Bradykinin Modulation of Tumor Vasculature: I. Activation of B$_2$ Receptors Increases Delivery of Chemotherapeutic Agents into Solid Peripheral Tumors, Enhancing Their Efficacy

Dwaine F. Emerich, Pamela Snodgrass, Reginald L. Dean, Denise Lafreniere, Mary Agostino, Tania Wiens, Hua Xiong, Brant Hasler, Joanne Marsh, Melissa Pink, Byong Su Kim, and Raymond T. Bartus

Preclinical Research and Development, Alkermes, Inc., Cambridge, Massachusetts

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ABSTRACT

Delivery of chemotherapeutic agents to solid peripheral tumors is compromised because the impaired microvasculature within and surrounding tumors limits diffusion and convection of agents from the vasculature to the tumor. Using a variety of rat tumor models, we show that intravenous administration of a vasoactive bradykinin B$_2$ receptor agonist (Cereport, or labradimil; formerly RMP-7) enhances by nearly 3 times the delivery of the chemotherapeutic agent carboplatin, as well as the larger 70-kDa marker dextran, into ectopic and orthotopic solid tumors. This effect was selective for tumor tissue, with little or no increase seen in nontumor tissues and organs. Additionally, the increased carboplatin levels observed in tumors persisted for at least 90 min (the longest time point measured). In contrast to the consistent effects with hydrophilic compounds, delivery of the lipophilic, high protein-binding chemotherapeutics paclitaxel and 1,3-bis[2-chloroethyl]-1-nitrourea (BCNU) was not enhanced. Administration of Cereport with either carboplatin or another hydrophilic chemotherapeutic agent, doxorubicin, significantly increased efficacy of both agents, manifested by suppression of tumor growth and prolonged survival in tumor-bearing rats. These data demonstrate that delivery of chemotherapeutics to tumors can be pharmacologically increased (by stimulating bradykinin B$_2$ receptors) without increasing the systemic exposure, or therefore, the toxic liability associated with higher chemotherapeutic doses.

Successful pharmacotherapy of solid tumors remains an unfulfilled medical goal, despite increased understanding of the molecular biology of tumor cells, the identification of novel cellular targets, and the availability of increased numbers of potential therapeutic agents. Chemotherapy often fails because adequate cytotoxic concentrations are not achieved, due to poor penetration and nonuniform distribution of the drug within the tumor (Jain, 1990, 1991). The inability to effectively deliver drugs to tumors is largely explained by the unique features of the microcirculation of the vasculature supplying and surrounding tumors, which conspire to limit both flow to the tumor mass and diffusion and convection into the tumor interstitium (Jain, 1990, 1991). The inability to effectively deliver drugs to tumors is largely explained by the unique features of the microcirculation of the vasculature supplying and surrounding tumors, which conspire to limit both flow to the tumor mass and diffusion and convection into the tumor interstitium (Jain, 1990, 1991).

Cereport$^1$ (labradimil or RMP-7) is a nonapeptide derivative of bradykinin that was designed to offer a longer plasma half-life with selectivity to the B$_2$ receptor (Straub et al., 1995), thus providing an improved research tool and potential therapeutic. Although quantitative in vivo pharmacokinetic data for Cereport are difficult to obtain (because the enzyme-linked immunosorbent assays cannot distinguish the parent compound from its metabolic products), Cereport’s half-life is known to be less than 10 min and estimated to be less than 3 to 4 min. Additionally, direct comparisons of Cereport and several of the peptide amino acid fragments demonstrated that Cereport binds preferentially to the B$_2$ receptor (Bartus et al., 1996b), but that its metabolic products are without significant binding activity across a range of peptide receptor types (Alkermes, Inc., unpublished data). Although Cereport has initially been developed as a means to increase delivery of chemotherapeutic agents to brain tumors (Bartus, 1999; Emerich et al., 2001a), due to its ability to temporarily disengage the tight junctions comprising the blood-brain barrier (BBB) (Sanovich et al., 1995), it (like bradykinin) has a range of vasoactive effects, in both central and peripheral blood vessels. It has been known for several decades that vasoactive compounds can change the hemodynamics of solid tumors, leading to changes in tumor perfusion (although typically decreases in blood flow have been noted) (Quinn et al., 1992). Because bradykinin is an important endogenous mediator of microvascular flow (Dewhirst et al., 1992), we reasoned that Cereport might modify the characteristics of the tumor vasculature in ways that could improve

ABBREVIATIONS: BBB, blood-brain barrier; BCNU, 1,3-bis[2-chloroethyl]-1-nitrourea; PDE-V, phosphodiesterase V.

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$^1$ Cereport is a registered trademark of Alkermes, Inc.
delivery of chemotherapeutics to the tumor interstitium. We tested this hypothesis using i.v. infusions of Cereport, along with chemotherapeutic agents and radiolabeled compounds to a variety of rat solid tumor models. We assessed the ability of Cereport to increase levels of compounds in the tumor, as well as enhance the efficacy of chemotherapeutic agents against tumor growth and survival in tumor-bearing rats.

Materials and Methods

Animals

Male and female Fischer (F344) rats or male Wistar rats (N = 579) (170–220 g; Taconic Farms, Germantown, NY) were housed in polypropylene cages with free access to food and water. The vivarium was maintained on a 12-h light/dark cycle with a room temperature of 22 ± 1°C and relative humidity levels of 50 ± 5%. All studies were approved in advance by Alkermes’ Institutional Animal Care and Use Committee and were conducted in a manner that met or exceeded National Institutes of Health guidelines.

Cell Culture

A rat ascites mammary adenocarcinoma cell line (MATB-III; ATCC CRL-1666) and a lymphoid sarcoma cell line (Walker 256; ATCC CRL-38) were used in the following studies. These cell lines were chosen because our preliminary studies indicated they survive and grow consistently following implantation into F344 (MATB-III) and Wistar (Walker 256) rats. Moreover, since these cell lines were derived from F344 and Wistar rats, this provides a syngeneic model of tumor growth while avoiding the interpretive issues raised when using an immunologically incompatible tumor line. Cells were grown and maintained at 37°C in a 95% O2, 5% CO2 humidified atmosphere using either McCoy’s medium 5A (MATB-III) or M199 medium (Walker 256) supplemented with 20 mM HEPES, 1/2 penicillin-streptomycin/Fungizone and either 5% heat-inactivated horse serum (MATB-III) or 10% heat-inactivated fetal bovine serum (Walker 256). Before implantation, cells were collected and washed briefly in serum-free media followed by phosphate-buffered saline. Following counting using a hemocytometer and trypan blue exclusion, cells were suspended in serum-free media containing 1.2% methyl cellulose at a density of 5 × 10⁶ cells/ml.

Tumor Models

Subcutaneous Tumor Models. Cell suspensions of either MATB-III or Walker 256 cells (200 µl containing 1 × 10⁶ cells) were injected, using a 22-gauge needle, into the rear flank of male and female rats. All tumors were palpated and measured daily until they reached a size of 1 cm² (7–10 days), at which time the animals were used in dosing studies.

Mammary Pad. Female Fischer rats were anesthetized with a 1-ml/kg intramuscular injection of a solution containing 74% ketamine, 23% xylazine, and 0.04% acepromazine. The cell suspension (1 × 10⁶ cells) was injected into the mammary pad of rats using either a 22-gauge needle, into the rear flank of male and female rats. All tumors were palpated and measured daily until they reached a size of 1 cm² (7–10 days), at which time the animals were used in dosing studies.

Liver Tumor Model. Male Fischer rats received a small incision in the abdomen and MATB-III cells (1 × 10⁶) in 200 µl) were injected into a single site in the left lobe of the liver, using a 25-gauge needle. The skin and underlying fascia were sutured closed using routine procedures. Preliminary studies were performed to characterize growth of tumors in the liver. Based on these experiments all tumors were allowed to grow until they reached a size of approximately 1 cm² (14 days), at which time the animals were used in dosing studies.

Lung Tumor Model. Under ketamine, xylazine, and acepromazine anesthesia, a small incision was made in the neck to isolate the jugular vein of male Fischer rats. A 22-gauge needle was inserted into the jugular vein and 1 × 10⁶ MATB-III cells were injected as a 200-µl bolus into the circulatory system. Based on pilot experiments that characterized the growth of the tumors in the lungs, dosing experiments, using either saline (N = 8) or Cereport infusions (N = 7), were carried out at day 21 following tumor injections.

Bradykinin B2 Immunocytochemistry

MATB-III tumors were removed and quickly frozen in chilled isopentane (−30°C). Tumors were then sectioned on a cryostat (20 µm), thaw-mounted onto microscope slides, and processed for B2 receptor immunohistochemistry as follows: 1) slides washed 6 × 10 min in dilution media (Triton X-100 and Tris buffer) followed by 0.1 M sodium periodate for 1 h; 2) slides washed 6 × 10 min in dilution media followed by 0.1 M sodium 3 × 10 min in dilution media, followed by normal horse serum and bovine serum albumin for 1 h; 3) slides incubated for 48 h (24 h at 22°C and 24 h at 4°C) in the primary monoclonal antibody to the B2 receptor (1:100; Transduction Laboratories, Lexington, KY); 4) slides washed 6 × 10 min in dilution media followed by a 1-h incubation in the appropriate biotinylated secondary IgG antibody (1:200; Vector, Burlingame, CA); 5) slides washed 6 × 10 min in dilution media, slides rinsed; 6) slides incubated with avidin-biotin complex (1:1000; Vector) for 1.25 h; 7) slides rinsed 3 × 10 min in imidazole/acetic acid buffer; 8) slides incubated in a solution containing 3,3'-diaminobenzidine and nickel ammonium sulfate dissolved in imidazole/acetic acid buffer with hydrogen peroxide for 8 min; and 9) reaction terminated by rinsing 3 × 10 min in imidazole/acetic acid buffer. Sections were dehydrated in alcohol and cover slipped. Control sections were processed in an identical manner except the primary antibody vehicle was substituted for the primary antibody.

Quantification of Drug Delivery to Peripheral Tumors

Studies examining delivery of compounds to peripheral tumors were conducted using previously published protocols (Bartus et al., 1996a, 2000; Elliott et al., 1996; Emerich et al., 1999). Briefly, animals were anesthetized with urethane (1.8 g/kg i.p.), and a cannula was placed in the jugular vein for drug administration. Cereport (RMP-7; Alkermes, Inc., Cambridge, MA) and [14C]carboplatin (mol. wt. = 371, specific activity = 144 µCi/mg), [14C]dextran (mol. wt. = 70 kDa; specific activity = 1.14 nCi/g), [14C]BCNU (mol. wt. = 214; specific activity = 73 µCi/mg; Amersham, Arlington Heights, IL), or [3H]paclitaxel (mol. wt. = 854; specific activity = 6.5 Ci/mmol; Moravek Biochemicals, Brea CA) were infused at a rate of 0.05 ml/min. For all studies, the radiolabel (100 µCi/kg) was infused for 15 min followed by a 10-min infusion of 0.9% saline or Cereport.

At the end of drug administration, rats were killed and the peripheral tumor was rapidly removed. A 1- to 2-mm-thick slice from the center of the tumor was divided into two equal parts comprising the center of the dissected piece (inner tumor) and the outside edge (outer tumor). Tumors grown in the liver were dissected free, divided into inner and outer portions, and processed with equal portions of the normal tissue immediately surrounding the tumor, defined as two consecutive 1-mm-wide rings of tissue, and tissue from a completely different liver lobe. For tumors that formed in the lungs following intrajugular cell injections, 15 of the discrete 2- to 3-mm-diameter tumors were pinched free with forceps and processed with equal amounts of adjacent, normal-appearing tissue. Tumor and tissue samples were weighed and placed into scintillation vials and the amount of radioactivity (nCi/g) was computed for each region using scintillation counts. The effect of pharmacologically blocking the bradykinin B2 receptor was examined by administering the selective B2 receptor antagonist HOE-140 (Research Biochemicals International, Natick, MA) before the Cereport infusion. [14C]Carboplatin was infused i.v. for 15 min followed by a 10-min i.v. infusion of saline or Cereport (0.1 µg/kg/min). HOE-140 was given as an i.v. bolus (100 µg/kg) 5 min before beginning the Cereport infusion and then as a 15-min infusion (5 µg/kg/min) over the last 5 min of the infusion.
animals with a tumor greater than 900 cm² were euthanized via CO₂ asphyxiation and that date recorded for calculating survival data.

### Variation in Temporal Dosing Parameters

To gain additional information regarding delivery of [¹⁴C]carboplatin to peripheral tumors the relative timing of Cereport and [¹⁴C]carboplatin was varied [the latter given as a bolus (i.e., within 2–3 s) in this instance to precisely control the Tₚₜ]. Two different doses of Cereport were tested (0.1 and 0.5 µg/kg/min for 10 min), in addition to a saline control group. Three dosing variations were directly compared in which the [¹⁴C]carboplatin bolus was administered either 2 min before initiating the Cereport infusion, at the start of the infusion or 2 min into the Cereport infusion.

### Suppression of Growth of Subcutaneous Tumors

Male Fischer rats received subcutaneous implants of MATB-III cells as described above. Either 9 or 10 days later, at which time the tumors had grown to a size of 1 cm², animals were anesthetized using a solution of ketamine, xylazine, and acepromazine and received a chronic indwelling intrajugular cannula for drug administration as previously described (Bartus et al., 2000; Emerich et al., 2001a). Immediately following surgery, the animals were placed in polystyrene buckets for intrajugular infusions using a syringe pump interfaced with a swivel-linked infusion line. Based on pilot studies, a dose of 5 mg/kg carboplatin (Sigma, St. Louis, MO) combined with the dose of Cereport (1.5 µg/kg) is shown in the current studies to significantly enhance diffusion of [¹⁴C]carboplatin into tumor. Animals were divided into one of three treatment groups: 1) saline infused from 0 to 25 min (N = 16), 2) carboplatin infused from 0 to 15 min followed by a 10-min saline infusion (N = 12), and 3) carboplatin infused from 0 to 15 min followed by a 10-min Cereport infusion (N = 14). One week after the first treatment (16 or 17 days following tumor implant), all animals received a second treatment, identical to the first, under awake, lightly restrained conditions.

A second series of studies combined Cereport with another hydrophilic chemotherapeutic agent, doxorubicin. Animals received saline (N = 8), doxorubicin (N = 12) plus saline, or Cereport plus doxorubicin (N = 12). Based on pilot studies, and consistent with a more concentrated treatment schedule in humans, animals received doxorubicin (2.5 mg/kg) every 3 to 4 days beginning 9 or 10 days following tumor injection. Tumor sizes were recorded every 2 to 3 days and any animals with a tumor greater than 900 cm² were euthanized via CO₂ asphyxiation.

### Survival Following Intrajugular Cell Injections

To determine whether the combination of carboplatin and Cereport enhances survival over that achieved with carboplatin alone, animals bearing lung tumors received a chronic indwelling cannula as described above. On days 16 and 23 following tumor implantation, animals were divided into groups and received i.v. infusions of either saline, carboplatin alone, or carboplatin plus Cereport (1.5 µg/kg/min), as described above for tumor suppression studies. These time points were selected to bracket the day (21 days following cell injection) at which delivery of [¹⁴C]carboplatin into lung tumors was examined. All animals were monitored daily for signs of ill health and any animal showing signs of morbidity was euthanized via CO₂ asphyxiation and that date recorded for calculating survival data.

### Results

#### Immunocytochemistry

Immunocytochemistry using an antibody to the B₂ receptor clearly revealed the presence of large and small immunostained blood vessels within MATB-III tumors (Fig. 1). Although the intense staining in blood vessels was consistently observed throughout the tumor, staining was virtually absent in the tumor cells surrounding the blood vessels. The localization of the B₂ receptor in blood...
vessels was confirmed in adjacent sections using an antibody specific for endothelial cells in blood vessels (CD-31) (Emeric et al., 2001b). This analysis revealed that the immunostaining for the B2 receptor was colocalized to vessels that were immunopositive for the CD-31 antibody. Immunostaining was eliminated in control sections with the primary antibody deleted.

Quantitation of Enhanced Drug Delivery. As shown in Fig. 2A, i.v. administration of the bradykinin B2 agonist Cereport significantly enhanced the delivery of radiolabeled carboplatin to subcutaneously grown MATB-III tumors in male rats. This effect was dose-dependent and was observed uniformly in both the inner and outer portions of the tumor. Delivery was maximally enhanced by infusions of 0.1 and 0.2 μg/kg/min Cereport, which more than doubled tumor levels of [14C]carboplatin (p < 0.01). The Cereport dose-response curve was nonmonotonic (i.e., an inverted U shape), with the highest dose tested (0.5 μg/kg/min) producing a lesser but still significantly enhanced (p < 0.05) delivery of carboplatin to the tumor (>50% increase). Separate studies demonstrated that administering the selective B2 antagonist HOE-140 completely blocked the enhanced uptake produced by Cereport. Cereport alone enhanced delivery of i.v. [14C]carboplatin by 140% (102.8 ± 23.6 nCi/g), relative to saline (84.0 ± 34.1 nCi/g) (p < 0.01). In contrast, delivery to peripheral tumors was decreased 14% (p > 0.1), relative to saline, when HOE-140 was coadministered with Cereport (72.0 ± 4.1 nCi/g).

Based on the dose-response curve generated with [14C]carboplatin (Fig. 2A) and extensive pilot experiments (data not shown), a series of representative Cereport doses were tested for their ability to enhance delivery of the larger molecule, 70-kDa [14C]dextran. Scintillation studies revealed that, like [14C]carboplatin, [14C]dextran delivery significantly benefited from Cereport (Fig. 2B) (p < 0.01) in both the inner and outer portions of the subcutaneously grown MATB-III tumor. Significant 3-fold increases in [14C]dextran were seen at the same Cereport doses (0.1 and 0.2 μg/kg/min) shown to be maximally effective for increasing [14C]carboplatin delivery (Fig. 2A), with the highest dose (0.5 μg/kg/min) producing a significant (p < 0.05), but lesser, effect on [14C]dextran delivery.

In contrast to the effects with [14C]carboplatin and [14C]dextran, delivery of [3H]paclitaxel and [14C]BCNU were not enhanced (p > 0.1) with a dose of Cereport (0.1 μg/kg/min) shown to be effective with those other agents (Table 1). Paclitaxel and BCNU are both distinguished as lipophilic compounds with high protein-binding characteristics (Fischer et al., 1997).

Generality of Enhanced Drug Delivery. To determine the generality of the phenomenon of enhanced delivery of [14C]carboplatin, Cereport was tested in a variety of solid peripheral tumor models (Table 1). Using female rats, we found that Cereport (0.1 μg/kg/min) significantly enhanced delivery of [14C]carboplatin in MATB-III tumors placed in the flank, as well the orthotopic mammary pad. Consistent with the effects observed in male rats (Fig. 2A), delivery of [14C]carboplatin was enhanced 196 to 210% (outer and inner tumor, respectively) in the subcutaneous tumors and 244 to 219% (outer and inner tumor, respectively) in the orthotopically grown tumors (Table 1). When a very different cell type (Walker 256) was implanted into the flank of male rats, similar effects were observed (132% increase in the inner tumor and 106% in the outer portion of the tumor).

Another series of experiments directly compared the effect of Cereport on delivery of carboplatin to MATB-III cells implanted directly into the liver (Fig. 3), versus noninjected, normal liver tissue. Cereport increased delivery of [14C]carboplatin to the liver tumors, with equivalent increases observed in the inner and outer portions of the tumor (p < 0.05). These effects were selective in that Cereport did not significantly alter delivery of [14C]carboplatin to either normal liver tissue adjacent to the implanted tumor within the same liver lobe (inner and outer rings) or in tissue from a completely...
mean 6

[14C]carboplatin into intravenously infused, spontaneously of Cereport were seen in other studies, see below.)

tumor by 143% relative to saline controls (p, tumor tissue implanted into liver, with virtually no effect on healthy liver tissue. Second lobe refers to liver tissue from a completely separate lobe (second lobe) (p > 0.1). (Similar selective effects of Cereport were seen in other studies, see below.)

Finally, the ability of Cereport to enhance delivery of [14C]carboplatin into subcutaneous mammary pad and liver tumors, Cereport enhanced [14C]carboplatin levels in tumor tissue (51%, p < 0.05). These effects were selective in that [14C]carboplatin levels in normal adjacent liver tissue were not affected by Cereport administration (15% increase relative to saline, p > 0.1).

Selectivity and Persistence of Enhanced Drug Delivery to Tumor Tissue. A wide range of tissues was examined to determine the differential effects of Cereport on tumor tissue versus normal, nontumor bearing, peripheral organs. Using male Fischer rats bearing MATB-III tumors, Cereport was shown to enhance delivery of [14C]carboplatin into the tumor by 143% relative to saline controls (p < 0.0001). How-

ever, only marginal effects were observed in other peripheral tissues from the same animals (Table 2). For instance, the greatest increases in [14C]carboplatin levels were in liver (23%), heart (27%), and lung (39%) (p values <0.05). It is important to note that in contrast to the effects observed in tumors, those seen in nontumor tissue were less consistent and robust, with replication studies revealing only a 20% change in lung tissue and an 11% increase in liver tissue (Fig. 3, p > 0.05).

The enhanced delivery of [14C]carboplatin was measured over time to determine whether it was transient or persistent, and how this temporal pattern compared with the more modest effects observed in other peripheral tissues and organs. Quantitation of [14C]carboplatin in tumor tissue demonstrated that the enhanced absolute levels persisted for at least 30 min, and remained significantly higher than baseline for as long as 90 min (the longest time point measured) (Fig. 4). This was in clear contrast to the time course for healthy lung tissue. Although several different nontumor tissues were evaluated, healthy lung was selected for this comparison because, among the peripheral tissues examined, it exhibited the greatest increase in [14C]carboplatin levels (see above). As shown in Fig. 4, [14C]carboplatin levels returned to baseline within 15 min in lung (the earliest time point measured). No other peripheral tissue demonstrated any elevation beyond the earliest time point (data not shown).

TABLE 1

Levels of radiolabeled compounds following intravenous Cereport: additional tumor models and agents

These data extend those depicted in Figs. 2 and 3 by establishing the phenomenon of enhanced delivery of carboplatin in an orthotopic model (i.e., mammary carcinoma cells implanted into the mammary pad) as well as with a different tumor line (Walker 256). Also, these data show that, in contrast to the consistent effects seen with hydrophilic compounds, no consistent effect of Cereport is seen on the delivery of two lipophilic compounds with high-protein binding characteristics. Data presented as mean nanocuries per gram of tissue ± S.E.M.

<table>
<thead>
<tr>
<th>Tumor Cell Type/Location</th>
<th>Gender/Strain</th>
<th>Treatment (N)</th>
<th>Radiolabel</th>
<th>Inner Tumor</th>
<th>Percentage of Change</th>
<th>Outer Tumor</th>
<th>Percentage of Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATB-III/flank</td>
<td>Female Fischer</td>
<td>Saline (6)</td>
<td>[14C]Carboplatin</td>
<td>131.3 (43.3)</td>
<td>+210</td>
<td>119.7 (40.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Female Fischer</td>
<td>Cereport 0.1</td>
<td>[14C]Carboplatin</td>
<td>407.2 (50.3)**</td>
<td>+210</td>
<td>354.7 (57.9)**</td>
<td>+196.3</td>
</tr>
<tr>
<td>MATB-III/mammary pad</td>
<td>Female Fischer</td>
<td>Saline (4)</td>
<td>[14C]Carboplatin</td>
<td>92.7 (27.4)</td>
<td></td>
<td>93.4 (30.8)</td>
<td></td>
</tr>
<tr>
<td>Walker 256/flank</td>
<td>Male Wistar</td>
<td>Cereport 0.1</td>
<td>[14C]Carboplatin</td>
<td>142.1 (24.0)</td>
<td>+132</td>
<td>161.9 (33.5)</td>
<td>+106</td>
</tr>
<tr>
<td>MATB-III/flank</td>
<td>Male Fischer</td>
<td>Saline (8)</td>
<td>[14C]Carboplatin</td>
<td>329.7 (45.2)**</td>
<td>+132</td>
<td>332.9 (35.9)**</td>
<td>+106</td>
</tr>
<tr>
<td>MATB-III/flank</td>
<td>Male Fischer</td>
<td>Cereport 0.1</td>
<td>[14C]Carboplatin</td>
<td>42.3 (12.2)</td>
<td></td>
<td>43.2 (12.6)</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.01, ** p < 0.001.

Fig. 3. Effect of Cereport on delivery of [14C]carboplatin to MATB-III cells implanted directly into the liver of male F344 rats, compared with nontumor, liver tissue. Inner ring and outer ring refer to liver tissue adjacent to and more distal to the implanted tumor, respectively, within the same liver lobe. Second lobe refers to liver tissue from a completely separate and normal, live lobe. Note the selective effects of Cereport on tumor tissue implanted into liver, with virtually no effect on healthy liver tissue. Similar selective effects of Cereport were seen in other studies (Table 2). Group sizes were saline (N = 8), Cereport (N = 9). Data are presented as mean ± S.E.M. nanocuries per gram of tissue. * p < 0.05 versus saline.

TABLE 2

Selective effects of Cereport on delivery of [14C]carboplatin into tumor versus peripheral organs

Note that while Cereport enhanced delivery of [14C]carboplatin into tumor nearly 2.5 times, only marginal effects were seen in nontumor tissue. Moreover, while significant effects in tumor were demonstrated in each of several separate experiments, subsequent experiments testing liver and lung tissue produced effects even more modest than those shown here (e.g., only an 11% change in liver and a 20% change in lung; p < 0.05). Data presented as nanocuries per gram of tissue ± S.E.M.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Saline (N = 16)</th>
<th>Cereport (N = 17)</th>
<th>Percentage of Change</th>
<th>p Value (vs. Saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>80.0 (10.0)</td>
<td>195.0 (25.1)</td>
<td>143</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Heart</td>
<td>75.1 (15.8)</td>
<td>108.4 (5.2)</td>
<td>27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>197.9 (28.3)</td>
<td>279.3 (26.9)</td>
<td>39</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Brain</td>
<td>11.8 (2.6)</td>
<td>9.3 (1.1)</td>
<td>-22</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td>Liver</td>
<td>111.64 (9.5)</td>
<td>151.45 (13.9)</td>
<td>33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Kidney</td>
<td>2199.9 (241.8)</td>
<td>1872.8 (171.5)</td>
<td>-15</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>
Variation in Temporal Dosing Parameters. A series of studies varied the relative timing of the Cereport and [14C]carboplatin infusions (Table 3). Maximal effects were achieved when the [14C]carboplatin $T_{\text{max}}$ preceded the Cereport infusion by 2 min, with infusions of 0.1 and 0.5 mg/kg/min Cereport enhancing [14C]carboplatin delivery by 300 and 149%, respectively. When the two were given simultaneously, the effects of Cereport were modestly reduced, with 0.1 and 0.5 mg/kg/min Cereport enhancing [14C]carboplatin delivery by 152 and 88%, respectively. Finally, when the initiation of the Cereport infusion preceded the [14C]carboplatin $T_{\text{max}}$ by 2 min, the effect of Cereport was further dampened. Although 0.1 mg/kg/min Cereport still significantly enhanced [14C]carboplatin delivery (increased 152%), the higher (0.5 mg/kg/min) dose did not impact delivery of [14C]carboplatin (−13% relative to saline). These data illustrate the complex and dynamic nature of this phenomenon, most likely related to time-related changes in Cereport’s hemodynamic effects during the course of the infusion.

**Suppression of Growth of Subcutaneous Tumors.** The effects of carboplatin on tumor growth were investigated in male F344 rats bearing subcutaneously implanted MATB-III tumors (Fig. 5A). On days 9 or 10 following tumor cell injection, the animals received i.v. infusions of carboplatin (5 mg/kg) with or without Cereport (0.15 µg/kg/min). One week later, the animals received a second identical treatment. The

**TABLE 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Carboplatin 2 min before Cereport</th>
<th>Carboplatin 2 min after Cereport</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>73.1 (9.3)</td>
<td>89.6 (8.6)</td>
</tr>
<tr>
<td>Cereport (0.1)</td>
<td>297.2 (52.2)**</td>
<td>226.0 (38.5)**</td>
</tr>
<tr>
<td>Cereport (0.5)</td>
<td>148.7 (20.9)**</td>
<td>168.2 (21.5)**</td>
</tr>
</tbody>
</table>

**Days Post-treatment**

**Fig. 5.** A, effect of Cereport (0.15 µg/kg/min for 10 min) combined with carboplatin (5 mg/kg) on growth of subcutaneously implanted MATB-III tumors in male F344 rats. Note that although carboplatin, alone, produced an initial but transient suppression of tumor growth, relative to saline, within a few days these tumors were growing at a rate that paralleled the saline-treated rats and were unresponsive to a second carboplatin administration. When the same dose of carboplatin was combined with Cereport, however, a much more robust and persistent effect on tumor growth was achieved, extending well beyond the time of the second drug treatment. B, effect of Cereport combined with doxorubicin (2.5 mg/kg) on growth of subcutaneously implanted MATB-III tumors in male F344 rats. As shown with carboplatin, doxorubicin alone modestly suppressed tumor growth. However, the combination with Cereport was much more effective. Vertical arrows depict timing of doses, whereas vertical lines indicate S.E.M.
tumors of control rats (saline-treated) grew rapidly and due to the size of the tumors, all animals were sacrificed for humane reasons within 1 week of the first treatment. Carboplatin alone produced an initial but transient suppression of tumor growth, relative to saline (day 4, post-treatment), but within several days, these tumors were growing at a rate that paralleled the saline-treated rats and were unresponsive to a second carboplatin administration. When the same dose of carboplatin was combined with Cereport, a much more robust and persistent effect was achieved, with suppression of tumor growth extending well beyond the time of the second drug treatment (Fig. 5A).

Similar beneficial effects of Cereport were observed when combined with the chemotherapeutic agent doxorubicin (2.5 mg/kg). Consistent with typical treatment regimens for doxorubicin in humans, animals were treated more frequently (every 3–4 days) relative to the carboplatin-dosing protocol, for a total of five treatments (Fig. 5B). As seen in the previous carboplatin study, all saline-treated animals exhibited rapid tumor growth and had to be killed for humane reasons within 9 days of the first treatment. Although i.v. doxorubicin alone produced modest suppression of tumor growth, the combination with Cereport robustly slowed the growth of the tumor.

Enhanced Delivery and Survival Following Intrajugular Cell Injection. We next evaluated the ability of Cereport to enhance delivery of carboplatin and increase the survival effects of carboplatin, using an intravenously infused, spontaneously seeded, lung metastatic tumor model. Cereport infusions significantly \( p < 0.05 \) enhanced delivery of \(^{14} \text{C}\)carboplatin to lung tumors (Fig. 6). This enhanced delivery was further associated with increased survival beyond that achieved with carboplatin alone. Kaplan-Meier survival curves shown in Fig. 7 demonstrate that although carboplatin produced a significant \( p < 0.05 \), but relatively modest increase in survival, the combination of carboplatin plus Cereport roughly doubled carboplatin’s effects \( (p < 0.01) \). For example, the median survival with carboplatin increased by 29% (from 31 to 40 days), whereas when combined with Cereport, it increased by 48% (to 46 days). Similarly, maximum survival with carboplatin increased by 30% (from 36 to 37 days), whereas with Cereport, it increased by 60% (to 61 days).

Discussion

The data presented in this manuscript demonstrate that i.v. infusion of the bradykinin B\(_2\) receptor agonist Cereport significantly enhances the delivery of hydrophilic compounds (including chemotherapeutics) to solid tumors. A wide variety of tumor models were studied, establishing the generality of this phenomenon. Using carboplatin as a prototypical chemotherapeutic agent, the increases achieved were shown to be selective for tumor tissue, persist for at least 1.5 h and manifest biologically as suppression of tumor growth and enhanced survival in tumor-bearing rats. Similar enhanced efficacy on suppression of tumor growth was seen when Cereport was combined with doxorubicin. In contrast to the robust and reliable increases in tumor levels seen with both carboplatin and the much larger 70-kDa dextran, no reliable increases were observed with the chemotherapeutic agents paclitaxel and BCNU. Both of these drugs are highly lipophilic and exhibit high protein binding, suggesting that the novel phenomenon achieved with bradykinin stimulation may be specific for water-soluble agents and/or drugs with low protein-binding characteristics.

These experiments used a rodent model of solid peripheral tumors that bears considerable homology to the vasculature in human solid tumors [see Emerich et al. (2001b) for discussion]. Immunocytochemical analysis using endothelial-specific markers demonstrated that like human tumors, the vasculature in this model is heterogeneous, with some regions of little infiltration and other regions containing a dense, tortuous plexus of small and large blood vessels (Emerich et al., 2001b). The variable density of the vasculature in the MATB-III tumor was confirmed in studies using fluorescent markers to quantify both pore size and perfusion of the tumor vasculature. The pore-size analysis also demon-

![Fig. 6. Effect of Cereport (1.5 μg/kg/min) on delivery of \(^{14} \text{C}\)carboplatin to spontaneously seeded lung tumors following intrajugular injections of MATB-III cells. Tumor tissue was obtained by “pinching off” 15 discrete 2- to 3-mm-diameter tumors from the surface of the lungs. Normal lung tissue refers to nontumor-bearing tissue adjacent to the lung tumors. Note the relatively selective effect of Cereport on tumor tissue, with no effect on healthy lung tissue. Data are presented as mean ± S.E.M. nanocuries per gram of tissue. \( * p < 0.005 \) versus saline.

![Fig. 7. Standard Kaplan-Meier plot depicting the effect of Cereport plus carboplatin on survival of rats with intravenously injected MATB-III lung metastatic tumors. Although carboplatin (5 mg/kg), alone, produced a modest increase in survival (relative to the saline and Cereport-alone, control groups), the combination of Cereport and carboplatin produced a significantly more robust effect.

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\text{Survival curves shown in Fig. 7 demonstrate that although carboplatin produced a significant (} p < 0.05, \text{but relatively modest increase in survival, the combination of carboplatin plus Cereport roughly doubled carboplatin’s effects (} p < 0.01). \text{For example, the median survival with carboplatin increased by 29% (from 31 to 40 days), whereas when combined with Cereport, it increased by 48% (to 46 days). Similarly, maximum survival with carboplatin increased by 30% (from 36 to 37 days), whereas with Cereport, it increased by 60% (to 61 days).}
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strated the relative leakiness of the tumor vasculature. The observation that the vessels contain pores large enough to permit the passage of beads as large as 0.2 μm is consistent with ultrastructural studies in animal and human tumors that demonstrate wide interendothelial junctions, numerous fenestrae, and large transendothelial channels (Jain, 1987, 1989). Another similarity between the MATB-III tumors and human peripheral tumors is the presence of increased transvascular pressure gradients. Relative to normal, nontumor tissue, the interstitial pressure in the present model and in human tumors is severalfold greater (Yuan, 1998; Emerich et al., 2001b). The increased pressure presumably occurs because of the proliferation of tumor cells within a confined space. It presents a formidable obstacle to delivery of chemotherapeutics from the vasculature to the tumor interstitium. Finally, recent evidence indicates that the endothelial cells of blood vessels within human solid peripheral tumors express a high preponderance of genes commonly found in normal endothelial vascular cells and those undergoing angiogenesis (St. Croix et al., 2000). These genetic data offer strong evidence for the qualitative similarities between the vascularatures across a range of tumors, raising the likelihood that the genetic similarity holds true across most, if not all blood vessels, including those supplying peripheral tumors in both humans and in rodent models. Although the cell line used in these studies grows more rapidly relative to most human tumors, the genetic, structural, and permeability characteristics of the MATB-III tumor in the present model would seem to provide a valid model for predicting the effects of Cereport, or any compound intended to modify the vasculature of tumors in humans.

An interesting quality of the phenomenon reported here is the nonmonotonic (inverted U) dose-response function. Although no definitive explanation is yet possible, several possibilities exist, including biochemical changes such as bradykinin receptor desensitization, internalization, and/or uncoupling (Roberts and Gallick, 1990; Munoz et al., 1993; Wolsing and Rosenbaum, 1993; Pradadutae et al., 1995), as well as depletion of bradykinin-induced second messengers (Sugita and Black, 1998). All of these have been well characterized for the B2 receptor system, are reportedly dose-related, and could conceivably have occurred within the time parameters of these studies (Munoz et al., 1993; Wolsing and Rosenbaum, 1993). Interestingly, we were not successful in modifying the dose-response function with zaprinast (Emerich et al., 2001b), a PDE-V inhibitor that is able to prolong the action of the B2-mediated second messenger cGMP (Sugita and Black, 1998). These data suggest that if a simple biochemical change is responsible for the shape of the dose-response function, it more likely involves an upstream event at the receptor level. An equally plausible explanation may involve the complex pattern of hemodynamic changes induced by Cereport. It is conceivable that the pattern of events responsible for the increased delivery of drugs into solid tumors may not be optimal at the highest end of the Cereport dose range. Support for this possibility is derived from studies demonstrating that the relative timing of Cereport administration and the infusion of carboplatin are very important. Using bolus injections of [14C]carboplatin to precisely control the timing of the maximal plasma concentrations (i.e., \( T_{max} \)), maximal delivery to tumor occurred when pharmacological stimulation of the B2 receptor closely followed (i.e., 2-min delay) the \( T_{max} \) of [14C]carboplatin. Significant, but reduced effects were seen when the two occurred simultaneously, whereas even smaller effects were seen at the highest dose when the \( T_{max} \) of [14C]carboplatin followed B2 receptor activation by 2 min (Table 3). These data not only demonstrate the importance of precisely timing the infusions of Cereport and carboplatin, but highlight the very dynamic and likely complex physiological responses (involving changes in systemic blood pressure, tumor interstitial fluid pressure, tumor vessel diameter, and tumor blood flow) induced in the solid tumor by bradykinin receptor stimulation (Emerich et al., 2001b). The decrease in tumor blood flow reported with Cereport, along with the decreased systemic blood pressure at the highest doses, may combine to counter the other physiological changes that might otherwise enhance delivery (e.g., decrease in interstitial fluid pressure and increase in vessel diameter). It is interesting that it was at the highest Cereport dose tested (1.0 µg/kg/min), where no enhanced delivery occurs and the blood pressure and blood flow changes are likely maximal, that no decrease in interstitial fluid pressure within the tumor was seen (Emerich et al., 2001b).

Another aspect of the phenomenon reported here, which remains incompletely understood, is the selectivity for tumor tissue. Although there exists precedence for selective bradykinin-mediated effects in both brain tumors (Inamura et al., 1994; Bartus et al., 1996a, 2000; Elliott et al., 1996; Bartus, 1999; Emerich et al., 2001a) as well as solid, peripheral tumors (Dewhirst et al., 1992), no clearly convincing explanation has yet been established. Our immunocytochemical analyses demonstrated the presence of B2 receptors of the vasculature of the MATB-III tumor and our pharmacological studies demonstrated that the B2 receptor antagonist could block the effects of Cereport. Although these studies reveal a clear B2 receptor-related mechanism in the enhanced delivery to tumors following Cereport, the possibility that the tumor vasculature contains a higher density of B2 receptors or increased concentrations of relevant second messenger systems (e.g., prostaglandin \( E_{2} \), nitric oxide) remain to be confirmed in future studies using more quantitative methodology. Adding to the intrigue of Cereport's selective effects on tumor vasculature is the relatively low plasma concentrations required to achieve the effects reported here for solid tumors in the periphery. The effective dose of 0.1 µg/kg/min has been estimated to produce plasma concentrations of Cereport of only 3 to 5 nM (Bartus et al., 2000). This is well below the \( K_i \) established for Cereport at the B2 receptor (i.e., 10–50 nM) (Bartus et al., 1996a). These data, therefore, raise the suggestion that bradykinin may serve some unknown and unappreciated physiological role in modulating blood and nutrient access to solid tumor interstitium. If this proves to be true, our attempts to increase delivery of chemotherapeutics using a therapeutic bradykinin agonist like Cereport represents an exploitation of a natural biological phenomenon in the classic manner of the Trojan horse.

Although the data reported here appear generally reminiscent of prior data with Cereport on brain tumors, where increased delivery of chemotherapeutic agents into those tumors was achieved, important phenomenological differences exist. For example, in the present results with solid, peripheral tumors, the dose-response curve for Cereport is shifted significantly to the left (i.e., the optimal doses were 0.1 and
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Send reprint requests to: Raymond T. Bartus, Ph.D., Preclinical R&D, Alkermes, Inc., 64 Sidney St., Cambridge, MA 02139. E-mail: rtbartus@alkermes.com

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0.2 µg/kg/min, whereas in brain tumors they were half an order of magnitude higher. Additionally, the dose-response studies with solid peripheral tumors consistently reveal a nonmonotonic, inverted U-shape, whereas the prior studies with gliomas revealed a more traditional, negatively accelerating, monotonic function (i.e., eventually achieving a flat, asymptote at the higher doses). Another difference involves the optimal timing of B2 receptor stimulation and the delivery of the chemotherapeutic agent. With glioma, optimal effects occur when the Cmax of the agent temporarily overlaps (i.e., follows) the stimulation of the bradykinin receptor (Emerich et al., 1999), whereas in the present studies, maximal effects are achieved when the Cmax clearly precedes activation of the bradykinin B2 receptor [most likely so that adequate concentrations of chemotherapeutic agent are achieved in the tumor vessel lumen before the reduction in tumor blood flow that occurs soon after Cereport infusion (Emerich et al., 2001b)]. Together, these data indicate that although the increased delivery achieved with Cereport in brain tumors shares certain characteristics with that reported here for solid, noncentral nervous system tumors, a number of clear and potentially important differences in the two phenomena nonetheless exist.

In summary, the phenomenon reported here is both novel and of potential practical value. The possibility of significantly increasing levels of chemotherapeutic agents into solid tumors without increasing systemic exposure seems intriguing. Most investigators agree that increasing the concentrations of chemotherapeutic agents to tumors could significantly improve treatment outcome. For this reason, expensive and difficult adjuvant therapies, involving autologous bone marrow and stem cell transplantation, as well as exogenous cytokine administration, continue to be investigated experimentally in an effort to help the patient tolerate an escalation in chemotherapeutic dose that would otherwise be lethal. Extensive preclinical animal toxicity tests and human clinical trials have previously demonstrated that Cereport is safe over a wide range of doses. The new data presented in this manuscript suggest it may be possible to significantly enhance the concentration of chemotherapeutic agents in solid tumors through coadministration with Cereport, without increasing systemic toxicity or incurring other safety risks. Thus, if the phenomenon reported here in animal models holds true in human oncology patients, a noticeable change in the treatment approach, as well as therapeutic response of these patients, might be expected.