Enhancement of Vincristine Cytotoxicity in Drug-Resistant Cells by Simultaneous Treatment With Onconase, an Antitumor Ribonuclease

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Background: Onconase, a protein isolated from oocytes and early embryos of the frog Rana pipiens, shares extensive homology with bovine pancreatic ribonuclease (RNase A) and possesses similar enzyme activity. Onconase is cytotoxic toward cancer cells in vitro and exhibits antitumor activity in animal models. In addition, Onconase has been shown to enhance the cytotoxic activity of some chemotherapeutic agents in vitro. Purpose: We studied interactions between the cytotoxic effects of Onconase and vincristine (VCR) in the treatment of drug-sensitive and multidrug-resistant human colon carcinoma cells in vitro and in mice. Methods: Transplantable human colon carcinoma cells (HT-29par cells) were infected with a retrovirus containing human mdrl (also known as MDRI and PGY1) complementary DNA (encoding P-glycoprotein [P-gp]), and clones that were cross-resistant to colchicine, doxorubicin, and vinblastine were selected (HT-29mdrl cells). Drug-resistant HT-29mdrl cells and drug-sensitive HT-29par parental cells were treated with Onconase and/or VCR in vitro at varying concentrations to measure the effects on protein synthesis and cell viability. The impact of Onconase on VCR accumulation in both types of cells was determined in the presence or absence of MRK-16, an anti-P-gp monoclonal antibody capable of reversing the multidrug-resistant phenotype. The antitumor effects of Onconase and/or VCR treatment were assessed in nude mice bearing established HT-29par or HT-29mdrl intraperitoneal tumors. IC50 values (drug concentrations resulting in 50% inhibition of protein synthesis or cell viability) for Onconase and VCR were determined from semilogarithmic dose–response curves; interactions between the cytotoxic effects of these two agents were evaluated using data from protein synthesis inhibition experiments and a two-way analysis of variance. Survival distributions from in vivo experiments were compared using Cox proportional hazards models. Results: The combination of Onconase and VCR yielded enhanced cytotoxicity in vitro that was independent of P-gp expression. Evaluation of the effects of these two compounds on protein synthesis over a wide range of drug concentrations indicated possible synergistic interactions (i.e., greater than additive effects) in both drug-resistant and drug-sensitive cells. The enhancement of VCR cytotoxicity was dependent on Onconase enzyme activity and was not associated with increased intracellular levels of VCR. Simultaneous treatment of mice bearing HT-29par tumors with Onconase and VCR did not extend their median survival time (MST) significantly (MST with VCR = 66 days; MST with VCR plus Onconase = 69 days; two-tailed P = 0.57); however, the MST of mice with HT-29mdrl tumors was extended significantly by this treatment (MST with VCR = 44 days; MST with VCR plus Onconase = 66 days; two-tailed P < .001). Conclusion: Combined administration of Onconase and VCR yields enhanced cytotoxicity in vitro and in vivo against human colon carcinoma cells that overexpress the mdrl gene. [J Natl Cancer Inst 1996;88:747-53]

Onconase is a protein, isolated from the oocytes and early embryos of the frog Rana pipiens, that exhibits antiproliferative and cytotoxic activity toward cancer cells in vitro (1-3). Onconase also displays antitumor activity in animal models (4). A phase I clinical trial evaluating the efficacy of Onconase given as a single therapeutic agent on a weekly basis has recently been completed (5). Phase II trials are currently in progress. The primary sequence of Onconase and that of bovine pancreatic ribonuclease (RNase A) are highly homologous (1); furthermore, Onconase possesses RNase activity. The RNase activity of Onconase is essential for the observed cytotoxic and antitumor effects, since inactivation of this activity completely blocks the protein’s inhibitory effects in cell-free systems (6) and in cultured cells (7). The mechanism of Onconase action in cell-free systems and after injection into Xenopus oocytes involves RNA degradation and subsequent protein synthesis inhibition (6). Thus, Onconase, with its unique mode of action, has been found to enhance the activities of standard chemotherapeutic agents such as cisplatin (8) and tamoxifen (3). A phase I/II clinical trial evaluating the combination of Onconase and tamoxifen in patients with advanced pancreatic carcinoma has recently been conducted (9).

In the study reported here, we evaluated cytotoxic effect interactions between Onconase and vincristine (VCR), a chemotherapeutic agent that is commonly associated with the multidrug-resistant phenotype caused by expression of the 170 000-d membrane protein P-glycoprotein (P-gp; encoded by the MDRI or PGY1 gene) [reviewed in (10-12)]. The rationale for the study was derived from previous investigations that demonstrated additive and, in some cases, even synergistic effect interactions, with regard to antitumor activity, for combinations of

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See "Notes" section following "References."
standard chemotherapeutic agents and interferons (13). Recently, we showed that recombinant human interferon α could potentiate reversal of the multidrug-resistant phenotype when it was used in combination with the anti-P-gp monoclonal antibody, MRK-16 (14). Since one of the mechanisms of interferon action is the inhibition of protein synthesis, we reasoned that Onconase might display a similar cytotoxic effect.

Materials and Methods

Materials

Onconase (previously named P-30 protein) is a registered trademark of the AffaCell Corporation (Bloomfield, NJ). Onconase and alkylated Onconase were provided as lyophilized powders, which were subsequently dissolved in phosphate-buffered saline (PBS). The concentrations of Onconase and RNase A (Calbiochem, San Diego, CA) in solution were determined with the aid of a spectrophotometer and use of the extinction coefficients E 1%277 nm = 7.3 for RNase A and 8.8 for Onconase and alkylated Onconase, respectively. Stock solutions of at least 1 mg protein/mL each were kept frozen at -20 °C until dilutions were prepared for assays. The murine monoclonal antibody MRK-16 was provided by Hoescht Japan Limited (Kawagoe-City, Saitama, Japan); the production and characterization of MRK-16 were described previously (15). Colchicine was purchased from Sigma Chemical Co. (St. Louis, MO); VCR and doxorubicin were purchased from Whistell's Pharmacy (Frederick, MD). Plastic 96-well microtiter plates were obtained from Nunc, Inc. (Gaithersburg, MD), and all other cell culture supplies were obtained from Life Technologies, Inc. (GIBCO BRL) (Grand Island, NY).

Mice

Female, pathogen-free, athymic NCR-nu/nu mice (6-8 weeks of age) were obtained from the Animal Production Area, National Cancer Institute (NCI)-Frederick Cancer Research and Development Center. The mice were kept in cages with filter bonnets under sterile conditions and a laminar air flow unit. They were maintained on a diet of sterilized mouse pellets and water ad libitum. Animal care procedures were in accordance with standards described in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Cell Lines and Cell Culture

The HT-29 human transplantable colon carcinoma cell line and the MDA-MB-231human breast carcinoma cell line were obtained from the American Type Culture Collection (Rockville, MD). S. Bates (NCI, Bethesda, MD) provided the MCF-7 human breast adenocarcinoma cell line and its doxorubicin (Adr)-resistant derivative, Adr 7-MCF-7 (originally selected by Fairchild et al. (16)). All cell lines were maintained in tissue culture medium (Dulbecco’s minimum essential medium supple-mented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1x nonessential amino acids, 1 mM sodium pyruvate, 50 IU/mL penicillin, and 50 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Infection of HT-29 cells with an mdrl complementary DNA (cDNA)-containing retrovirus, yielding transfectants that overexpress P-gp by at least 15-fold, has been described (17). Briefly, the parental HT-29 cells (HT-29mdrl), as well as MDA-MB-231 cells (MDA-MB-231mdrl), were infected with phAmdr/A, a retrovirus carrying the human mdrl cDNA, and clones that were cross-resistant to colchicine, doxorubicin, and vinblastine were isolated. These clones, designated HT-29mdrl and MDA-MB-231mdrl, were expanded and maintained in culture medium containing 10 ng/mL colchicine.

Cytotoxicity Assays

Protein synthesis was measured as described previously (18). Briefly, cells were plated (at densities given in the figure legends) in 96-well microtiter plates in 100 μL of Dulbecco’s minimum essential medium (supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, and nonessential amino acids); additions were made in a total volume of 10 μL (i.e., approximately 10% of the final volume), and the plates were incubated at 37 °C for the times detailed for each experiment. PBS containing 0.1 μCi of [3H]leucine (310 mCi/mmol; Du Pont NEN, Boston, MA) was added for 2-4 hours, and the cells were then collected onto glass fiber filters using a PHD cell harvester (MELCO Scientific, Arnold, MD), where they were lysed and washed with water to remove uncorrelated [3H]leucine. The filters were subsequently dried with ethanol and counted. Results obtained with this assay are expressed as percentages of [3H]leucine incorporation in mock-treated wells. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (19), measuring cellular cytotoxicity, was also used in some experiments; in these experiments, the cells were treated and the assays were performed as previously described (17).

Cellular Uptake and Flux of [3H]VCR

A modification of the technique described by Rittmann-Gruener et al. (20) was used. Cultures of tumor cells in log-phase growth were harvested and then seeded at 5 x 106 cells per well in 2 mL of medium in 12-well, flat-bottom, plastic tissue culture plates (Falcon, Cambridge, MA). Following attachment to the plastic, the cells were washed, and 2 mL of fresh culture medium containing MRK-16 (50 μg/mL), with or without Onconase (0.8 μM), was added to the wells. The cells were incubated for 1 hour at 37 °C before the addition of 0.1 μCi [3H]VCR (specific activity, 7 Ci/mmol; Amersham Life Science, Inc., Arlington Heights, IL; final concentration, 14 nM) per well. Triplicate cultures were incubated for an additional 1, 2, 4, or 6 hours at 37 °C. The cultures were then washed three times with fresh medium, followed by lysis of the cell monolayers with 0.4 mL 0.1 N NaOH. Cell-associated radioactivity was determined by liquid scintillation counting of the entire sample. Data were expressed as total cell-associated VCR.

Human Colon Carcinoma Xenograft Model

In vivo animal studies were performed using a colon carcinoma xenograft model essentially as described (17). In this model, untreated animals possess solid peritoneal tumors and large numbers of intraperitoneal ascitic tumor cells with no evidence of distant metastases. In this study, intraperitoneal treatment with VCR, Onconase, or a combination of the two agents was initiated 10 days after the intraperitoneal injection of colon carcinoma cells (5 x 106 cells); treatments were administered weekly, with a total of three treatments given. Eight and 11 animals per treatment group were used for experiments with the sensitive and the resistant colon carcinoma cell lines, respectively. All animals had detectable, malignant ascitic tumor cells at the start of treatment.

Statistical Methods

IC50 values (i.e., drug concentrations resulting in 50% inhibition of either cell viability or protein synthesis) for Onconase and VCR were determined directly from semilogarithmic dose-response curves. The statistical significance of some in vitro comparisons was assessed using the two-tailed Student’s test. Survival distributions from in vivo experiments were compared by use of Cox proportional hazards models (21); computations were performed using the computer program developed and described by Thomas et al. (22). Testing for the presence of synergy in the effects of VCR and Onconase against colon carcinoma cells involved a two-way analysis of variance of the logarithms of the residual fractions (of protein synthesis) observed at each concentration of the two agents, alone or in combination. Tests for synergy incorporated data from experiments done in triplicate microtiter plates, each plate containing a complete replicate of all factorial combinations. The significance of the interaction of the effects of the two agents at each combination was determined by a one-tailed t test. Thus, at any combination of VCR and Onconase, an observed geometric mean fraction significantly less than that predicted by the product of the marginal fractions was interpreted as evidence of synergy. This fractional product method formalized by Webb (23), outlined by Valeroite and Lin (24), and applied by Hu et al. (25) is valid for agents whose mechanisms of action are mutually nonexclusive (i.e., totally independent of one another) and whose dose-effect curves are hyperbolic (26). To the extent that deviations from this assumption of mutual nonexclusivity exist for the two agents tested, the significance of any conclusions regarding synergy will be lessened.

Results

In Vitro Sensitivity of HT-29par and HT-29mdrl Cells to Onconase and VCR

Onconase has been shown to inhibit cell-free (6) and cellular protein synthesis (7); its inhibition of cellular protein synthesis can result in cell death. We as-
sessed the effects of VCR and/or Onconase on protein synthesis in HT-29par and HT-29mdrl cells by monitoring changes in [14C]leucine incorporation. The data presented in Fig. 1, top panel, were derived from the pooled results of several experiments. There was a substantial difference in the estimated IC50 values obtained for VCR with the parental and the drug-resistant cell lines (estimated IC50s of 6 × 10⁻¹⁰ M and 5 × 10⁻⁸ M, respectively). In contrast, the cytotoxic effect of Onconase was unrelated to P-gp expression levels (IC50 of approximately 6 × 10⁻⁵ M in both cell lines). Since similar dose-response curves were obtained for Onconase with HT-29par cells and HT-29mdrl cells, we next evaluated the ability of a fixed concentration of Onconase to modulate the toxicity of VCR in both cell types. When used alone at a concentration of 0.8 μM, Onconase did not inhibit protein synthesis (1536 ± 162 cpm [mean ± standard error of the mean] versus 1525 ± 51 cpm of radiolabeled leucine incorporated in the presence and absence of Onconase, respectively); however, it enhanced the protein synthesis inhibition caused by VCR in both cell lines (Fig. 1, bottom panel). In the presence of Onconase, the estimated IC50 of VCR on HT-29par protein synthesis was less than 1.3 × 10⁻¹⁰ M (the lowest concentration tested), compared with 3 × 10⁻¹⁰ M without Onconase. With HT-29mdrl cells, the estimated IC50 values were 7 × 10⁻¹⁰ M and 7 × 10⁻⁸ M, respectively. Thus, Onconase enhanced VCR cytotoxicity in multidrug-resistant human colon cancer cells as well as in the corresponding drug-sensitive parental cells.

The results obtained with protein synthesis inhibition measurements were consistent with those obtained using the MTT assay to measure cell viability. Parallel experiments conducted using both assays and HT-29mdrl cells yielded similar IC50 values for Onconase: 5.7 × 10⁻⁵ M with the protein synthesis assay and 7.1 × 10⁻⁵ M with the MTT assay; for VCR, the IC50 values obtained with the two assays were 3 × 10⁻⁹ M and 2.2 × 10⁻⁸ M, respectively. Similarly, the IC50 values for mixtures of Onconase and VCR were very nearly identical whether measured by protein synthesis inhibition or by the MTT cytotoxicity assay (data not shown). Therefore, in these experiments, the combined means of these individual experiments and standard errors (when they are greater than the symbol size) are shown. VCR in HT-29par cells (solid squares) and VCR in HT-29mdrl cells (open squares); Onconase in HT-29par cells (solid circles); Onconase in HT-29mdrl cells (open circles). Bottom panel: Enhancement of VCR cytotoxicity in HT-29par and HT-29mdrl cells by simultaneous treatment with Onconase. In these experiments, a fixed concentration of Onconase (0.8 μM) was used with varying concentrations of VCR. Duplicate analyses were performed for each data point. Two separate analyses were combined to obtain the data points for VCR plus Onconase. The combined means of these individual experiments and standard errors (when they are greater than the symbol size) are shown. VCR (solid squares) and VCR + Onconase (open squares) in HT-29par cells; VCR (solid circles) and VCR + Onconase (open circles) in HT-29mdrl cells.
ments, protein synthesis inhibition accurately reflects overall cytotoxicity.

As seen in Fig. 1 (top panel), the response of HT-29 colon carcinoma cells to 0.8 μM Onconase was variable (75%-100% of the protein synthesis measured for the control); therefore, combinations of Onconase and VCR were examined over a wider range of concentrations and tabulated (Table 1). These data were evaluated using a two-way analysis of variance and the one-tailed t test.

Mechanistic studies (7) and evaluation in the National Cancer Institute antitumor drug screen (Rybak SM: unpublished data) indicate that Onconase and VCR are agents whose mechanisms of action are totally independent of one another. Therefore, the fractional product method (23) was applied to analyze the combined drug effects. This analysis showed that some combinations of the two agents yielded significantly greater inhibition of [14C]leucine incorporation experimentally than was predicted from the additive effects of the individual compounds, suggesting synergistic effect interactions. With the drug-sensitive HT-29par cell line (Table 1), possible synergistic effect interactions (one-tailed P<.05), as well as interactions that were less than expected, were observed to be clustered around VCR concentrations of 1.3 nM or less (11 [73.3%] of 15 agent combinations). In contrast, with the resistant HT-29mdr1 cell line, possible synergy (one-tailed P<.05) plus interactions that were less than expected appeared over a broader range of VCR doses, with 61.9% (13 of 21) of these interactions occurring at concentrations of VCR above 1.3 nM. These data indicate that the effect interactions of VCR and Onconase may differ, with respect to the VCR concentration, in the parental and the drug-resistant cells.

Although this study focused on effect interactions using VCR and Onconase in human colon carcinoma cells, the in vitro modulatory activity of Onconase also extended to other carcinoma cell lines and other P-gp substrates (Rybak SM: unpublished data).

Lack of Effect of Onconase on Cellular Levels of VCR

P-gp expression results in decreased cellular accumulation of VCR, and treatment with the monoclonal antibody MRK-16 (anti-P-gp) increases the amount of drug in P-gp-expressing cells (14,15). Therefore, the effect of Onconase on the uptake of VCR, in the presence or absence of MRK-16, was examined in HT-29par and HT-29mdr1 cells. The accumulation of VCR (14 nM) was time dependent in HT-29par cells and was not affected by the presence of MRK-16, Onconase, or a combination of the two agents (Fig. 2, top panel). The accumulation of VCR in the parental cells was then compared with that observed in HT-29mdr1 cells. As shown in Fig. 2, bottom panel, the accumulation of VCR in the drug-resistant cells was impaired, and the addition of MRK-16 antibody increased the drug level in these cells. However, treatment with Onconase, either alone or in the presence of MRK-16, did not affect the rate of VCR accumulation. These results are consistent with the data in Fig. 1 that established that Onconase cytotoxicity is independent of processes associated with the multidrug-resistant phenotype. Taken together, the results imply that Onconase does not directly affect the function of the P-gp drug transporter and does not affect drug accumulation indirectly.

Requirement of Onconase Enzymatic Activity to Enhance VCR Cytotoxicity

Onconase’s ability to enhance VCR cytotoxicity appears to require the specific enzymatic activity of the protein, since alkylated Onconase, with 2% residual activity (I), does not sensitize tumor cells to the drug. Protein synthesis was measured after treating HT-29mdr1 cells with VCR (5 × 10⁻⁹ M) in the presence or absence of either Onconase or alkylated Onconase (0.8 μM) for 48 hours. As expected, the protein synthesis

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**Table 1. Interaction between Onconase and vincristine (VCR) in HT-29par human colon carcinoma cells in vitro**

<table>
<thead>
<tr>
<th>Onconase concentration, μM</th>
<th>0</th>
<th>0.007</th>
<th>0.13</th>
<th>1.3</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitive HT-29par cells</strong></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>1.000</td>
<td>0.972</td>
<td>0.906</td>
<td>0.758</td>
<td>0.262</td>
<td>0.238</td>
<td>0.234</td>
<td>0.196</td>
<td>0.175</td>
</tr>
<tr>
<td>0.008</td>
<td>0.974</td>
<td>0.930</td>
<td>0.891</td>
<td>0.360</td>
<td>0.293</td>
<td>0.285</td>
<td>0.229</td>
<td>0.205</td>
<td>0.191</td>
</tr>
<tr>
<td>0.08</td>
<td>0.983</td>
<td>0.888t</td>
<td>0.768t</td>
<td>0.306t</td>
<td>0.167t</td>
<td>0.167t</td>
<td>0.190t</td>
<td>0.178t</td>
<td>0.152t</td>
</tr>
<tr>
<td>0.8</td>
<td>0.788</td>
<td>0.672t</td>
<td>0.682t</td>
<td>0.290t</td>
<td>0.211</td>
<td>0.175</td>
<td>0.207</td>
<td>0.154</td>
<td>0.105t</td>
</tr>
<tr>
<td>4</td>
<td>0.322</td>
<td>0.301</td>
<td>0.256t</td>
<td>0.139t</td>
<td>0.115</td>
<td>0.121</td>
<td>0.114</td>
<td>0.113</td>
<td>0.096</td>
</tr>
<tr>
<td>40</td>
<td>0.154</td>
<td>0.119t</td>
<td>0.115t</td>
<td>0.133</td>
<td>0.114</td>
<td>0.091</td>
<td>0.167</td>
<td>0.115</td>
<td>0.111</td>
</tr>
</tbody>
</table>

| **Resistant HT-29mdr1 cells** |      |       |      |     |     |     |     |     |     |
| 0                          | 1.000| 1.003 | 0.747| 0.977| 0.998| 0.850| 0.652| 0.415| 0.319|
| 0.008                      | 0.831| 0.786t| 0.726| 0.845| 0.869| 0.838| 0.647| 0.451| 0.304|
| 0.08                       | 0.896| 0.681t| 0.935| 0.390| 0.953| 0.681t| 0.600| 0.236t| 0.199t|
| 0.8                        | 0.702| 0.640t| 0.733| 0.800| 0.809| 0.619| 0.433| 0.219t| 0.137t|
| 4                          | 0.452| 0.312t| 0.180t| 0.271t| 0.193t| 0.236t| 0.148t| 0.130t| 0.078t|
| 40                         | 0.147| 0.114t| 0.072t| 0.092t| 0.128t| 0.087t| 0.086t| 0.064t| 0.075t|

*Interaction between various combinations of Onconase and VCR was examined using a two-way analysis of variance. The one-tailed t test, there was a statistically significant synergistic interaction at the combinations indicated in the tables with bold type, where a P<0.05, b P<0.01, and c P<0.001.

†Interactions less than expected from the additive effects of the two agents. Results were obtained from triplicate determinations of [14C]leucine incorporation into cells treated with VCR, Onconase, or combinations of the two agents for 48 hours as described in the "Materials and Methods" section and are presented as the geometric means.
observed in cells treated with a combination of VCR and Onconase was less than that measured in cells treated with VCR alone. However, in parallel cultures, the protein synthesis measured in cells treated with VCR or a combination of VCR and alkylated Onconase was virtually identical (% protein synthesis: VCR, 100% ± 3% [mean ± standard error of the mean]; VCR + Onconase, 48% ± 9% [versus VCR alone, two-tailed P<.01]; VCR + alkylated Onconase, 92% ± 4% [versus VCR alone, two-tailed P = .21]).

**Effectiveness of the Simultaneous Addition of VCR and Onconase In Vitro**

To determine whether Onconase (0.8 μM) had to be added simultaneously with VCR (0.02 μM) to detect enhanced cytotoxicity, the order of administration of these agents was varied in vitro. Pretreatment with VCR or with Onconase for 24 hours followed by addition of the complementary agent was less effective than simultaneous addition (data not shown).

**Survival of Mice With HT-29<sup>par</sup> and HT-29<sup>mdr1</sup> Xenografts After Treatment With VCR, Onconase, or a Combination of VCR and Onconase**

Nude mice were given intraperitoneal injections of 5 x 10<sup>6</sup> ascites cells (either HT-29<sup>par</sup> cells or HT-29<sup>mdr1</sup> cells). Ten days after tumor cell injection (when ascitic tumors were well established), specific groups of mice were treated for 3 weeks with weekly intraperitoneal injections of either VCR at the maximum tolerated dose (MTD) (1.08 μmol), Onconase (5 nmol), or a combination of the two agents. Preliminary experiments determined that VCR could be administered at its MTD with this concentration of Onconase, suggesting that Onconase, at the dose used, does not increase the toxicity of VCR treatment. As shown in Fig. 3, top panel, the median survival time (MST) of HT-29<sup>par</sup> tumor-bearing animals treated with VCR was 66 days, compared with 39 days for control animals treated with PBS vehicle alone (two-tailed P<.001). Onconase treatment alone did not extend survival significantly (MST = 41 days; in comparison with control animals, two-tailed P = .057), and Onconase combined with VCR was not significantly better than VCR alone (MST = 69 days; two-tailed P = .57). Consistent with previous reports (14,17), VCR did not extend the survival of animals bearing HT-29<sup>mdr1</sup> tumor cells (Fig. 3, bottom panel; MST = 44 days, compared with 42 days for vehicle-treated animals; two-tailed P = .67). The administration of Onconase alone was also ineffective in these animals (MST = 44 days; in comparison with control animals, two-tailed P = .60). However, treatment of these mice with a combination of Onconase and VCR increased their survival time to the same extent as that observed for VCR treatment of animals bearing drug-sensitive tumors (MST = 66 days; in comparison with control animals, two-tailed P<.001). Thus, Onconase can sensitize P-gp-expressing colon carcinoma cells to VCR in vitro and in vivo.
Fig. 3. Effect of vincristine (VCR), with or without Onconase, on survival of nude mice bearing tumors derived from HT-29par cells (top panel, eight animals per treatment group) or HT-29mdrl cells (bottom panel, 11 animals per treatment group). Intraperitoneal therapy was initiated 10 days after intraperitoneal injection of the colon carcinoma cells; therapy was administered weekly for a total of three treatments. Control, saline (open circles); Onconase, 5 nmol/mouse (solid circles); VCR, 1.08 μmol/kg (solid squares); Onconase and VCR (open squares). MST = median survival time in days.

Discussion

We have demonstrated that Onconase, an antitumor ribonuclease now in clinical trials, can enhance the antitumor effects of VCR, even in the presence of P-gp. We found that Onconase cytotoxicity was not dependent on the multidrug-resistant phenotype and that Onconase did not affect cellular accumulation of VCR. Therefore, the mechanism by which Onconase acts to enhance VCR toxicity is not related to mdrl gene expression or function. To our knowledge, the only other agent known to enhance the sensitivity of drug-resistant cells without directly affecting drug binding to P-gp (and subsequent efflux) is phenytoin (24). Yet, unlike phenytoin, which enhances only VCR cytotoxicity (27), Onconase also modulates the sensitivity of HT-29 and MDA-MB-231 parental and drug-resistant cells to doxorubicin, a P-gp substrate with a different mechanism of cytotoxic action (Rybak SM: unpublished data).

Although the precise mechanism by which Onconase acts to enhance the effects of VCR is not known, the ribonuclease activity of the protein appears to be required (7). A previous study (7) demonstrated that Onconase binds to cultured tumor cells and that it is internalized into the cytosol, where it causes RNA degradation. This RNA degradation is associated with the cellular cytotoxicity of Onconase. To determine whether ribonuclease activity is also required in sensitizing HT-29 cells to VCR, we tested an alkylated derivative of the protein, which retained only 2% of the normal activity toward Onconase RNA substrates (7). Alkylated Onconase was not cytotoxic to either HT-29par or HT-29mdrl cells, and it was ineffective in enhancing the effects of VCR. These results demonstrate that the mechanism by which Onconase sensitizes HT-29 cells to VCR is dependent on its ribonuclease activity. Since Onconase did not affect the function of P-gp by increasing intracellular VCR levels, our results suggest that the effects of Onconase on RNA degradation and cell metabolism lower the threshold for cell responsiveness to VCR.

We showed that the effect interactions of Onconase and VCR also occurred in vivo. Co-administration of these two agents allowed mdrl-mediated drug resistance to be overcome in mice bearing HT-29mdrl xenografts. The MST of mice with HT-29mdrl tumors was 42 days, a period that was not extended by treatment with VCR (44 days) or Onconase (44 days) as single agents. Treatment with a combination of VCR and Onconase, however, improved the survival of these mice substantially (MST, 66 days), yielding the same MST (66 days) that was achieved with VCR treatment in mice bearing drug-sensitive HT-29par tumors. These results have been replicated in other experiments and clearly show an interaction of the effects of Onconase and VCR that overcomes MDR1-mediated resistance in vivo. Treatment with Onconase and VCR (MST, 69 days) did not significantly extend the survival of mice bearing VCR-sensitive HT-29par tumors (in comparison with mice treated with VCR alone [MST, 66 days]).

Onconase-VCR effect interactions at the cellular level in vitro indicate that drug-resistant cells and the parental cells
from which they are derived may differ in their response to the two agents when the concentration of VCR is varied. As shown in Fig. 1, bottom, Onconase enhanced the cytotoxicity of VCR over the entire range of VCR concentrations in HT-29MDR cells (i.e., protein synthesis continued to decrease with increasing concentrations of VCR [in the presence of a fixed concentration of Onconase]). In contrast, a plateau in protein synthesis inhibition was observed in drug-sensitive HT-29PAR cells treated with Onconase and VCR (i.e., protein synthesis did not continue to decrease as concentrations of VCR were increased; however, the same concentrations of VCR without Onconase did lead to decreased protein synthesis). Furthermore, the data in Table 1 suggest that the distributions of cytotoxic effect interations are different between HT-29MDR and HT-29PAR cells. Although conclusions about cytotoxicity based on the data in Table 1 are only tentative because of limitations implicit in the partial product method of analysis (26), the results do provide one possible explanation for the failure of Onconase to enhance VCR toxicity in mice bearing VCR-sensitive tumor cells. If our hypothesis that Onconase causes enhanced sensitivity to VCR through its effect on cellular metabolism is correct, we would predict that cells will become less responsive to Onconase as they become saturated with VCR. Because sensitive cells become saturated with VCR at lower concentrations than resistant cells, we would expect that Onconase synergy will be evident in sensitive cells primarily at lower concentrations of VCR. Although a cellular interpretation of results achieved in whole animals is difficult (24), the data suggest that treatment of mice bearing drug-sensitive HT-29PAR tumors at the MTD of VCR may have obscured the detection of synergistic effect interactions with Onconase that might have been detectable at lower VCR doses.

In conclusion, our results demonstrate that a novel RNase is capable of overcoming the P-gp-mediated multidrug resistance of human colorectal HT-29MDR cells. In addition, they indicate a potential for prolonged survival when Onconase is combined with other anticancer agents in the treatment of tumors that may be resistant to those agents. These results may have clinical relevance, since Onconase can be administered intravenously in repeated weekly doses without evidence of true immunologic sensitization (5).

References


Notes

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