Effect of RasGAP N2 Fragment–Derived Peptide on Tumor Growth in Mice

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Peptides that interfere with the natural resistance of cancer cells to genotoxin-induced apoptosis may improve the efficacy of anticancer regimens. We have previously reported that a cell-permeable RasGAP-derived peptide (TAT-RasGAP$_{317–326}$) specifically sensitizes tumor cells to genotoxin-induced apoptosis in vitro. Here, we examined the in vivo stability of a protease-resistant D-form of the peptide, RI·TAT-RasGAP$_{317–326}$, and its effect on tumor growth in nude mice bearing subcutaneous human colon cancer HCT116 xenograft tumors. After intraperitoneal injection, RI·TAT-RasGAP$_{317–326}$ persisted in the blood of nude mice for more than 1 hour and was detectable in various tissues and subcutaneous tumors. Tumor-bearing mice treated daily for 7 days with RI·TAT-RasGAP$_{317–326}$ (1.65 mg/kg body weight) and cisplatin (0.5 mg/kg body weight) or doxorubicin (0.25 mg/kg body weight) displayed reduced tumor growth compared with those treated with either genotoxin alone (n = 5–7 mice per group; P = .004 and P = .005, respectively; repeated measures analysis of variance [ANOVA, two-sided]). This ability of the RI·TAT-RasGAP$_{317–326}$ peptide to enhance the tumor growth inhibitory effect of cisplatin was still observed at peptide doses that were at least 150-fold lower than the dose lethal to 50% of mice. These findings provide the proof of principle that RI·TAT-RasGAP$_{317–326}$ may be useful for improving the efficacy of chemotherapy in patients.

Chemotherapeutic drugs kill tumor cells by activating apoptosis. If this activation is compromised (eg, through additional gene mutations), a tumor may develop resistance to the anticancer drugs (1,2). Consequently, strategies to restore tumor sensitivity to apoptosis are promising approaches for treating cancer. Much is now known about the mode of action of proteins that regulate cell death in cancer cells. This knowledge has led to the design of peptides that, in vitro, can perturb the resistance of cancer cells to anticancer agents (3,4). However, few studies have examined the efficacy of those peptides as anticancer compounds in vivo (3).

We have previously reported that fragment N2, a caspase-generated polypeptide from RasGAP, a regulator of Ras- and Rho-dependent pathways, strongly sensitizes tumor cells, but not normal cells, to genotoxin-induced apoptosis (5,6). The tumor sensitization property of the N2 fragment is contained within a 10–amino acid sequence (amino acids 317–326) (7). A synthetic peptide (called TAT-RasGAP$_{317–326}$) corresponding to this sequence fused to a cell-permeable peptide derived from the human immunodeficiency virus TAT protein was found to efficiently increase the extent of apoptosis induced by a variety of genotoxins (7) and other anticancer treatments (8) in several tumor cell lines. Although it is now known that tumor cells must be able to activate the p53 and p53-upregulated modulator of apoptosis (PUMA) pathway for them to undergo TAT-RasGAP$_{317–326}$ peptide–mediated sensitization to genotoxin-induced apoptosis (9), the molecular mechanisms underlying the apoptosis sensitization property of TAT-RasGAP$_{317–326}$ remain to be characterized in detail. Here, we assessed the ability of TAT-RasGAP$_{317–326}$ to increase the efficacy of genotoxins in an in vivo context.

We first examined the functional stability of the peptide in biological fluids because it has been shown that peptides that are susceptible to proteolytic degradation can rapidly lose their function (10,11). Indeed, the ability of the natural L-form of TAT-RasGAP$_{317–326}$ (L·TAT-RasGAP$_{317–326}$) to sensitize cisplatin-treated human p53-positive osteosarcoma U2OS cells (12) to undergo apoptosis [assessed by scoring the percentage of cells displaying pycnotic nuclei (9)] decreased as a function of increasing peptide preincubation time in serum-containing medium, suggesting that this peptide is sensitive to degradation by serum proteases (Figure 1, A). This sensitivity to degradation is likely to adversely affect the antitumor activity of the peptide in vivo. One way to render a peptide more resistant to proteolytic degradation is to convert its amino acids from the natural L-form to the protease-resistant D-form. To best mimic the structure of the natural peptide, the sequence of the D-peptide is generally inverted, generating the so-called retro-inverso form (13,14). After preincubation in serum-containing medium for up to 2 days, the retro-inverso form of TAT-RasGAP$_{317–326}$ (RI·TAT-RasGAP$_{317–326}$) showed almost no decrease in its ability to sensitize U2OS cells to cisplatin-induced apoptosis (Figure 1, A). This finding suggests that RI·TAT-RasGAP$_{317–326}$ is a more stable compound than L·TAT-RasGAP$_{317–326}$. Dose–response analyses indicated that RI·TAT-RasGAP$_{317–326}$ was more effective than L·TAT-RasGAP$_{317–326}$ in sensitizing U2OS cells to the apoptosis-inducing action of cisplatin (Figure 1, B and C), a likely reflection of its higher stability compared with L·TAT-RasGAP$_{317–326}$.

Another parameter that can affect the efficacy of an anticancer compound in vivo is its rate of clearance from the circulation. We used liquid chromatography coupled with mass spectrometry to examine the persistence of RI·TAT-RasGAP$_{317–326}$ in blood samples taken at various times from

a 7-week-old female NMRI Nu/Nu (nude) mouse (Janvier, Le Genest-St-Isle, France) that received a single intraperitoneal injection with the peptide at 3.3 mg/kg body weight. All experiments involving mice were approved by the State of Vaud Veterinary Office (Lausanne, Switzerland). RI·TAT-RasGAP$_{317-326}$ was detectable in the circulation as early as 15 minutes after injection and for at least 90 minutes after injection; peak concentrations were observed 1 hour after the injection of the peptide (Supplementary Figure 1, A and B, available online). By contrast, we could not detect the peptide in blood samples obtained from two mice that were similarly injected with L·TAT-RasGAP$_{317-326}$ (data not shown), consistent with the notion that the L-peptide is less stable, or more rapidly cleared, than the retro-inverso form. The observation that RI·TAT-RasGAP$_{317-326}$ could be detected in the circulation for relatively long periods of time suggested that it might be able to reach distant tissues, including tumors, after it is injected into mice. To test this possibility, a nude mouse bearing a visible tumor derived from subcutaneous injection of human colon cancer HCT116 cells was injected intraperitoneally with RI·TAT-RasGAP$_{317-326}$ (4.8 mg/kg body weight). The mouse was killed approximately 1 hour after peptide injection, and its liver, lungs, brain, and the tumor were removed and processed for peptide detection by mass spectrometry. RI·TAT-RasGAP$_{317-326}$ was detectable in the liver and lungs of this mouse but was not found in the brain (Supplemental Figure 1, C, available online), suggesting that it may not cross the blood–brain barrier. Importantly, RI·TAT-RasGAP$_{317-326}$ was also detected in the subcutaneous tumor.

We next evaluated the toxicity of RI·TAT-RasGAP$_{317-326}$ in nude mice that were injected intraperitoneally twice per week for 4 weeks with RI·TAT-RasGAP$_{317-326}$ or RI·TAT (a control peptide consisting of the retro-inverso form of the TAT peptide) (dose range = 0.8–7.2 mg peptide/kg body weight; n = 3–4 mice per group). Intraperitoneal injection of RI·TAT-RasGAP$_{317-326}$ at a dose of 4.8 mg/kg of mouse body weight was lethal (three of the three injected mice died, all within 45–60 minutes after the first injection), whereas a dose of 2.4 mg/kg body weight was not,

even after repeated injections (three of the three injected mice were alive after the eighth injection) (Supplementary Table 1, available online). These results indicate that the dose of RI·TAT-RasGAP$_{317-326}$ that is lethal to 50% of mice (ie, the LD$_{50}$) is between 2.4 and 4.8 mg/kg body weight. The lethality induced by high doses of RI·TAT-RasGAP$_{317-326}$ did not seem to be due to the presence of the cell-permeable TAT peptide because none of the three mice injected intraperitoneally with RI·TAT at a dose of 4.8 mg/kg body weight died. Necropsy revealed that the cause of death in mice injected with lethal doses of RI·TAT-RasGAP$_{317-326}$ appeared to be massive hemorrhages in the lungs and kidneys (Supplementary Figure 1, D, available online). No damage to the liver, pancreas, or spleen was observed (data not shown).

We next conducted two types of in vivo experiments to examine the effect of RI·TAT-RasGAP$_{317-326}$ on the efficacy of cisplatin against tumors, in particular under conditions in which the doses of cisplatin had poor or marginal effects. In the first experiment, 7-week-old female nude mice were injected intraperitoneally with 1.5×10^6 HCT116 cells. Beginning the next day, the mice were injected intraperitoneally twice per week for 4 weeks with phosphate-buffered saline (PBS), cisplatin (1 mg/kg body weight; catalog no. P4394, Sigma-Aldrich, St Louis, MO), RI·TAT-RasGAP$_{317-326}$ (2.4 mg/kg body weight), or cisplatin plus RI·TAT-RasGAP$_{317-326}$ (n = 15–24 mice per group). At the end of the treatment period, the mice were killed by cervical dislocation, and their tumors were removed, weighed (when possible), and scored empirically as follows: tumors weighing more than 1 mg (score 5), tumors weighing more than 0.5 mg up to 1 mg (score 4), tumors weighing more than 0.1 mg up to 0.5 mg (score 3), vascularized tumors grouped in clumps but too small to be weighed (score 2), or nonvascularized dispersed tumors too small to be weighed (score 1). Mice treated with RI·TAT-RasGAP$_{317-326}$ plus cisplatin developed statistically significantly fewer tumors with higher scores than mice treated with cisplatin alone (P = .032, two-sample location exact two-sided Wilcoxon test; all statistical analyses were performed using SAS/STAT software v9.1.3, SAS Institute, Inc. (Cary, NC) (Figure 2, A). This finding indicates

CONTEXT AND CAVEATS

Prior knowledge
Peptides that interfere with the natural resistance of cancer cells to genotoxin-induced apoptosis in vitro, such as TAT-RasGAP$_{317-326}$, a cell-permeable RasGAP-derived peptide, may improve the efficacy of anticancer regimens in vivo.

Study design
Examination of the in vitro and in vivo stability of a protease-resistant D-form of the peptide, RI·TAT-RasGAP$_{317-326}$, and its effect on tumor growth in nude mice bearing subcutaneous human colon cancer HCT116 xenograft tumors.

Contribution
RI·TAT-RasGAP$_{317-326}$ was stable in biological fluids, and after injection into mice, it persisted in the bloodstream for more than 1 hour, reached distant tissues and subcutaneous tumors, was effective at doses at least 150-fold below the dose lethal to 50% of mice, and improved the efficacy of genotoxins.

Implications
RI·TAT-RasGAP$_{317-326}$ may be useful for improving the efficacy of chemotherapy in patients.

Limitations
Tumors in nude (ie, immunocompromised) mice may not behave the same as syngeneic tumors in immunocompetent mice.

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that cisplatin in combination with the peptide hampers tumor growth better than cisplatin alone.

In the second type of experiment, HCT116 cells were injected subcutaneously into the left and right upper flanks of 7-week-old female nude mice (2.5×10^5 cells injected per flank). One week later, when visible tumors had developed, the mice were injected intraperitoneally once per day for 1 week with PBS, RI·TAT-RasGAP$_{317-326}$ (1.65 mg/kg body weight), or cisplatin (0.5 mg/kg body weight) or doxorubicin (0.25 mg/kg body weight; catalog no. D1515, Sigma-Aldrich), alone or in combination with RI·TAT-RasGAP$_{317-326}$ (n = 4–7 mice per group). We tested doxorubicin because it is structurally different from cisplatin and has a different mode of action (15). The orthogonal dimensions of

Figure 1. Effects of the L and the RI forms of TAT-RasGAP$_{317-326}$ on cisplatin-induced apoptosis in U2OS cells. Sequences of the peptide used in the figure: RI·TAT-RasGAP$_{317-326}$, DTRLNTVWM-WGGRRRQRRKKRG (D-amino acid); L·TAT-RasGAP$_{317-326}$, GRKKRRQRRRGGWMWVTNLRTD (L-amino acid); RI·TAT, RRRQRRKKRG (D-amino acid); and L·TAT, GRKKRRQRRR (L-amino acid). Apoptosis was assessed by counting the cells that displayed a pycnotic nucleus. The results presented in each panel correspond to the mean percentage of apoptotic cells for three independent experiments; **error bars** correspond to 95% confidence intervals. **A**) Peptide functional stability assay. U2OS cells were incubated for 20 hours with 15 μM cisplatin plus the indicated peptides, which were previously preincubated for the indicated times in Dulbecco's modified Eagle medium containing 10% newborn calf serum. Apoptosis was then assessed. **B**) Tumor cell sensitization in response to increasing peptide concentrations. U2OS cells were treated for 20 hours with increasing concentrations of the indicated peptides in the absence (**left panel**) or presence (**right panel**) of 15 μM cisplatin. Apoptosis was then assessed. **C**) Tumor cell sensitization induced by the peptides in response to increasing cisplatin concentrations. U2OS cells were treated for 20 hours without (Control) or with (20 μM final concentration) the indicated peptides in the presence of increasing concentrations of cisplatin. Apoptosis was then assessed. The values for L·TAT and RI·TAT are slightly offset for better visualization. L=natural; RI=retro-inverso.
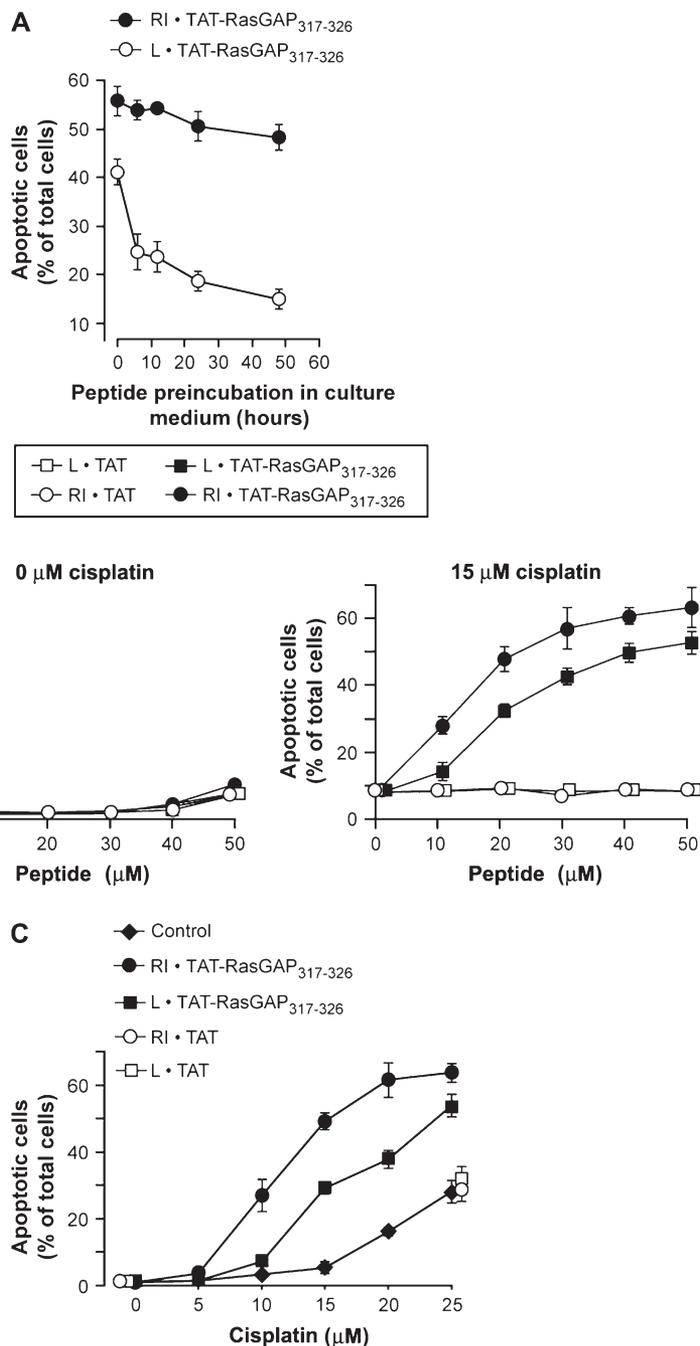
the tumors were measured daily with the use of a caliper on isoflurane-anesthetized mice, beginning on the first day of treatment injection (day 0, ie, when tumors were just visible), for up to 13 days. Tumor volume was calculated as [(largest orthogonal dimension)^2 × (smallest orthogonal dimension) × (π/6)]. When a mouse developed tumors on both flanks, the volumes of the two tumors were averaged to get a single value for that mouse to allow statistical comparisons with mice that developed only one tumor. All mice in a given experiment

were killed by cervical dislocation while still anesthetized when the largest tumors exceeded a specific threshold volume (500–1000 mm^3, depending on the experiment). Mice treated with RI·TAT-RasGAP$_{317-326}$ plus either cisplatin or doxorubicin developed statistically significantly smaller tumors than mice treated with RI·TAT-RasGAP$_{317-326}$ ($P=.005$ and $P<.001$, respectively) or with the respective genotoxin alone ($P=.004$ and $P=.005$, respectively; repeated measures analysis of variance [ANOVA, two-sided]) (Figure 2, B and C).

Compared with PBS, RI·TAT-RasGAP$_{317-326}$ by itself had no effect on tumor growth ($P=.89$ and $P=.41$ for the cisplatin and doxorubicin experiments, respectively; repeated measures ANOVA [two-sided]). These data demonstrate that RI·TAT-RasGAP$_{317-326}$ can inhibit the growth of already formed and detectable tumors when used in combination with a genotoxin (cisplatin or doxorubicin).

Given our finding that one injection of a lethal dose of TAT-RasGAP$_{317-326}$ led to hemorrhages in lungs and kidneys

Figure 2. In vivo chemosensitizing efficacy of RI·TAT-RasGAP$_{317-326}$. **A)** Effect of RI·TAT-RasGAP$_{317-326}$ on intraperitoneal tumors. Nude mice (7-week-old females) were injected with 1.5 million HCT116 cells intraperitoneally. The following day and thereafter twice a week, the mice were injected intraperitoneally with PBS, 2.4 mg/kg of RI·TAT-RasGAP$_{317-326}$, 1 mg/kg cisplatin, or a combination of cisplatin and RI·TAT-RasGAP$_{317-326}$. After 28 days, the mice were killed and the development of the tumors scored (score 5, tumors weighing more than 1 mg; score 4, tumors weighing between 0.5 and 1 mg; score 3, tumors weighing between 0.1 and 0.5 mg; score 2, vascularized tumors grouped in clumps but too small to be weighed; and score 1, nonvascularized dispersed tumors too small to be weighed); examples of tumors with scores 1 and 2 are shown on the right in (**A**). Mice that did not show any presence of tumors (ie, mice in which the tumors did not "take") were excluded from the analysis (PBS: four mice excluded of 15 injected; RI·TAT-RasGAP$_{317-326}$: three mice excluded of 15 injected; cisplatin: one mouse excluded of 21 injected; cisplatin + RI·TAT-RasGAP$_{317-326}$: four mice excluded of 24 injected); the proportion of excluded mice did not differ statistically significantly among the groups (Fisher exact test, P=.225). The **dots** represent individual mice. Statistical analysis for the difference between the cisplatin and the cisplatin + RI·TAT-RasGAP$_{317-326}$ groups was performed with a two-sample location exact two-sided Wilcoxon test. **B and C)** Cisplatin- and doxorubicin-sensitizing effect of RI·TAT-RasGAP$_{317-326}$ on subcutaneous tumors. Nude mice (7-week-old females) were injected subcutaneously with 250000 HCT116 cells on the left and right upper flanks. Seven days later, the mice that developed visible tumors were injected each day for 7 consecutive days (in **red** in the figure) with PBS (300 µL), RI·TAT-RasGAP$_{317-326}$ (1.65 mg/kg in 300 µL PBS), cisplatin (0.5 mg/kg in 300 µL PBS), or RI·TAT-RasGAP$_{317-326}$ plus cisplatin (**B**). In the experiment described in (**C**), cisplatin was replaced with 0.25 mg/kg doxorubicin. Tumor volume was plotted as a function of time (for mice that developed tumors on both flanks, the two tumor volumes were averaged). The number of mice analyzed is indicated in the figure. Mean values are plotted, and **error bars** correspond to 95% confidence intervals. **D)** Dose–response analysis of the in vivo sensitizing effect of the peptide. Nude mice were treated and analyzed as in (**B**) but with the conditions indicated in the figure. **E)** Peptide injection frequency. Nude mice were injected subcutaneously with 500000 HCT116 cells and treated as described in (**D**), except that 0.16 mg/kg body weight RI·TAT-RasGAP$_{317-326}$ was injected on the indicated days [**open diamonds**, **triangles**, and **squares**; cisplatin was injected every day as in (**D**)]. The P values in (**B–E**) are from repeated measures ANOVA (two-sided). ANOVA=analysis of variance; PBS=phosphate-buffered saline; RI=retro-inverso.
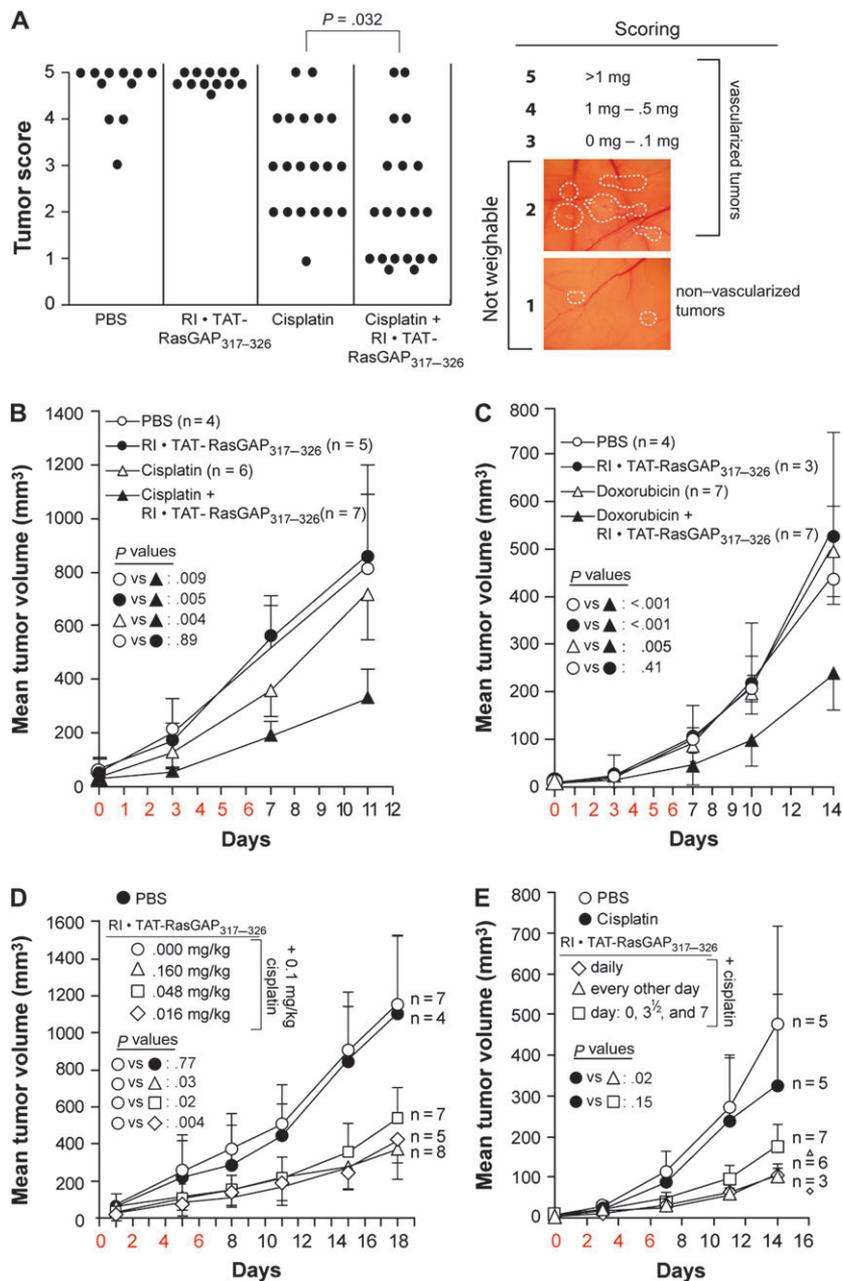
(Supplementary Figure 1, D, available online), we assessed whether the nonlethal dose of RI·TAT-RasGAP$_{317-326}$ that enhanced the sensitivity of tumors to cisplatin and doxorubicin in the previous experiment had any effect on these organs. Non–tumor-bearing mice were injected with PBS, RI·TAT-RasGAP$_{317-326}$, cisplatin, or RI·TAT-RasGAP$_{317-326}$ plus cisplatin as described above (n=2 mice per group); the mice were killed by cervical dislocation after the last injection (ie, 8 days after the first injection), and histological sections of

their lungs and kidneys were prepared. We observed no damage to these organs in any of the mice (Supplementary Figure 1, E, available online). Thus, a nonlethal dose of RI·TAT-RasGAP$_{317-326}$ (1.65 mg/kg body weight), which was only approximately two- to three fold lower than the lethal dose (~5 mg/kg body weight), did not appear to cause visible alterations in the lungs or kidneys, even when it was administered in the presence of cisplatin.

To examine if RI·TAT-RasGAP$_{317-326}$ doses lower than 1.65 mg/kg body weight

could exert a genotoxin-sensitizing effect on tumor growth in mice, we used the experimental design described above but with decreasing doses of injected RI·TAT-RasGAP$_{317-326}$ and with a dose of cisplatin that is at the threshold of inducing an inhibitory effect on tumor growth by itself. This dose of cisplatin was determined by injecting mice with various doses of cisplatin only (range=0.005–1 mg/kg body weight) using the injection protocol shown in Figure 2, B. The highest dose of cisplatin tested that did not reduce tumor growth was found to be

0.1 mg/kg body weight (data not shown). HCT116 tumor–bearing mice were treated with PBS or RI·TAT-RasGAP$_{317-326}$ (at doses of 0, 0.16, 0.048, or 0.160 mg/kg body weight) combined with cisplatin at 0.1 mg/kg body weight (n = 4–8 mice per group). Cisplatin alone (0.1 mg/kg body weight) had no effect on the growth of HCT116 tumors compared with PBS ($P = .77$; repeated measures ANOVA [two-sided]) (Figure 2, D). However, cisplatin plus RI·TAT-RasGAP$_{317-326}$ at 0.16 mg/kg body weight efficiently inhibited tumor growth compared with cisplatin alone ($P = .03$; repeated measures ANOVA [two-sided]). Importantly, RI·TAT-RasGAP$_{317-326}$ at 0.016 mg/kg body weight, a dose more than 150-fold lower than the estimated LD$_{50}$ (ie, between 2.4 and 4.8 mg/kg body weight), also improved the efficacy of cisplatin to inhibit the growth of HCT116 tumors compared with cisplatin alone ($P = .004$; repeated measures ANOVA [two-sided]).

We also examined the effect of varying the frequency of RI·TAT-RasGAP$_{317-326}$ peptide injection on tumor growth. HCT116 tumor–bearing mice were injected for 1 week with RI·TAT-RasGAP$_{317-326}$ (0.16 mg/kg body weight), daily, every other day, or every 3.5 days, in combination with a daily injection of cisplatin (0.1 mg/kg body weight) (n = 3–7 mice per group). Control mice were injected daily with PBS or cisplatin alone. Tumor volumes were measured every 3–4 days. Injection of the peptide every other day sensitized tumor cells to cisplatin (every-other-day peptide injection + cisplatin vs cisplatin only: $P = .02$; repeated measures ANOVA [two-sided]) as efficiently as daily injection of the peptide (every-other-day peptide injection + cisplatin vs daily peptide injection + cisplatin: $P = .94$; repeated measures ANOVA [two-sided]). Injection of the peptide every 3.5 days also sensitized the tumors somewhat to cisplatin (every 3.5 days peptide injection + cisplatin vs cisplatin only: $P = .15$; repeated measures ANOVA [two-sided]) but with reduced efficiency compared with daily or every-other-day injections of the peptide. These results indicate that RI·TAT-RasGAP$_{317-326}$ does not need to be injected every day along with cisplatin to exert its sensitization property, probably because of its increased resistance to degradation in biological fluids.

A limitation of this study is that tumors in immunocompromised mouse models (eg, nude mice) may not behave as syngeneic tumors in immunocompetent mice. Indeed, there is now clear evidence that the immune system positively modulates some anticancer therapies (16). For example, doxorubicin-induced death of colon carcinoma cells implanted in syngeneic mice elicits an immune response that favors the elimination of the tumor cells (17). The efficacy of RI·TAT-RasGAP$_{317-326}$ against human tumors implanted in nude mice might therefore be attenuated by the lack of an intact immune system in nude mice.

The properties of RI·TAT-RasGAP$_{317-326}$ described here in mouse tumor models indicate that this peptide has the potential to be used in humans to sensitize tumor cells to genotoxin treatments (ie, to enhance the antitumor effect of genotoxins): it is stable in biological fluids, it persists in the bloodstream for more than 1 hour after intraperitoneal injection, it reaches distant tissues and organs (including subcutaneous tumors), its efficacious doses are at least 100-fold below the LD$_{50}$, and it greatly improves the efficacy of genotoxins. To our knowledge, this peptide is the only compound that has been shown to improve the efficacy of genotoxins and that behaves strictly as a chemosensitizer, that is, it has no effect on tumors by itself (4). This compound would therefore have the potential to improve the efficacy of chemotherapeutic agents that are currently used in the clinic, particularly in situations in which doses of genotoxin have to be lowered to reduce side effects.

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