G-Quadruplex Ligand RHPS4 Potentiates the Antitumor Activity of Camptothecins in Preclinical Models of Solid Tumors

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Abstract **Purpose:** The formation of G-quadruplex structures at telomeric DNA sequences blocks telomerase activity, offering an original strategy to design and develop new antitumor agents. The pentacyclic acridinium salt RHPS4 is one of the most effective and selective G4 ligands able to rapidly disrupt telomere architecture, resulting in apoptosis of cancer cells. Here, we studied the therapeutic index of RHPS4 and its integration with chemotherapeutics in preclinical model of solid tumors.

Experimental Design: The antitumoral activity of RHPS4 was evaluated on human xenografts of different histotypes and compared with that of standard antineoplastic agents. Moreover, the effect of RHPS4/chemotherapeutics combinations on cell survival was studied and the most favorable combination was evaluated on tumor-bearing mice.

Results: RHPS4 was active *in vivo* as single agent and showed a high therapeutic efficacy when compared with conventional drugs. Moreover, RHPS4 had antitumoral activity in human melanoma xenografts inherently resistant to chemotherapy and exhibited antimetastatic activity. RHPS4 also showed a strong synergistic interaction with camptothecins and this effect was strictly dependent on the drug sequence employed. Treatment of mice with irinotecan followed by RHPS4 was able to inhibit and delay tumor growth and to increase mice survival.

Conclusions: Our data show that RHPS4 has a good pharmacodynamic profile and in combination therapy produces a strong antitumoral activity, identifying this drug as promising agent for clinical development.

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Functional telomeres are required for the long-term proliferation of cancer cells, and without mechanisms maintaining telomeres, cells activate pathways leading to cell cycle arrest or apoptosis. Telomerase overexpression is required for telomere maintenance in the majority of cancer cells (1). In preclinical studies, telomerase inhibitors have shown promise as effective antitumor agents against a variety of xenografts. These translational advances have resulted in the first antitelomerase agent, the oligonucleotide-based GRN163L targeting the telomerase RNA template, entering clinical evaluation (2). Other possible targets for the disruption of telomere maintenance are specific DNA structures that can form from telomeric sequences. Telomere ends in a 3′ single-stranded overhang, also called G-overhang, which may be involved in different DNA conformations. Due to repetition of guanines, the G-overhang is prone to form four-stranded DNA structures, termed G-quadruplexes (G4), in either intramolecular or intermolecular conformations. There is now reason to believe that G4 structured DNA is not merely an *in vitro* artifact, strongly supporting the physiologic relevance of this nucleic acid structure at the telomeres (3). The inability of telomerase to use a G4 folded telomeric substrate has led to the emergence of a novel strategy for cancer therapy (4). G4-interacting agents are small molecules able to bind to, and stabilize, the telomeric DNA in a quadruplex conformation, thereby inhibiting telomere extension by telomerase (2–4). Also, results from different groups indicate that G4 ligands might disrupt telomere architecture, in both telomerase-positive and ALT-positive tumors, causing immediate and profound effects on cell proliferation (5). Over the past decade, many chemical classes of G4 ligands have been described. Several agents reduce the growth of various cancer cells *in vitro* after a few weeks of exposure to low micromolar concentrations, often accompanied by cellular senescence and/or apoptosis (2). Significantly, several G4 ligands also induce a short-term effect associated

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Translational Relevance

New antitelomere strategies represent important goals for development of selective cancer therapies because most human cancers acquire the ability to activate telomerase and possess altered telomeres compared with normal somatic cells. From this point of view, G-quadruplex ligands are promising compounds because they both inhibit telomerase activity, limiting long-term proliferation of cancer cell, and directly target components of the protective cap of telomere, leading to immediate effects on cancer cell proliferation. We recently showed that one of them, the RHPS4 molecule, has an antitumoral effect by targeting telomeric chromatin in xenografted tumor models. This report uncovers a specific link between RHPS4 and chemosensitivity toward camptothecins in colorectal tumors and identifies DNA damage response factors (phosphorylation of H2AX) as surrogate marker of tumor response. Our studies provide a compelling argument to suggest that the telomere pathway is a well-validated target at the preclinical level and encourage the development and evaluation of therapeutic combined option in future clinical protocols, especially for colon cancers.

with telomere uncapping; moreover, some of them exhibit antitumoral activity in mice bearing various human tumor xenografts (2).

RHPS4 (3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate) is a pentacyclic acridine (Fig. 1A) showing a high binding affinity for quadruplex DNA structures (6) and inhibits telomerase at submicromolar levels (7, 8). RHPS4 possesses classic telomerase inhibitory properties at low dosage (9, 10) and a growth-inhibitory effect at high concentration (9). Interestingly, the biological effects of RHPS4 occurred in malignant cells but not in their normal counterparts, which were unaffected by the treatment, suggesting that this agent would preferentially kill cancer cells (11). Moreover, RHPS4 had antitumoral activity linked to its ability to rapidly induce telomere damage and cell death (11). Telomere injury plays a critical role in the antitumoral properties of this compound and activation of damage response proteins (phosphorylation of H2AX) may be a surrogate marker of tumor response in clinical trials (11). At present, there is little known about the potential toxicities of this class of compound and their interaction with cytotoxics in preclinical models. These studies will be extremely informative in helping to guide rational clinical telomere-targeted therapeutic strategies.

Materials and Methods

Tumor cell lines. Human M14, LP, LM, and M20 melanoma, HT29 colorectal adenocarcinoma, and CG5 breast cancer cell lines were obtained and maintained as reported previously (12 – 16). Human PC-3 prostate, H460 non-small lung, and HCT116 colorectal cancer lines were obtained from the American Type Culture Collection.

Drugs. RHPS4 was synthesized as described previously (17). The following antineoplastic agents were used: (*S*)-(+)-camptothecin (Sigma), 7-ethyl-10-hydroxycamptothecin (Alexis), Adriamycin (Adriblastina; Pharmacia), gemcitabine (Gemzar; Eli Lilly), paclitaxel (Taxol; Bristol-Myers Squibb), cisplatin (DDP; Prontoplatamine; Pharmacia), irinotecan (CAMPTO; Pfizer), bleomycin (Bleomicina; Euro Nippon Kayaku), docetaxel (Taxotere; Aventis Pharma), and 5-fluorouracil (Fluorouracile Teva; Teva Pharma).

In vitro ***treatment.*** Cells were seeded at a density of 5×10^4 cells/plate and exposed 24 h later to the following drugs: RHPS4 (1-4 μmol/L for 96 h), DDP (3-12 μmol/L for 2 h), Adriamycin (0.1-0.4 μmol/L for 2 h), gemcitabine (4-16 nmol/L for 24 h), paclitaxel (1-8 nmol/L for

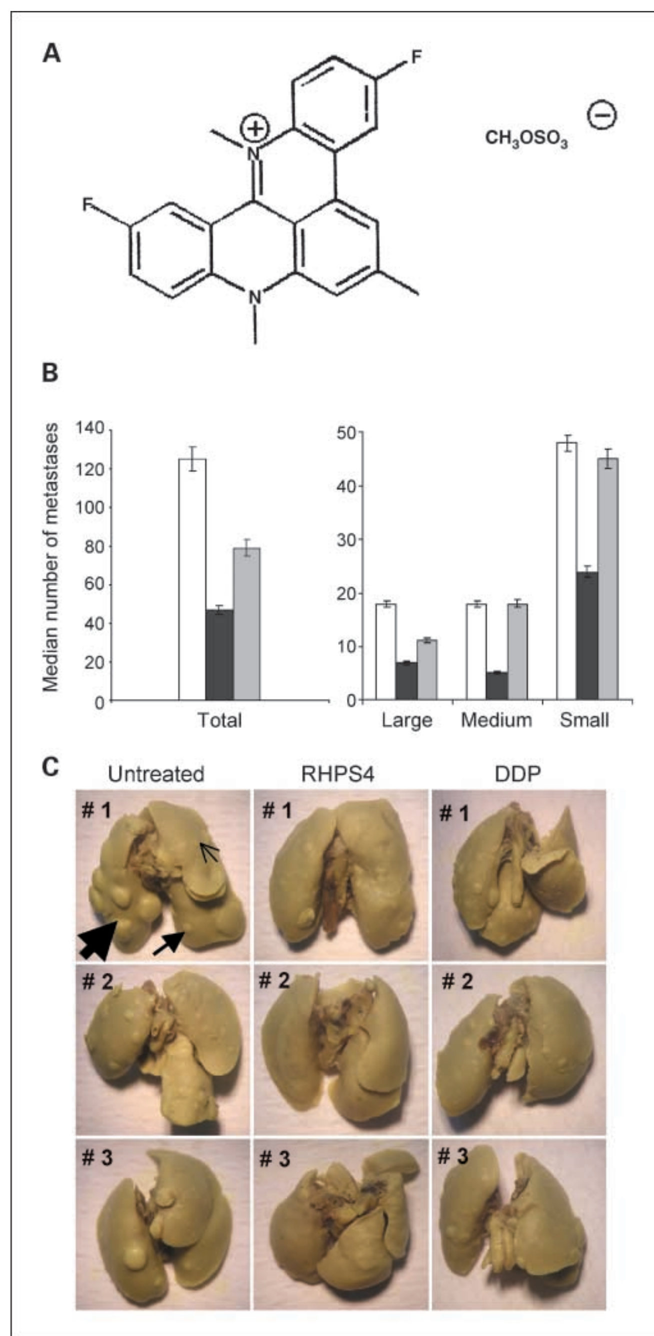

Fig. 1. Antimetastatic effect of RHPS4 or DDP on human melanoma. Mice bearing M20 tumors were treated with RHPS4 or DDP. At day 28 after tumor cell injection, lungs were removed and the number of metastases was determined. *A,* chemical structure of the pentacyclic acridine RHPS4. *B,* median number of lung nodules observed in control (*white column*), RHPS4-treated (*black column*), or DDP-treated (*gray column*) mice. *C,* representative images of lungs from the different groups. Large (↑), medium (↑), or small (↑) metastases are indicated.

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Table 1. Antitumoral activity of RHPS4 in comparison with antineoplastic drugs on different xenografts histotypes

Tumor histotype	Tumor line*	Drug †	TWI ‡ (%)	T-C§ (d)
Melanoma	M14	RHPS4	51	15
		DDP	32	7
Melanoma	LP	RHPS4	48	14
		DDP	31	6
Melanoma	LM	RHPS4	40	6
		DDP	14	1
Prostate	PC-3	RHPS4	44	8
		Docetaxel	39	5
Lung	H460	RHPS4	50	6
		Gemcitabine	30	2
Breast	CG5	RHPS4	52	7
		Paclitaxel	23	1
Colon	HT29	RHPS4	41	6
		5-Fluorouracil	30	1

*Mice were injected with the different tumor cell lines and treatment with RHPS4 or with the antineoplastic drugs started when a tumor mass of about ~300 mg was evident in mice.
†All the drugs were given at the maximum tolerated doses as reported in Materials and Methods.
‡TWI, calculated at the nadir of the effect as [1 - (mean tumor weight of treated mice / mean tumor weight of controls)].
§T-C, where T and C are the median times for treated and control tumors, respectively, to achieve equivalent size.

24 h), camptothecin (0.01-1 μmol/L for 2 h), 7-ethyl-10-hydroxycamptothecin (0.5-2 μmol/L for 2 h), and bleomycin (10-40 nmol/L for 2 h). In the combination experiments, the medium containing the first drug was removed and replaced with fresh medium containing the second drug. Colony-forming ability was evaluated as reported previously (12).

In vivo *experiments.* CD-1 male and female nude (*nu/nu*) mice, ages 6 to 8 weeks, were purchased from Charles River Laboratories. All procedures involving animals and their care were conducted as reported previously (12).

RHPS4 toxicologic profile was analyzed in healthy mice. Mice were evaluated for toxic deaths, body weight loss, white blood cells (WBC) and platelet number by microscopic count. Bone marrow cells were stained with May-Grünwald/Giemsa. For histologic analysis, mice were euthanized 2 days after the end of treatments and organs were collected and stained with H&E.

Systolic blood pressure, heart rate, and transthoracic echocardiography were evaluated in conscious mice as described previously (18). The antitumor effect of RHPS4, given intravenous at 10 mg/kg/d for 15 consecutive days, was evaluated on xenografts of different tumor histotypes and compared with conventional drugs. In particular, nude mice were injected with M14 or PC-3 cells at 5×10^6 per mice, HT29 or CG5 at 3×10^6 per mice, H460, LP, or LM cells at 2×10^6 per mice, and M20 at 1×10^6 per mouse. The drugs were given intraperitoneally at maximum tolerated dose assessed in previously experiments (12–16). In particular, tumor-bearing mice were treated with DDP (3.3 mg/kg/d for 3 consecutive days), docetaxel (5 mg/kg/d for 3 consecutive days), paclitaxel (10 mg/kg every 3 days × 3), 5-fluorouracil (19 mg/kg/d for 5 consecutive days), gemcitabine (80 mg/kg every 4 days × 3), and irinotecan (15 mg/kg/d for 5 consecutive days). All the treatments were started when a tumor mass of 300 to 350 mg was evident in the mice. In the combination experiments, the two drugs were administered with an interval of 24 h. Mice received drugs in one or two cycles of treatment with an interval time of 4 days between each cycle. Antitumor efficacy was evaluated as described previously (12) in terms of tumor weight inhibition (TWI), tumor growth delay (T-C), and antimetastatic activity.

The animals were euthanized for ethical reasons when tumors reached a mean of 3.5 g in weight or when they became moribund during the observation period (the time of euthanization was recorded as the time of death).

Immunohistochemistry. *In situ* detection of apoptosis was done by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay (Enzo Life Sciences, DakoCytomation) as reported previously (14). Apoptosis was counted in eight high-power fields (400× magnification) per section and reported as apoptotic index. Two independent observers did the counts in blinded fashion. Activation of damage response was determined immunohistochemically by using monoclonal antibody anti-γ-H2AX (Upstate) as reported previously (11).

Statistical analysis. Synergism, additivity, and antagonism were assessed by isobologram analysis as reported previously (16). Combination index (CI) values <0.9, > 0.9 < 1.2, and >1.2 indicate synergism, additivity, and antagonism, respectively. The statistical difference of tumor weight and apoptotic index among the different groups was determined by Student's *t* test assuming unequal variances. Survival

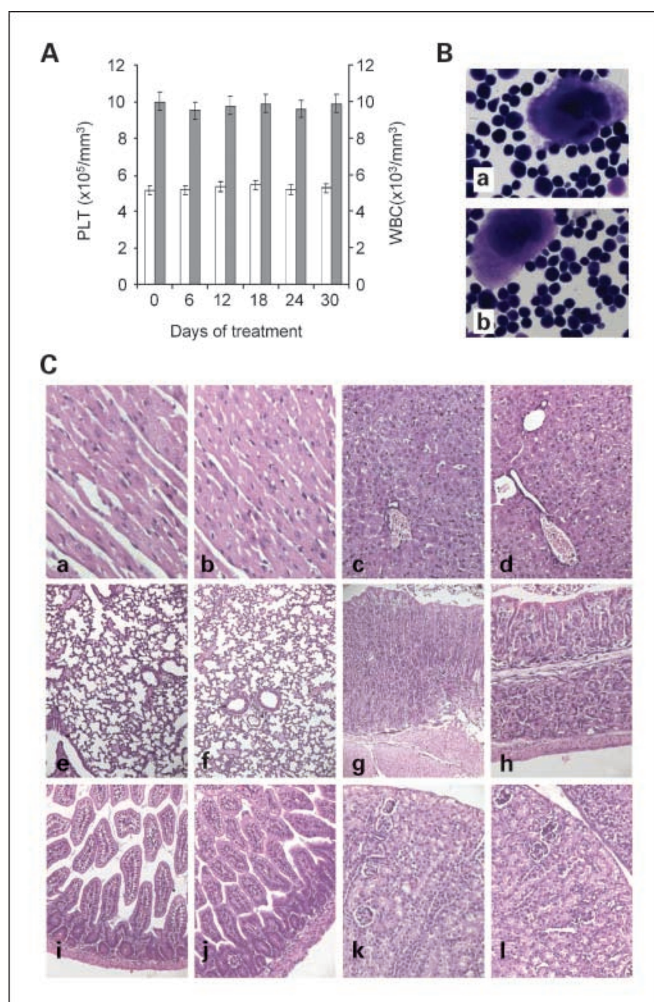

Fig. 2. Hematologic and histologic features following RHPS4 treatment. *A,* median number of WBC (*white column*) and platelet (*PLT*; *gray column*) calculated at day 0 (before treatment) during and after the end of treatment. *B,* May-Grünwald/Giemsa-stained cytospins from bone marrow of control (*a*) or RHPS4-treated (*b*) mice. Original magnification, ×20. *C,* H&E staining of tissue sections from heart (*a* and *b*; original magnification, ×400), liver (*c* and *d*; original magnification, ×200), lung (*e* and *f*; original magnification, ×100), stomach (*g* and *h*; original magnification, ×100), intestine (*i* and *j*; original magnification, ×100), and kidney (*k* and *l*; original magnification, ×200).

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curves of mice were generated by Kaplan-Maier product-limit estimate as described previously (15). Differences were considered statistically significant when $P < 0.05$.

Results

Antitumor activity of RHPS4 in comparison with standard chemotherapeutics on different xenografts. With the purpose to assess the potential use of RHPS4 as antineoplastic drug, we evaluated its ability to reduce the growth of human tumors xenografted in mice. Table 1 reports the antitumoral efficacy of RHPS4 in comparison with that of antineoplastic drugs commonly used in the management of human tumors. RHPS4 was more effective than DDP in melanoma xenografts. Indeed, RHPS4 treatment produced ~50% decrease of tumor mass, as evaluated at the nadir of the effect, with a T-C of 15 days. In contrast, DDP treatment resulted in a maximum of 30% TWI accompanied by 7 days of T-C. More interestingly, by using two melanoma lines derived from the primary tumor (LP) and the

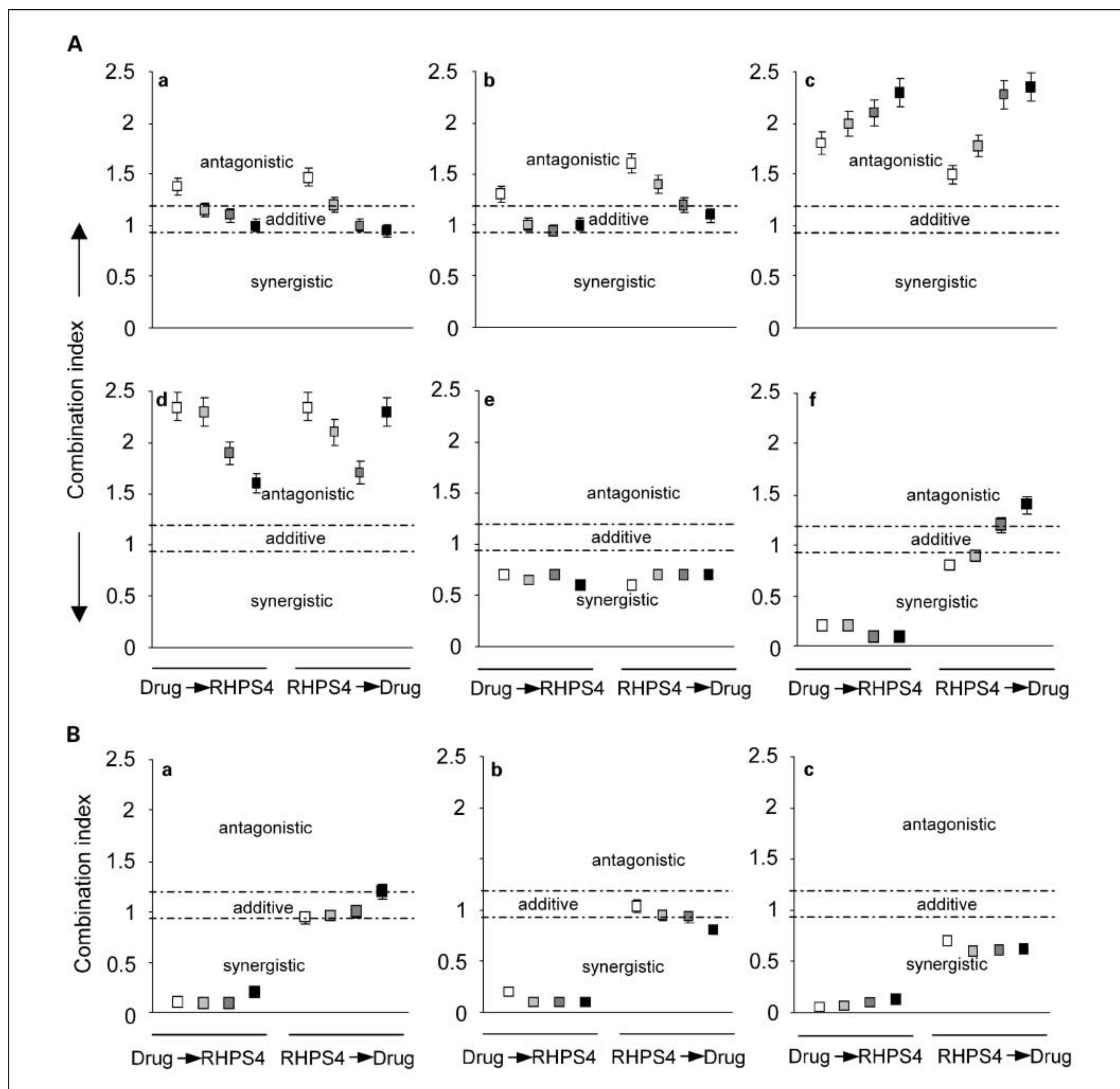

Fig. 3. Effect of RHPS4/drug combinations on cell survival. *A,* M14 cells were exposed to RHPS4 in combination sequence with DDP (*a*), Adriamycin (*b*), gemcitabine (*c*), paclitaxel (*d*), bleomycin (*e*), and camptothecin (CPT; *f*) and CI was calculated by the Chou-Talalay method. *B,* HCT116 cells were exposed to RHPS4 and camptothecin in combination (*a*). HT29 cells were treated with RHPS4 and camptothecin (*b*) or 7-ethyl-10-hydroxycamptothecin (SN-38; *c*). Data plotted are CI at 50% (*white squares*), 75% (*light gray squares*), 90% (*dark gray squares*), and 95% (*black squares*) fraction killed. Mean ± SD of three independent experiments.

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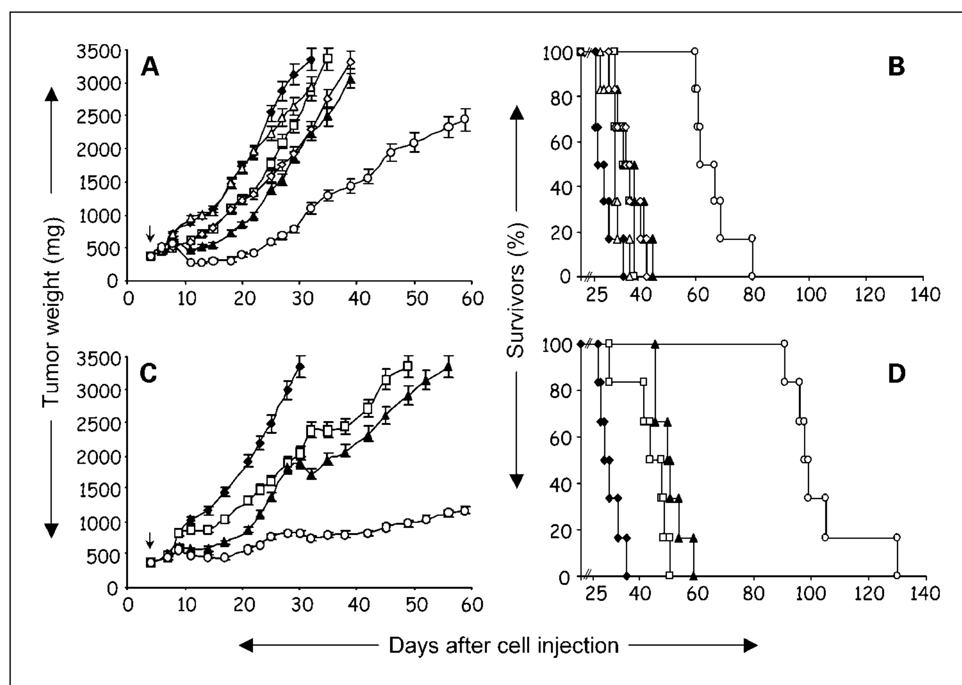

Fig. 4. Antitumor efficacy of RHPS4 in combination with irinotecan on HT29 xenografts. Tumor-bearing mice treated with RHPS4 and irinotecan alone or in combination given in one (*A* and *B*) or two (*C* and *D*) cycles of administration. *A* and *C*, mean ± SD tumor weight (mg). *Arrows*, start of treatments. *B* and *D*, survival curves. ♦, saline solution; □, RHPS4 (days 4-18); ▲, irinotecan (days 4-8); ○, irinotecan (days 4-8) followed by RHPS4 (days 9-23); ●, RHPS4 (days 4-18) followed by irinotecan (days 19-23); △, irinotecan late (days 19-23). Tumor weights and survival of mice treated with the schedule irinotecan followed by RHPS4 are significantly different ($P < 0.001$) compared with control and all treated groups.

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metastatic lymph node (LM) of the same patient, we found that RHPS4 was also effective in a human melanoma line inherently resistant to DDP. The results reported in the Table 1 show that RHPS4 was more active than DDP in reducing the growth of LP xenografts. The LM line, originated from the metastasis, was almost completely resistant to DDP. Interestingly, RHPS4 resulted effective in inhibiting the growth of LM tumors with ~40% decrease of tumor weight, thus indicating that RHPS4 could overcome the chemoresistance that usually arises during the tumor progression. RHPS4 also showed antitumor efficacy in other tumor lines of different histotype; again, its antineoplastic activity was higher than that of standard chemotherapeutics.

Then, we studied the ability of RHPS4 to reduce spontaneous metastases. As reported in Fig. 1B, treatment with RHPS4 markedly decreased the number of metastases from the M20 tumor, a highly metastatic human melanoma line, the median number of total nodules from lungs of mice treated with RHPS4 being significantly reduced compared with control mice ($P = 0.018$). Moreover, the antimetastatic effect of RHPS4 was stronger than that elicited by DDP ($P = 0.05$). Figure 1C shows representative images of lungs from three different controls, RHPS4- and DDP-treated mice, where large, medium, and small metastases are evident.

Toxicologic profile of RHPS4 in mice. The treatment of mice with RHPS4 given intravenous at 10 mg/kg/d for 15 consecutive days was well tolerated, as no toxic deaths or body weight loss was observed during or after treatment. Nevertheless, having observed that mice, just after treatment, adopted a crouched position for some minutes, we investigated if RHPS4 induced changes in blood pressure and heart function. We realized that RHPS4 caused a marked but reversible hypotension. The duration was dose dependent with a normalization of systolic blood pressure values at 25, 50, and 120 min after RHPS4 dose of 5, 10, and 15 mg/kg, respectively. Echocardiographic studies revealed a remarkable lower heart rate (520 versus 716 beats/min) and cardiac output (16 versus 33 mL/min) in the treated compared with the control mice; the fractional shortening values instead were comparable (66.7% versus 64.3%). The decrease in cardiac output in the absence of depressed contractility may be attributed to decreased venous return due to vasodilatation. Heart rate was constant during the echocardiographic exams in both animals and no arrhythmias were recorded.

We also studied the effect of RHPS4 treatment on hematologic and histologic features (Fig. 2). As it is evident from Fig. 2A, RHPS4 did not affect hematopoietic cells, as no change in WBC and platelet number has been observed during and after the end of treatment. Fig. 2B reports representative cytospins from control (*top*) and RHPS4-treated mice, showing that RHPS4 did not induce alterations on bone marrow cells. Finally, histologic analysis done at the end of treatment (Fig. 2C) revealed no evidence of lesions or morphologic alterations in the organs examined, thus confirming the absence of RHPS4-related toxicity.

In vitro *interaction between RHPS4 and chemotherapeutics.* The favorable therapeutic index of RHPS4 has prompted us to investigate its role in combination therapy. We evaluated the ability of RHPS4 to increase the cytotoxic activity of antineoplastic drugs, chosen based on their different mechanisms of action (Fig. 3A). The results, reported in terms of CI, show that the treatment of M14 melanoma cells with RHPS4 in combination with DDP or Adriamycin (Fig. 3A, *a* and *b*) was not particularly effective exhibiting only an additive or even antagonistic interaction (CI ≥1) regardless of the drug sequence employed. When RHPS4 was administered with gemcitabine or paclitaxel (Fig. 3A, *c* and *d*), a strong antagonistic effect was observed (CI ≥1.5), making both these combinations unsuitable. Conversely, a slight synergistic interaction was elicited by the combination of RHPS4 and bleomycin (Fig. 3A, *e*); the CI

was below 1 for both sequences employed. Notably, when cells were treated with RHPS4 followed by camptothecin, a slight synergistic effect was observed (Fig. 3A, *f*); more interestingly, the administration of camptothecin followed by RHPS4 resulted the most effective in reducing the survival of M14 cells, as a highly synergistic interaction between the two drugs was observed (CI ≤ 0.2).

Because camptothecins are now considered cornerstone drugs for the management of advanced colorectal cancer, we have tested the effect of RHPS4 combined with camptothecins on colorectal carcinoma lines. The results obtained confirmed the high efficacy of the RHPS4/camptothecin combination (Fig. 3B). Indeed, when HCT116 or HT29 cells were treated with camptothecin followed by RHPS4, a strong synergistic effect between the two agents was observed with a CI < 0.2 (Fig. 3B, *a* and *b*). The inverse sequence was less effective in reducing the survival of tumor cells, eliciting only an additive or slight synergistic interaction. Comparable results have been obtained by using 7-ethyl-10-hydroxycamptothecin, the active metabolite of irinotecan, a drug currently approved worldwide for use as first-line therapy in metastatic colorectal cancer (Fig. 3B, *c*).

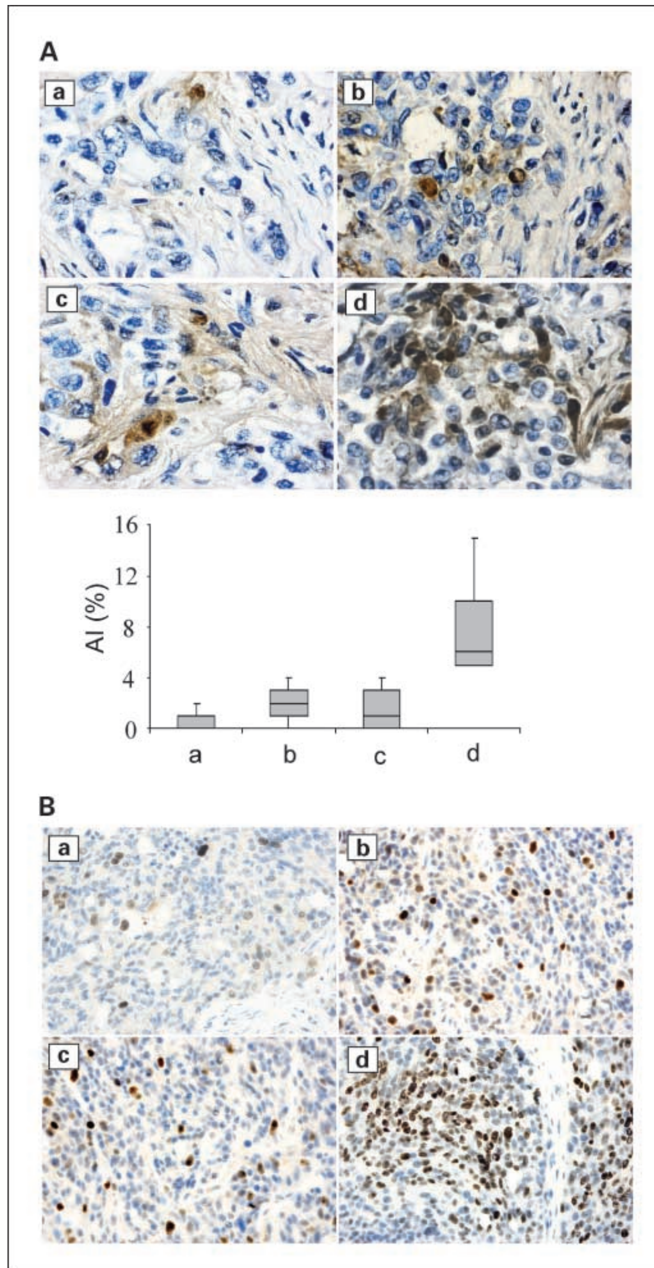

Fig. 5. Immunohistochemical analyses, in HT29 tumors from control and RHPS4/irinotecan-treated mice. Tumor-bearing mice were treated according the following schedules: *a,* saline solution; *b,* irinotecan; *c,* RHPS4; *d,* irinotecan followed by RHPS4. *A, upper,* representative images of apoptosis in tumor sections from mice treated with the different schedules. Original magnification, ×100. *Lower,* % of apoptotic index (AI), *lines,* median; *boxes,* 25th and 75th percentiles; *whiskers,* minimum and maximum values. *B,* immunohistochemical analysis of γ-H2AX. Magnification, ×40. Representative of three independent experiments with comparable results.

Therapeutic efficacy of RHPS4/irinotecan combination on HT29 xenografts. Based on the above reported *in vitro* experiments, the antitumoral efficacy of irinotecan/RHPS4 was studied on HT29-bearing mice. As shown in the Fig. 4, the treatment of mice with irinotecan→RHPS4 was more effective in reducing the growth of HT29 xenografts compared with the inverse sequence and with both drugs given alone (Fig. 4A). The tumor mass of mice treated with this schedule, as evaluated at the end of treatments, was significantly reduced ($P < 0.0001$) compared with all other groups. Indeed, treatment with irinotecan→RHPS4 produced the highest antitumor efficacy being ~80% TWI compared with control tumors. The TWI was accompanied by a T-C of 20 days, significantly increased ($P = 0.0005$) compared with mice treated with irinotecan alone (T-C = 10 days). The ability of irinotecan/RHPS4 combination in reducing the mass and in retarding the progression of tumor growth resulted in a marked improvement of mice survival (Fig. 4B). In fact, mice treated with the schedule irinotecan→RHPS4 exhibited a 139% increase in lifespan, significantly different ($P < 0.001$) compared with the inverse sequence and with all other groups.

Based on these results and in an effort to optimize the therapeutic index of this treatment, we evaluated if the administration of a second cycle of treatment could improve the response of HT29 xenografts to the therapy. As it is evident from Fig. 4C, the treatment of mice with irinotecan→RHPS4 given in two cycles of treatment exhibited an increased antitumor efficacy, especially in terms of duration of the response. Indeed, mice treated with the combination showed >80% TWI, with a tumor mass significantly reduced compared with control ($P < 0.0001$), irinotecan-treated ($P = 0.00013$), and RHPS4-treated ($P = 0.0003$) mice. Notably, the inhibitory effect persisted for ~2 months, as a T-C of 62 days has been observed compared with 15 and 8 days elicited by irinotecan and RHPS4 alone, respectively. This prolonged inhibitory effect on tumor growth led to an impressive increase of mice survival (~240%) significantly higher ($P < 0.0001$) compared with the other groups. Interestingly, no evidence of toxicity was noted in all treated mice, thus showing the favorable tolerability of this new antineoplastic strategy. Immunohistochemical analysis done in tumors sections showed that the highest therapeutic efficacy of the irinotecan/RHPS4 combination resulted from the activation of apoptosis and damage response (Fig. 5). In fact, the apoptotic index (Fig. 5A) and the percentage of γ-H2AX-positive cells (Fig. 5B) markedly increased ($P < 0.001$) in tumors from mice treated with the combination compared with saline solution or with irinotecan and RHPS4 alone.

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Discussion

The inability of telomerase to use a G4-folded telomeric substrate has led to the emergence of a novel avenue for cancer therapy based on the use of G4-stabilizing agents. In this context, by using RHPS4, we have recently validated telomere as pertinent drug target (11), providing a compelling rational to target the limitless replicative potential of malignant cells for broad-spectrum cancer therapy. Although questions remain regarding the *in vivo* mechanism of action of existing G4 stabilizers to predict what effects they have on noncancerous cells, the telomere-specific effects described by our group using RHPS4 (11) led us to further explore the translational process.

Here, we report that the telomere-interactive molecule RHPS4 has antitumor activity against a variety of human tumor xenografts in mice and its therapeutic efficacy is comparable, and often superior, with that of antineoplastic drugs commonly used in the human tumors. RHPS4 is also active on human melanoma inherently resistant to chemotherapy and inhibits the development of lung metastases. An antimetastatic activity has also been recently reported for the telomerase RNA template antagonist GRN163L and this effect was independent of telomerase inhibition and correlated with antiadhesive properties *in vitro* (19).

Interestingly, in view of clinical application, RHPS4 was well tolerated and has a good toxicologic profile. Indeed, RHPS4 does not induce alterations on hematopoietic/bone marrow cells and on major organs, thus showing the absence of drug-related toxicity, except for a dose-related hypotension that was marked but reversible even at the highest dose. The observation that RHPS4 has a high therapeutic index in xenografts should not depend on differences in telomere length and structure between mice and humans, because RHPS4 limits the growth of mouse cancer cells (data not shown) without affecting the viability of normal human cells (11). Whatever the reason will be, this differential response is intriguing and may open new avenues of interference.

The high therapeutic index of RHPS4 prompted us to warrant for further studies aimed at evaluating its role in combination therapy. Among the drugs analyzed, gemcitabine and paclitaxel show a strong antagonistic effect, making both these RHPS4 combinations unsuitable, although a slight additive or even antagonistic interaction was observed with DDP and Adriamycin. Antagonist drug effects observed between RHPS4 and the direct DNA-interacting agents (gemcitabine, DDP, Adriamycin, and the previously reported temozolomide; ref. 10) are not surprising considering that all the drugs can also react with telomeric DNA; therefore, the action of one drug could be sterically antagonized by the action of the other. Notably, a slight synergistic interaction was elicited by RHPS4/bleomycin combination; more interestingly, camptothecin acts clearly synergistically with RHPS4 and this effect was strictly depending on the sequence employed.

Several arguments suggest that the synergistic effect of the combination of camptothecin and RHPS4 results, at least in part, from an impaired telomere replication: (*a*) DNA replication plays an important role in the cytotoxicity of camptothecin; (*b*) both camptothecin and RHPS4 specifically target the G strand of telomeric DNA (11, 20); (*c*) telomere replication generates specific topologic aberrations, which might required more TOP1 to be resolved (21); and (*d*) the presence of G-quadruplex, either naturally occurring or stabilized by RHPS4, must be disrupted for replication elongation to proceed (21). The fact that telomeres are likely to be preferential targets of the two drugs, together with the sequence dependence of their synergistic effect, raise the interesting possibility that RHPS4 blocks the repair of the camptothecin-dependent damages formed during telomere replication. For instance, RHPS4 could prevent telomerase to act on abruptly shortened telomeres due to camptothecin-dependent cleavage of the G strand. Alternatively, camptothecin could modify the general sensitivity of the cell to RHPS4, for example, by increasing its intracellular concentration or by modifying the expression program or even by blocking cells at a particular stage of the cell cycle (22).

The effectiveness of the *in vitro* RHPS4/camptothecin combination has been confirmed *in vivo* showing that treatment of mice with irinotecan and RHPS4 was able to inhibit and delay the tumor growth of colon cancer and to increase the mice survival. Irinotecan is now considered a cornerstone drug in the management of colorectal cancer and in combination with 5-fluorouracil represents the standard chemotherapy for advanced stage of disease. However, resistance to irinotecan remains a major problem. Therefore, the use of new compounds able to improve the therapeutic efficacy of irinotecan without increasing the toxicity toward normal tissues could represent a promising strategy for the treatment of this neoplasia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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