Twice-weekly Intravenous Treatment of Pancreatic Cancer with Atrial Natriuretic Peptide and Vessel Dilator

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Abstract. Background: Atrial natriuretic peptide (ANP) and vessel dilator eliminate 80% and 33% of human pancreatic adenocarcinomas growing in athymic mice when given subcutaneously for 28 days via osmotic pumps. Materials and Methods: To determine if similar beneficial effects can be obtained by ANP and vessel dilator on a bi-weekly basis, bolus infusion via vascular ports bi-weekly for 4 weeks was given to athymic mice bearing human pancreatic adenocarcinomas. Results: Vessel dilator and ANP (each at 100 μM) (n=6 for each) resulted in a 33% (p<0.01) and 17% (p<0.05) elimination of human pancreatic adenocarcinomas, respectively, while the tumor volume increased 64-fold (p<0.001) in the placebo-treated mice (n=12). During the 4 weeks of treatment, the growth velocity decreased 92% and 68% with vessel dilator and ANP, respectively, compared to untreated mice. Conclusion: Biweekly vessel dilator and ANP both eliminate some human pancreatic adenocarcinomas in athymic mice.

A family of peptide hormones are synthesized within the heart and stored in the atrial myocyte as a 126 amino acid (a.a.) prohormone (1, 2). The atrial natriuretic peptide (ANP) gene synthesizes this prohormone, which contains four peptide hormones consisting of a.a. 1-30 (i.e. long-acting natriuretic peptide), a.a. 31-67 (vessel dilator), a.a. 79-98 (kaliuretic peptide) and ANP (a.a. 99-126) (1, 3). Treatment with cardiac hormones via subcutaneous pumps for 28 days results in elimination of 80% and 33% of the human pancreatic adenocarcinomas in athymic mice with ANP and vessel dilator, respectively (4). The human pancreatic adenocarcinomas which are eliminated never recur in the lifespan of the mice (4). Even in the treated animals which do not have a complete elimination of the human pancreatic adenocarcinomas, their tumor volume is decreased to 10% (and with vessel dilator to 2%) of that of untreated animals during the 28-day treatment period and throughout their lifespan (4).

ANP and vessel dilator affect several signaling pathways in cancer cells (5-11). They significantly (p<0.0001) inhibit 95% to 98% of the phosphorylation of 3 metabolic targets within cancer cells which are part of the GTP–Ras–mitogen-activated protein kinase kinases 1 and 2 -extracellular-signal regulated kinases 1 and 2 (Ras-MEK 1/2) (ERK 1/2) kinase cascade (5-11). Extracellular-signal regulated kinases (ERK) 1/2 are mitogen activated protein kinases (MAP kinase) important for the growth of cancer (12, 13). Growth factors such as epidermal growth factor (EGF), fibroblast growth factor, platelet-derived growth factor and vascular endothelial growth factor (VEGF) after binding to their specific receptor tyrosine kinases act via ERK 1/2 kinase to cause proliferation (13). Vessel dilator and ANP reduce the activation of ERK 1/2 over a concentration range of 0.01 μM to 1 μM. Vessel dilator and ANP (each at 1 μM) inhibit the phosphorylation of ERK 1/2 kinases by 96% (p<0.0001) and 94% (p<0.0001), respectively (5, 6). Their ability to inhibit ERK 1/2 is inhibited by cyclic GMP antibody and cyclic GMP itself inhibits ERK phosphorylation by 93% (8, 9). Vessel dilator and ANP over a concentration range of 0.1 μM to 10 μM inhibit 95% and
90%, respectively, of the binding of active Ras-GTP (10, 11). These cardiac hormones reduce the number of human pancreatic adenocarcinomas in cell culture while simultaneously reducing the DNA synthesis in these cancer cells by 90% (14). These two cardiac hormones, which have the most significant beneficial anticaner effects when given continuously for 28 days (4, 15), were administered over an identical 28-day treatment period but were given twice per week rather than continuously (4) to athymic mice with human pancreatic cancer to determine if these cardiac hormones could have beneficial effects when given less frequently.

Materials and Methods

**Human pancreatic adenocarcinoma cells.** Human pancreatic adenocarcinoma cells (HPAC; CRL-2119) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This pancreatic adenocarcinoma cell line was derived in 1994 from a nude mouse xenograft of a primary tumor removed from the head of the pancreas of a 64-year-old Caucasian female patient (16).

**Culture of pancreatic adenocarcinoma cells for tumor formation in vivo.** Cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (FBS) 10%, at a temperature of 37°C in 5% CO₂, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days. Only single-cell suspension with a viability of >90% was used for the injections. Cell pellets were resuspended in saline prior to injection.

**Human pancreatic adenocarcinomas in the athymic mice.** Homozygous (nu/nu) athymic nude mice with vascular access ports were purchased from The Jackson Laboratory, Bar Harbor, Maine, USA. Twenty-gram mice were given subcutaneous injections of 1x10⁶ of human pancreatic adenocarcinoma cells in 250 μl of phosphate-buffered saline, pH 7.4, on the left side of the back. The control mice for the cancer cell injection received 250 μl of phosphate-buffered saline on the left side of their backs (n=6 for control mice for the cancer cell injection received 250 μl of phosphate-buffered saline, pH 7.4, on the left side of the back. The control mice for the cancer cell injection received 250 μl of phosphate-buffered saline on the left side of their backs (n=6 for each group). The untreated (i.e. control) mice who developed pancreatic cancer (n=12) received 0.9% saline intravenous bolus biweekly for 4 weeks, which was what vessel dilator and kaliuretic peptide were diluted in prior to their intravenous bolus infusions. Each mouse had induction of anesthesia with carpofen at 10 mg/kg body weight followed by isoflurane at 2 to 4% inhaled for the duration of the procedure. Tumor growth was followed by electronic digital Vermier caliper measurements every day with tumor volume recorded daily (17). Tumor volume (V) was calculated by the formula V=(a x b²)/2 where a=largest superficial diameter and b=the smallest superficial diameter (17).

**Research protocol.** The injected adenocarcinoma cells coalesced into well-defined tumors of at least 2 mm x 2 mm (volume=4 mm³) in approximately 28 days. These tumors were confirmed to be present by three different investigators. The human pancreatic adenocarcinomas were then treated twice weekly for 4 weeks with 100 μM bolus intravenously of ANP or vessel dilator through a vascular port placed by The Jackson Laboratory (Bar Harbor, Maine, USA). The peptide hormones utilized in the experiments were synthesized by Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). This research protocol was approved by the Institutional Animal Care and Use Committee of the University of South Florida and the James A. Haley Veterans Medical Center and followed the “Guiding Principles for Research Involving Animals and Human Beings” (18).

**Statistical analysis.** Univariate statistics (percentage of treated mice or tumor volume) were calculated to describe the ability of the cardiac hormones to reduce or eliminate the growth of human pancreatic adenocarcinomas. To test whether the observed differences between groups over time were statistically significant, linear mixed (growth curve) models as described by Verbeke (19) were used. The dependent variable for the analysis was mean tumor volume. The independent variables for the analyses were group membership, which had three levels (2 experimental and 1 control groups), time, modeled as a continuous random variable, and group-by-time interaction term. This group-by-time interaction term compares the slope of means of the dependent variable by group across the follow-up period. In our first model, results from the experimental groups were compared with those from the control group. We followed this analysis with a second model, excluding the control group, to investigate whether the mean tumor volume was different over time for the experimental groups. All models employed restricted maximum likelihood estimations (REML). Fit statistics were compared for models using several covariance structures and the structure with the best fit (first-order autoregressive) was retained. All statistics analyses were conducted using SAS version 9.1 (SAS Institute, Inc., Cary, NC, USA). In all cases, p<0.05 was considered the criterion for statistical significance.

Results

**Vessel dilator with bi-weekly bolus infusion eliminates 1/3 of human pancreatic carcinomas.** One-third of the mice harboring human pancreatic carcinomas treated with vessel dilator on a twice weekly basis had their human pancreatic cancers eliminated (p<0.01; Table I). In the first of these animals to respond to vessel dilator, the tumor was no longer present 4 weeks after the treatment period ceased. This cancer never recurred in the lifespan of the mouse. In the second athymic mouse harboring a human pancreatic cancer, the pancreatic cancer disappeared after the 3rd week (i.e. the 6th vessel dilator bolus infusion via the port) and never returned. This animal, however, developed congestive heart failure and ascites 2 months after completion of 4 weeks of bi-weekly infusions. At autopsy, this animal had a large liver with ascites but no evidence of metastasis in the liver, lung or peritoneal area. There was no metastasis noted anywhere in this animal. With vessel dilator, the growth velocity decreased 92% during treatment versus that of the 12 simultaneously non-treated (control) animals, down to 4.2 mm³/week versus 53.4 mm³ per week in untreated controls (p<0.0001; Figure 1) during the 4 weeks of treatment but in the majority of the animals, the human pancreatic cancer grew to a large size 2 months post-treatment and the animals were euthanized. One animal’s (#3) pancreatic cancer grew slower post treatment...
but by 5 months post-treatment had grown to a large size (2183 mm$^3$) and the mouse was euthanized. Thus, during the 4-week period of bolus treatment with vessel dilator, tumor volume decreased to only 15% of that of the untreated animals but after the bolus infusion of vessel dilator ceased, the tumors in these animals began to grow again and at 2 months post-treatment their tumor volume was 72% of that of the untreated mice (Table I).

**Bi-weekly treatment with ANP eliminates 1 in 6 human pancreatic carcinomas.** ANP eliminated the human pancreatic adenocarcinomas in 17% (1 in 6) ($p<0.05$) of the athymic mice (Table I). In animal #6 (Table I), the human pancreatic adenocarcinoma began to decrease in size after 1 week of bi-weekly ANP bolus infusions and completely disappeared after the third week of treatment (Table I). This human pancreatic cancer never returned in post-treatment follow up of this athymic mouse. In a second athymic mouse treated with ANP (#3 in Table I) the human pancreatic cancer did not grow during the 4-week infusion and then grew slowly during the post-infusion period compared to those in the untreated animals. This cancer was not eliminated and did continue to grow rather than remaining stable in volume at 10 weeks after the 4 weeks of bi-weekly treatment (Table I).

### Table I. Human pancreatic carcinomas in nu/nu athymic mice treated with atrial natriuretic peptide and vessel dilator through bolus dosing.

<table>
<thead>
<tr>
<th>Volume (mm$^3$) of tumor</th>
<th>Treatment (weeks)</th>
<th>Post-treatment (weeks)</th>
<th>ANP</th>
<th>Vessel dilator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4</td>
<td>5 6 7 8 9 10 11 12 End (weeks)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14.9 19.2 80.3 34 27</td>
<td>40.3 54.6 84.6 148.2 273.2 567.1 1083 2081.8 5207.8 (13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.8 26.1 81.6 57.4 98.3</td>
<td>264.1 293.6 682.7 674.8 1051.2†</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
<td>14.4 75.7 52.7 64 146</td>
<td>239.5 388.4 618.2 634 1059 1998.7 3306.3 3738.5 (12)</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>26.1 65.9 68.9 73.9 174.8</td>
<td>258.4 317.9 586.3 634 824.7 787.1 2075.6 1807.2 1757.2 (13)</td>
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<td>5</td>
<td>65.9 81.3 116.8 364.4 329.5</td>
<td>584.8 919.5 1196.3†</td>
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<tr>
<td>6</td>
<td>10.3 53.7 55 54.3 27.3</td>
<td>106.4 69.4 85.2 104.5 186.2 254 333.3 357.1 2606.2 (18)</td>
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<td></td>
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<tr>
<td>7</td>
<td>28.8 28.8 58.5 74 60.3</td>
<td>125.2 247.5 263.3 466.3 439.6 489.6 687.5 1090.8 2415.8 (16)</td>
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<tr>
<td>8</td>
<td>42 49.8 57 95.6 96.8</td>
<td>205.9 188.9 186.6 207.6 315.8 422 408.7 529.5 1984.7 (16)</td>
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<tr>
<td>9</td>
<td>84.1 89.7 187.9 383 345.5</td>
<td>269.1 598 593.5 601.4 565.5 1060.9 1062.4 1603.9 3256 (14)</td>
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<tr>
<td>10</td>
<td>10 35 124 309 1003</td>
<td>1307 1783 2351†</td>
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<tr>
<td>11</td>
<td>130.6 184.4 159 178 260.7</td>
<td>541 538 540 538.2 1254 2019.5†</td>
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<tr>
<td>12</td>
<td>83.8 100.2 225.4 334.5 537.1</td>
<td>375 826.7 773 816.1 1057.7 1375.5 2026 2275</td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
<td>45.2 67.5 105.6 168.5 258.9</td>
<td>359.7 513.8 601.0 482.5 702.7 992.7 1372.9 1685.5 2871.3</td>
<td>122.1 117.5 159.1 164.2 191.1</td>
<td>265.3 280.7 469.9 592.0 653.1 896.4 492.4 618.0</td>
</tr>
<tr>
<td>±SEM</td>
<td>11.0 13.1 16.7 39.8 80.8</td>
<td>98.0 139.5 96.7 77.6 123.9 194.8 361.5 382.56</td>
<td>29.8 44.3 79.4 59.0 70.8</td>
<td>89.5 95.9 188.4 273.3 262.6 366.0 221.2 269.9</td>
</tr>
</tbody>
</table>

ANP, Atrial natriuretic peptide. Weeks 1-End beginning with start of infusion for 4 weeks followed up to 40 weeks post-infusion until euthanasia for tumor growing too large or for normal lifespan. ‘0’ week represents the baseline size of the tumor prior to infusion of the respective peptide hormones. *Metastatic lesion; †mouse euthanized and autopsy performed; 0, volume, tumor no longer present; ( ), weeks at time of euthanasia; End=volume of tumor if euthanasia is post 12 weeks in Table. Vessel dilator mouse #1 had congestive heart failure and ascites but no tumor at autopsy.
which time the tumor volume averaged 1686 mm$^3$ (Table I). 1 month, 22-fold in 6 weeks and 37-fold by 2 months, at untreated control animals (n=12), the tumors grew 10-fold in untreated controls (Figure 1; (Table I).

Growth of untreated human pancreatic carcinomas. In the untreated control animals (n=12), the tumors grew 10-fold in 1 month, 22-fold in 6 weeks and 37-fold by 2 months, at which time the tumor volume averaged 1686 mm$^3$ (Table I). The mean tumor volume increased further to 2871 mm$^3$ (64-fold increase) at a mean of 13 weeks for the control group (Figure 1; $p<0.001$)

**Discussion**

Vessel dilator and ANP can eliminate human pancreatic adenocarcinomas in athymic mice when given by bolus infusion on a bi-weekly basis for 4 weeks. The ability of vessel dilator to eliminate human pancreatic carcinomas by this regimen was similar (33%) to that of giving vessel dilator continuously for 4 weeks via subcutaneous pumps (4). The inhibition of the growth velocity during treatment with vessel dilator (92%, $p<0.0001$) and ANP (68%, $p<0.001$) was dramatic but when the treatment was completed, the mean decrease in cancer volume to 15% of the untreated animals increased to 72% of the volume of untreated animals by 2 months post treatment. The differences between the results of the two treatment regimens are i) with the bi-weekly regimen, one of the animals developed ascites with a big liver, which was not observed with subcutaneous treatment, and ii) with the bi-weekly regimen the human pancreatic adenocarcinomas which were not eliminated (two-thirds) continued to grow and became very large as opposed to the continuous 28-day treatment with vessel dilator where the tumors that were not eliminated decreased in volume to 2% of that of the untreated pancreatic carcinomas (4). This huge difference ($p<0.0001$) in outcome of treatment with twice weekly intravenous infusion for 4 weeks versus continuous subcutaneous infusion of vessel dilator for 4 weeks would suggest that with respect to vessel dilator, the preferred treatment would be *via* subcutaneous infusion for 4 weeks. This would suggest that human pancreatic adenocarcinomas being exposed continuously to treatment doses of vessel dilator has more beneficial anticancer effects than intermittent (bi-weekly) treatment with vessel dilator.

With respect to congestive heart failure and ascites developing in one of the vessel dilator treated animals, it could not be determined if this was due to a metastatic lesion. The liver was large in this animal but no metastatic lesion was observed in the liver, abdomen or chest. It is to be noted that in the 12 untreated animals which all developed large tumors, none developed ascites. Since vessel dilator has strong diuretic, natriuretic and beneficial effects in treating congestive heart failure in humans and animals (20-25) and its gene is up-regulated in the heart to help overcome ascites (26), it would be implausible that vessel dilator itself caused the congestive heart failure and ascites.

ANP given twice weekly *via* intravenous infusion for 4 weeks did have beneficial effects and eliminated 17% of its human pancreatic carcinomas in athymic mice treated in this manner. The response to ANP of the human pancreatic adenocarcinomas, however, was markedly ($p<0.0001$) less than the 80% elimination of human pancreatic adenocarcinomas in athymic mice when ANP is given by continuous subcutaneous infusion for 4 weeks (4). In the present investigation, where ANP was given intravenously twice weekly for 4 weeks, the majority of the tumors were not eliminated and continued to grow after ANP treatment. These findings are opposed to those in which all human pancreatic adenocarcinomas that were not eliminated decreased in volume to less than 10% of the untreated mice when ANP was given subcutaneously for 4 weeks (4). This information would suggest that for ANP, as with vessel dilator, the preferred method of treatment of human pancreatic adenocarcinomas is continuous exposure to these peptide hormones, synthesized mainly in the heart.

We conclude that giving vessel dilator and ANP bi-weekly intravenously for 4 weeks has beneficial effects in the treatment of human pancreatic adenocarcinomas in that both

![Graph showing growth velocity of human pancreatic adenocarcinomas in vivo](https://via.placeholder.com/150)
of these cardiac hormones eliminated some of the human pancreatic carcinomas in the athymic mice and the cancer did not return during the lifespan of the mice. Continuous subcutaneous treatment of human pancreatic adenocarcinomas in athymic mice, however, has superior beneficial anticancer effects (4) compared to bi-weekly intravenous infusion of ANP and vessel dilator in the treatment of pancreatic adenocarcinomas.

Conflict of Interest Statement

Dr. D.L. Vesely has given the patent rights to treating cancer with the cardiac hormones to the University of South Florida, which in turn has licensed one of the patents to Kalos Therapeutics, San Diego, CA, U.S.A. None of the other co-authors have any conflict of interest.

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References


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