high expression levels of BCRP, exhibited cross-resistance to topotecan and SN38. LoVo/DX cells, selected for resistance to doxorubicin and characterized by a typical multidrug resistant phenotype, showed a moderate cross-resistance to SN38 (Table 2). The $IC_{50}$ values of NiK-12192 itself were comparable in parental cells and resistant sublines**.** Using a concomitant 72 h-exposure, the combination of topotecan and subtoxic concentrations of Nik-12192 resulted in a sensitization of both the HT29 parental and resistant cells (Fig. 2). A sensitization to SN38 by NiK-12192, was found in LoVo/DX and their parental cells. The potentiation of camptothecins activity was found with NiK-12192 concentrations not affecting cell growth, while at concentrations inhibiting cell proliferation only additive effects were observed (not shown). An analysis of drug interaction performed with the Drewinko method (Zanchi et al., 2005) also supported the potentiation of camptothecin cytotoxicity. Being the sensitization more marked in combination with topotecan in HT29 and its mitoxantrone-resistant subline, such cell systems were chosen for further studies.

**Subcellular localization of topotecan.** To elucidate the cellular bases of the interaction between topotecan and NiK-12192, some aspects of the cellular accumulation and distribution of

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topotecan with or without NiK-12192 were investigated (Fig. 3). In HT29 and HT29/Mit cell lines, after exposure to topotecan, we observed an emission signal in the perinuclear region with polar cytoplasmic diffusion in some cells and the presence of some round vesicles. These patterns were already reported to indicate a topotecan localization in mitochondria and endoplasmic reticulum, on the basis of the comparative analysis with specific staining for these cell structures (Croce et al., 2004). The concomitant treatment with both compounds resulted in a diffuse distribution of the fluorescence in the cytoplasm, with an intense polar localization in some cells.

**Drug effect on staining with a lysosomotropic agent.** It has been reported that bafilomycin A1, a natural vacuolar-$H^+$-ATPase inhibitor, modulates pH (Bowman et al., 1988). Thus, we examined the effect of NiK-12192 on lysosome acidity induced by topotecan, using the lysosomotropic agent acridine orange whose protonated form accumulates in acidic compartments with a bright red fluorescence, whereas cytoplasm and nucleus showed a green fluorescence (Traganos and Darzynkiewicz, 1994). Cells treated with single or combined drugs for 48 h and then stained with acridine orange are shown in Fig. 4. Staining with acridine orange revealed the change of acidification. The appearance of red fluorescence in topotecan-treated cells indicated the acidification of cellular organelles. In contrast, untreated cells exhibited green fluorescence. FACScan analysis allows determination of changes in the degree of acidity in terms of red/green fluorescence ratio (Table 3), that indicates the degree of cellular acidity or of the fractional volume of the acidic compartment (Paglin et al., 2001). In HT29 cells the exposure to topotecan produced an increase of the number of cells with a high R/G ratio (25% of cells with a R/G ratio $\geq$ 6). This effect was partially reversed by the combination with NiK-12192. In HT29/Mit cells, the acidification induced by topotecan was more marked, because 70% of cells had a R/G ratio > 7. As observed with bafilomycin A1, NiK-12192 was able to increase the

percentage of cells with a low R/G ratio, i.e. with a higher pH. In the combination, NiK-12192 restored in topotecan-treated cells the pattern of acidification of untreated control cells. The results supported that HT29/Mit cells, characterized by higher levels of vacuolar-$H^+$-ATPase expression (not shown), were substantially more susceptible to drug-induced modulation of acidification.

**Drug effect on BCRP expression.** Since several drugs (in particular, substrates) could modulate the expression of BCRP, the effects of topotecan and NiK-12192 on BCRP protein expression were investigated in HT29 cells under the same conditions of antiproliferative activity experiments. Topotecan caused an appreciable upregulation of the protein (Fig. 5). This finding is not surprising, because topotecan is a substrate for BCRP (Perego et al., 2001). In contrast NiK-12192 caused a marginal (if any) downregulation of BCRP. A similar pattern of modulation of BCRP was found in HT29/Mit cells (not shown).

**Antitumor activity.** In all the *in vivo* studies both topotecan and NiK-12192 were delivered per os, by gavage. In the combination, NiK-12192 was always given daily x 3 times for repeated cycles, starting the same day of each topotecan treatment (about 1 h later). When the combination topotecan + NiK-12192 was tested against HT29/Mit human tumor xenograft, topotecan was given at its MTD (15 mg/kg) q4dx3 times, and NiK-12192 at the dose of 30 mg/kg. As expected for a BCRP-expressing tumor, topotecan was poorly active in inhibiting tumor growth (Table 4). The effect of NiK-12192 itself was negligible. The combination of the two drugs resulted in a clear therapeutic improvement, being the 75% TWI, observed 5 days after treatment end, much higher than the expected value based on the efficacy of each drug alone (58%). Moreover, the observed TWI significantly differed from that of topotecan ($p<0.001$). As

reflected by the LCK value, the growth inhibition lasted for long time, and 70% TWI was still assessable 10 days later, i.e. 28 days after tumor cells inoculum (not shown). One mouse treated with the combination died at day 16, and all mice of the group lost weight (20%), thus indicating some increase of toxicity of the combination in such conditions.

A parallel experiment was designed for mice implanted with the parental HT29 tumor line using a lower dose of NiK-12192, 20 mg/kg (Table 4). Against the parental tumor topotecan was active, and the combination of the two drugs achieved an improved antitumor effect. Indeed, 5 days after the last treatment the 88% TWI was significantly higher than that achieved by topotecan alone ($p \leq 0.05$) and higher than the expected (77%), considering that NiK-12192 alone exhibited minimal activity. Again, the inhibition of tumor growth lasted for long time, achieving a LCK value of 2.5. However, in spite of the reduced dose of NiK-12192, the combination still resulted partially toxic, and a relevant body weight reduction was achieved. Thus, a general toxic effect on the mouse might contribute to the tumor growth inhibition. Growth curves representative of the experiments reported in Table 4 are shown in Fig. 6.

In order to identify optimal conditions for the treatment with the combination of the two drugs, possibly without toxic effects, we designed further experiments in HT29 tumor-bearing mice, increasing the interval between each administration of the cytotoxic drug (Table 4). Topotecan, 15 mg/kg, delivered weekly (q7dx3), was active in inhibiting tumor growth. In the group treated with the combination, a therapeutic advantage was achieved, being the 82% TWI, significantly higher than that achieved by topotecan alone ($p<0.05$). Again, a higher value of LCK was achieved. In contrast to the results of the above reported experiments, in such experimental conditions the combination resulted well tolerated without relevant BWL% or lethality, whereas 1 mouse died in the group treated with topotecan alone. NiK-12192 alone, 30

mg/kg, remained poorly active against the HT29 tumor xenograft even when administered daily (5 days/week) for 3 weeks (not shown).

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## Discussion

Vacuolar-$H^+$-ATPase has been proposed as an useful target to be exploited for the improvement of antitumor chemotherapy, because modulation of pH is a protective mechanism activated in response to stress conditions, in particular following DNA damage (Torigoe et al., 2002; Paglin et al., 2001). Moreover, vacuolar-$H^+$-ATPase has a role as cooperating factor in the multidrug resistant phenotype mediated by overexpression of ATP-dependent membrane proteins that function as drug efflux pumps (Raghunand and Gillies, 2000). The results reported in this study show that NiK-12192, a novel synthetic vacuolar-$H^+$-ATPase inhibitor, was able to sensitize colon carcinoma cells to two camptothecins chosen for their therapeutic interest, i.e. topotecan and SN38 (the active metabolite of irinotecan). NiK-12192 resulted effective in sensitizing cells overexpressing P-glycoprotein (LoVo/DX) or BCRP (HT29/Mit) to topotecan and SN38 cytotoxicity. Other vacuolar-$H^+$-ATPase inhibitors derived from natural sources, such as bafilomycin A1 and concanamycin, have been reported to reverse multidrug resistant phenotype and to potentiate the activity of topoisomerase II inhibitors in cell systems (Ouar et al., 2003). Our findings showed that the NiK-12192 vacuolar-$H^+$-ATPase inhibitor was effective in modulating sensitivity of P-glycoprotein overexpressing cells to SN38. This finding may be of potential therapeutic interest, because irinotecan is used in clinical treatment of colon carcinoma. In addition, the results indicate the ability of NiK-12192 to increase topotecan effects in cell lines expressing an other transport system, the BCRP protein, which is present either in the parental HT29 cells or, at a much higher level, in the HT29/Mit cells (Perego et al., 2001; Croce et al., 2004).

The potentiation of the cytotoxic effects of camptothecins by NiK-12192 was reflected in a significant improvement in the antitumor activity *in vivo* of topotecan in combination with oral

NiK-12192. Although NiK-12192 itself was well tolerated at the tested dose, the combination of NiK-12192 with maximum tolerated doses of topotecan may result in an appreciable increase of drug toxicity. Indeed, with the administration sequence investigated in the study, i.e. 3 days of NiK-12192 after each dose of the cytotoxic drug, the schedule of topotecan administration was critical for the toxicity, which was observed when the drug (15 mg/kg) was delivered biweekly (q3/4d), but not when delivered weekly. The increase of toxicity could be expected considering that the target of NiK-12192 is not tumor specific. Thus, our results indicate that the choice of an appropriate treatment schedule is necessary to avoid undesirable effects. Moreover, preliminary results concerning the pharmacokinetics of NiK-12192 indicated a persistent plasma level, thus suggesting that less frequent administrations might be better tolerated.

The potentiation of antiproliferative/antitumor effects and the modulation of cellular acidification observed with the combined treatment of topotecan and NiK-12192 suggest a linkage between the two events. Indeed, NiK-12192, as well as bafilomycin A1, was effective in modulating the pattern of drug-induced acidification in acidic vesicular compartment, which can be a protective mechanism activated in response to DNA damage, as reported in cells treated with ionizing radiation (Paglin et al., 2001). Our study clearly showed that topotecan was able to enhance acidification in vesicular compartment of resistant cells and this behaviour was consistent with the ability of resistant cells to tolerate stress conditions. When NiK-12192 was present in topotecan-treated cells, the pH reversed to the value of untreated cells, with a pattern comparable to that found in sensitive cells. Although the fluorescence analysis did not allow a quantitation of the intracellular content of topotecan, the available data support a marked change in subcellular drug distribution. Indeed the concomitant exposure to topotecan and NiK-12192 resulted in an increased distribution of topotecan in nucleus and cytoplasm. A plausible explanation for this behaviour is that the acidification of the vesicular compartment influences the

subcellular localization of topotecan. It is unlikely that the sensitization of colon carcinoma cells was mediated by interaction of Nik-12192 with the transporters (BCRP or P-glycoprotein), because it was detected in cells expressing either low (parental) or high levels (resistant sublines) of these proteins. However, since changes of acidification could influence the expression of these transport systems (Adams et al., 2006), an indirect effect mediated by protein down-regulation could not be ruled out.

In the present study, NiK-12192 alone exhibited only a marginal activity against the two human tumor xenografts investigated. However, further studies will be necessary to better characterize the pharmacological profile of NiK-12192, and its efficacy when delivered by other routes and schedules. Noteworthy, in combination studies NiK-12192 was effective by oral route, and such property represents an important preclinical requisite of the compound, in perspective of a possible clinical use in combination with other antitumor agents. In previous studies, different proton pump inhibitors, i.e. pantoprazole and bafilomycin A1, have been reported to show antitumor activity in a human and in a rat tumor xenograft, respectively (McSheehy et al., 2003; Yeo et al., 2004). However, the two studies report different modalities of cell death in the different experimental systems, thus indicating that more investigations are needed to better elucidate the critical factors involved in the antitumor activity of proton pump inhibitors. Other classes of V-ATP-ase inhibitors are known, but results of *in vivo* antitumor activity are not available (Boyd et al., 2001; Beutler and McKee, 2003; Bowman et al., 2003; Niikura et al., 2004; Huss et al., 2005).

In conclusion, we have shown for the first time that NiK-12192, a small molecule vacuolar-$H^+$-ATPase inhibitor, was able to potentiate the antitumor activity of topotecan in preclinical tumor models, both in topotecan-sensitive and –resistant tumor xenografts. The synergistic interaction was more evident in the topotecan-resistant BCRP-overexpressing

HT29/Mit cell subline, thus supporting the potential interest of the novel inhibitor for the therapy of camptothecin-resistant tumors.

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**Footnotes**

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**Legends for Figures**

Figure 1. Chemical structure of NiK-12192.

Figure 2. Synergistic activity of NiK-12192 in combination with camptothecins on HT29 and LoVo parental and resistant-derived cell lines. Cell growth was evaluated by sulforodamine B assay after 72 h of drug exposure. Numbers indicate the combination index according to Drewinko (36). ●, topotecan or SN38 alone; ▲, topotecan + NiK-12192; ▼, SN38 + NiK-12192. Nik-12192 was used at subtoxic concentrations, i.e. 0.5 μM in HT29 and HT29/Mit and 1 μM in LoVo and LoVo/DX. Values are the mean (± SD) of three independent experiments.

Figure 3. Fluorescence images of HT29 and HT29/Mit cells incubated in topotecan (22 μM) with or without NiK-12192 (1 μM) for 40 min. Images were acquired by means of a processor digital system using a high sensitive ISIT camera coupled to a fluorescence microscope under excitation at 366 nm, and selected through a long pass filter 430 nm. Pictures were adjusted to comparable gray levels, to allow an easier comparison of the fluorescence patterns.

Figure 4. Fluorescence microscopy of HT29 and HT29/Mit cells exposed to topotecan or NiK-12192 or their combination. Cells were stained with the lysosomotropic agent acridine orange to detect the change of acidification in the acidic compartment.

Figure 5. BCRP expression in HT29 cells. Cells were exposed to $IC_{50}$ for each drug (topotecan or Nik-12192) for 72 h. Then the whole cells lysates were analyzed by western blotting with the

specific antibody. Actin is shown as control for protein loading. Lane 1, untreated cells; lane 2, cells treated with Nik-12192; lane 3, cells treated with topotecan.

Figure 6. Growth curves of HT29 and HT29/Mit tumor xenografts treated per os with solvent (X); topotecan, 15 mg/kg (△); NiK-12192, 20 mg/kg (□) or 30 mg/kg (■); topotecan+NiK-12192 (▲). Arrows indicate the days of treatment. Each point reports the mean values (± S.E.) of 9-10 tumors.

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**Table 1. Inhibition of vacuolar-$H^+$-ATPase activity by NiK-12192**

| Compound | Vacuolar-$H^+$-ATPase assay ($IC_{50}$, μM) | | | |
|---|---|---|---|---|
| | BCG[a] | hOc[b] | *hK*[c] | hL[d] |
| NiK-12192 | 0.017±0.003 | 0.025±0.001 | 0.22±0.10 | 0.54±0.10 |

[a]Bovine chromaffin granules

[b]Human osteoclastoma

[c]Human kidney

[d]Human liver

**Table 2. Antiproliferative activity of topotecan and SN38, alone or in combination with NiK-12192, on drug-sensitive and drug-resistant colon cancer cell lines**

| | $IC_{50}$ μM[a] | | | | |
|---|---|---|---|---|---|
| Cell line | NiK-12192 | topotecan | topotecan+Nik-12192[b] | SN38 | SN38+Nik-12192[b] |
| HT29 | 1.06 | 0.3 | 0.05 | 0.03 | 0.01 |
| HT29/Mit | 1.09 | 30 | 8 | 0.44 | 0.31 |
| LoVo | 2.51 | 13.3 | 4 | 0.06 | 0.002 |
| LoVo/DX | 3.12 | 17 | 10 | 0.15 | 0.05 |

[a]$IC_{50}$ were evaluated after 72 h of drug exposure, by sulforodamine B assay and represent the mean values of three independent experiments. Standard errors were lower than the 7% of each value.

[b]NiK-12192 was used at subtoxic conditions; 0.5 μM in HT29 and HT29/Mit, and 1 μM in LoVo and LoVo/DX cells.

**Table 3. Red/green fluorescence ratios in cells treated with NiK-12192, topotecan or both, for 48 h**

| | % of cells with a red/green fluorescence ratio of[a] | | | | | |
|---|---|---|---|---|---|---|
| | 2-3 | 3-4 | 4-5 | 5-6 | 6-7 | 7-8 |
| **HT29** | | | | | | |
| Control | | | 1 | 98 | 1 | |
| NiK-12192 | | | 2 | 82 | 16 | |
| Topotecan | | | 0 | 75 | 25 | |
| NiK-12192+topotecan | | | 8 | 78 | 14 | |
| **HT29/MIT** | | | | | | |
| Control | | | 23 | 23 | 54 | |
| NiK-12192 | | 35 | 18 | 47 | | |
| Bafilomycin A1 | 66 | 14 | | 20 | | |
| Topotecan | 14 | 3 | | 13 | | 70 |
| NiK-12192+topotecan | | | 4 | 48 | 48 | |

[a]Values were obtained from dot plot evaluation of FACScan analysis of acridine orange-stained cells. The intensity of red fluorescence is proportional to the degree of acidity and/or to the volume of the cellular acidic compartment.

**Table 4. Antitumor effects of the combination of topotecan and NiK-12192 against human colon carcinoma xenografts**

| | Drug[a] | Dose mg/kg | Days of treatment[b] | TWI%[c] observed | TWI%[c] expected | LCK[d] | Max[e] BWL% | Tox/Tot[f] (day) |
|---|---|---|---|---|---|---|---|---|
| HT29/MIT | NiK-12192 | 30 | 3-5, 7-9,11-13 | 26 | | 0.3 | 2 | 0/5 |
| | Topotecan | 15 | 3,7,11 | 43 | | 0.2 | 13 | 0/5 |
| | Topotecan<br>NiK-12192 | 15<br>30 | 3,7,11<br>3-5, 7-9, 11-13 | 75*** | 58 | 1.2 | 20 | 1/5 (16) |
| HT29 | NiK-12192 | 20 | 3-5, 7-9, 11-13 | 20 | | 0.2 | 0 | 0/5 |
| | Topotecan | 15 | 3,7,11 | 71 | | 1.6 | 6 | 0/5 |
| | Topotecan+<br>NiK-12192 | 15<br>20 | 3,7,11<br>3-5, 7-9, 11-13 | 88* | 77 | 2.5 | 19 | 1/5 (14) |
| | Topotecan | 15 | 3,10,17 | 70 | | 1.3 | 7 | 1/5 (23) |
| | Topotecan+<br>NiK-12192 | 15<br>30 | 3,10,17<br>3-5, 10-12, 17-19 | 82* | | 2.0 | 9 | 0/5 |

[a]Topotecan and NiK-12192 were delivered by oral route. In the combination treatment, topotecan was followed by NiK-12192 in about 1 h.
[b]Treatment started when mean tumor weight was around 100 mg.
[c]TWI% was calculated at day 18 or 28 after tumor cell inoculum when treatment ended at day 13 or 19, respectively. See Materials and Methods section for the assessment of observed and expected values.
[d]LCK=$Log_{10}$ Cell Kill (to 1 g of tumor).
[e]Maximum body weight loss in percentage
[f]Any death in treated mice occurring before any death in control mice.
*$p \leq 0.05$ and ***$p < 0.001$ vs topotecan-treated mice, by Student's t test.

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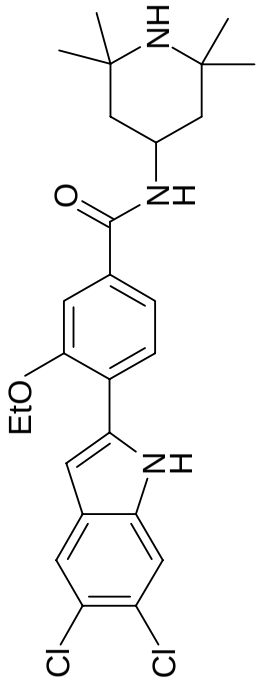


Figure 1

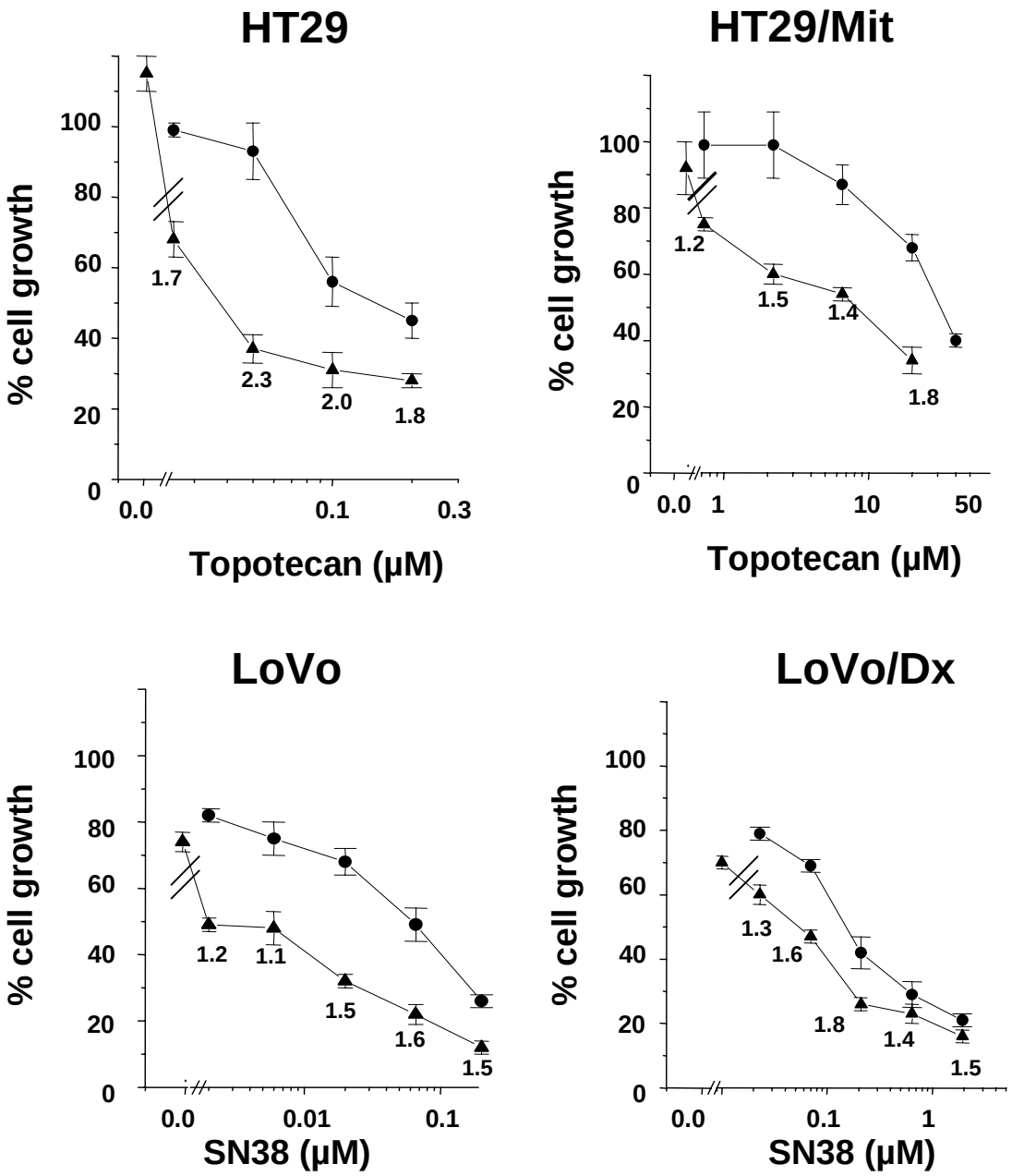


Fig. 2

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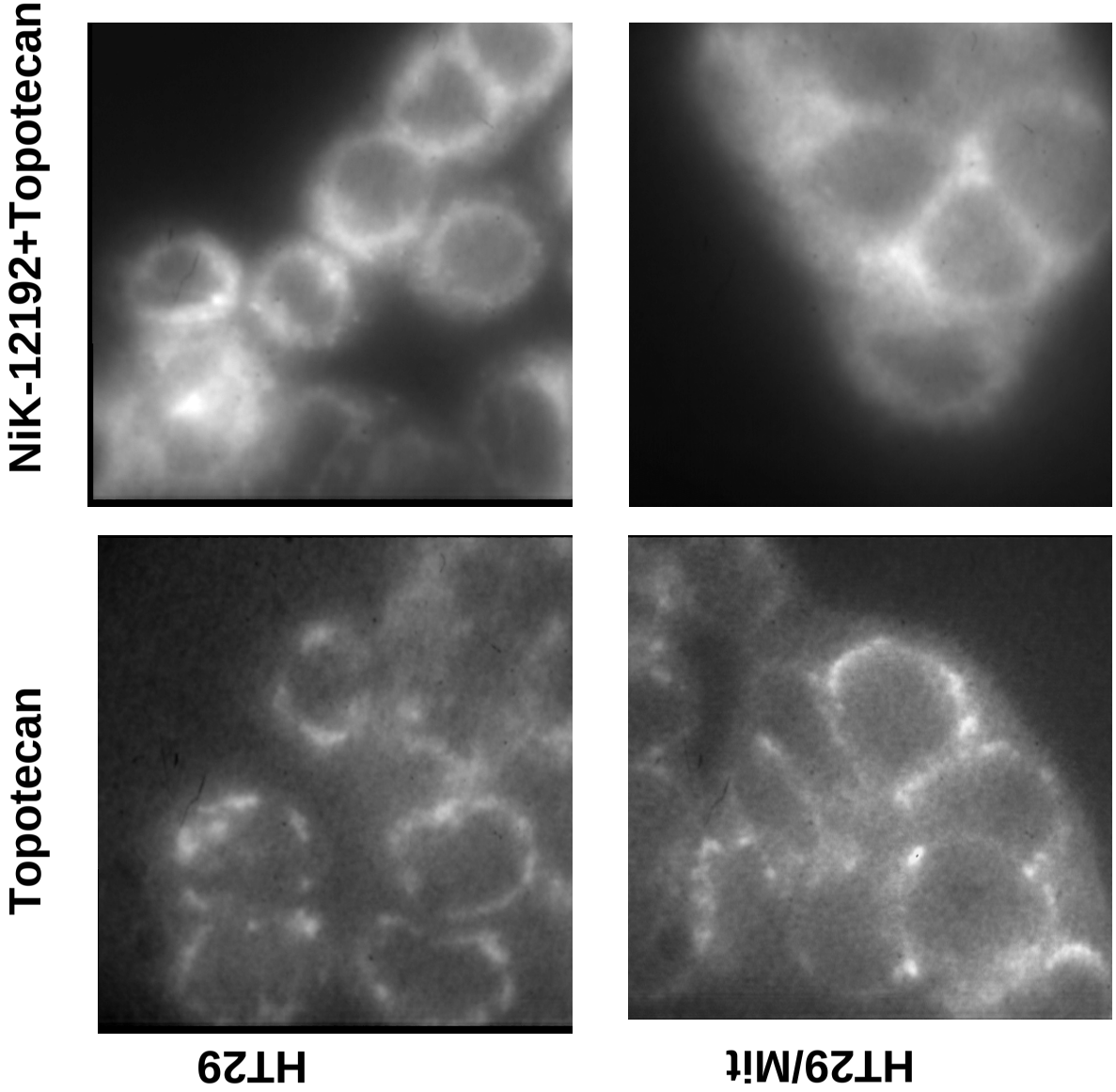


Fig. 3

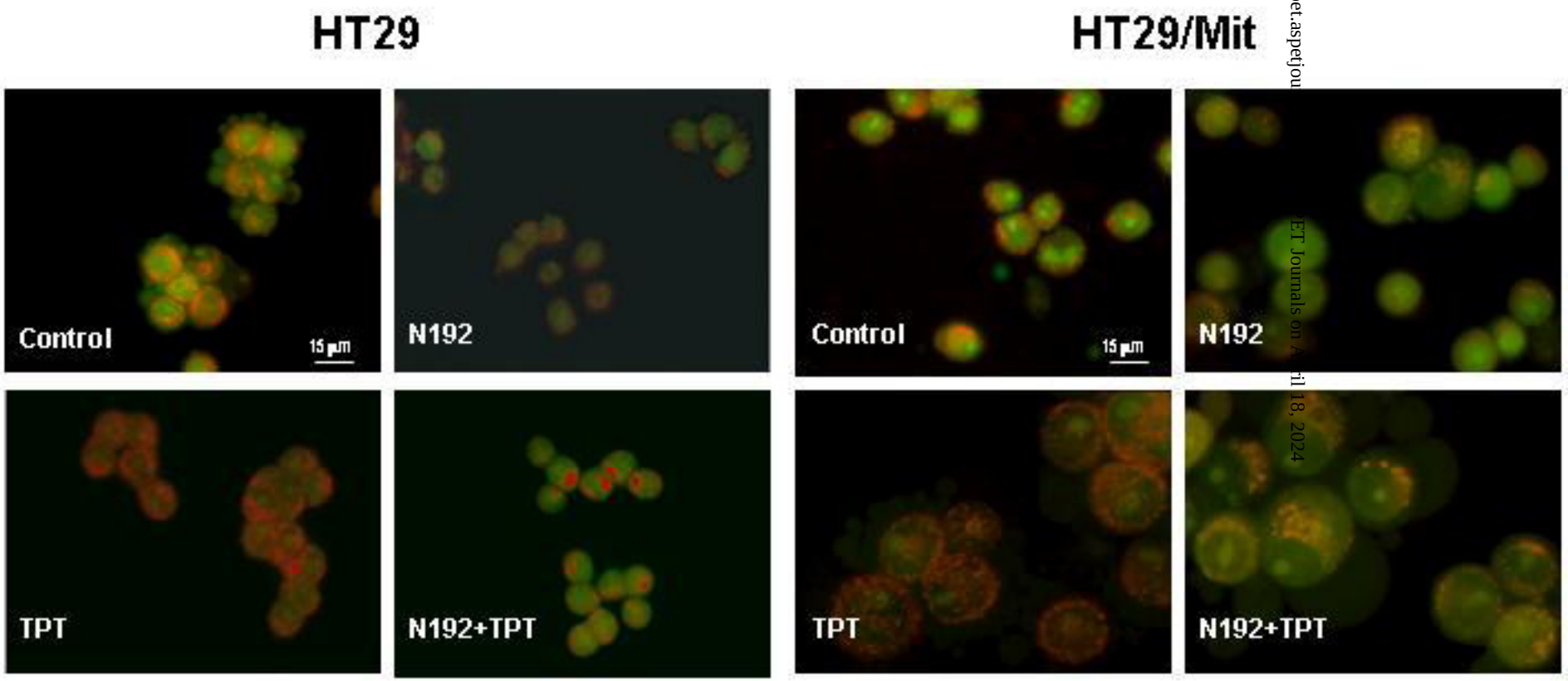


Fig. 4

Fig. 5

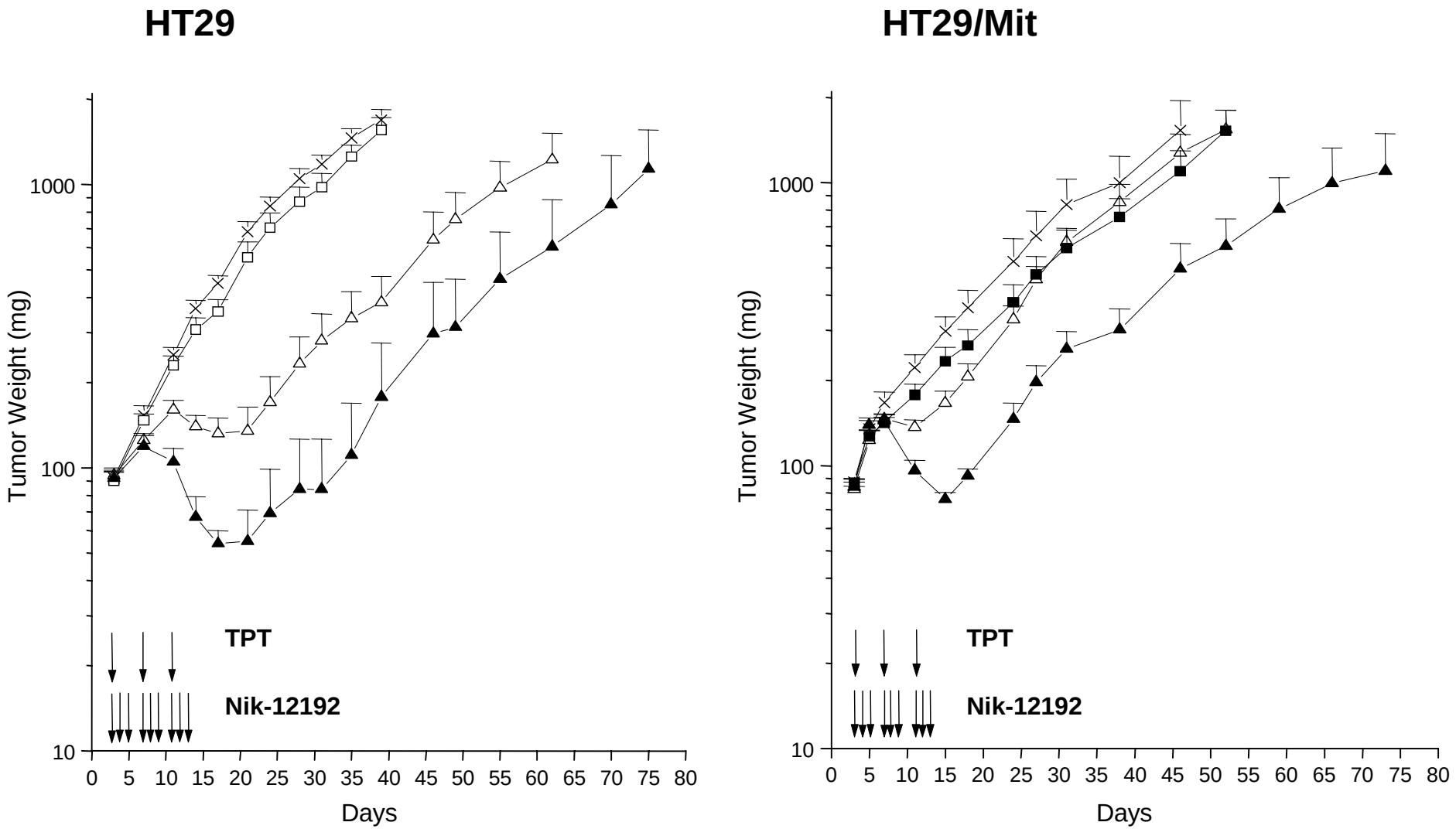


Fig. 6