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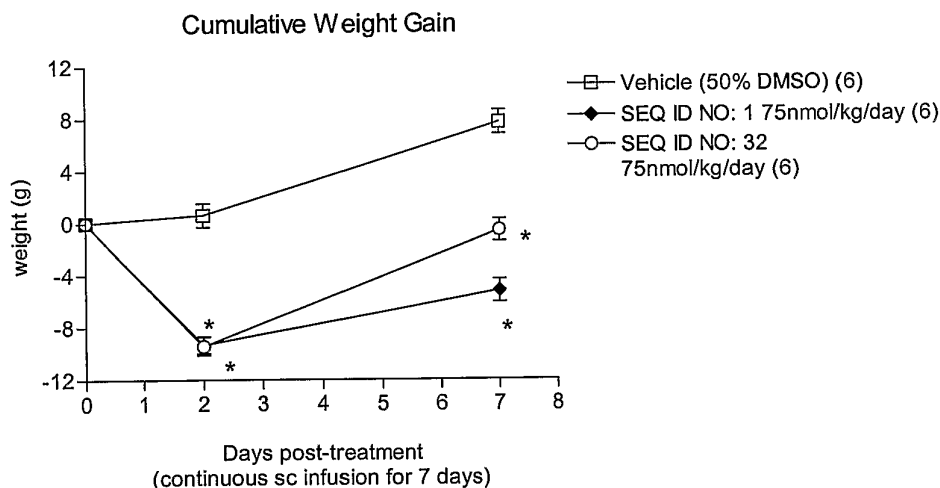
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(54) Title: NOVEL AMYLIN FAMILY POLYPEPTIDE-6 (AFP-6) ANALOGS AND METHODS OF MAKING AND USING THEM



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(57) Abstract: The present invention relates to Amylin Family Polypeptide-6 (AFP-6) analogs, which include derivatives and fragments, related nucleic acids, expression constructs, host cells, and processes for recombinant production of the AFP-6 analogs. The AFP-6 analogs of the invention include one or more amino acid sequence modifications. In addition, methods and compositions are disclosed to treat and prevent conditions such as metabolic and cardiovascular disorders, e.g., obesity, diabetes, metabolic syndrome, myocardial ischemia, and increased cardiovascular risk.

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NOVEL AMYLIN FAMILY POLYPEPTIDE-6 (AFP-6) ANALOGS AND
METHODS OF MAKING AND USING THEM

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. provisional patent application number 60/617,468, filed October 8, 2004, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

5 The present invention relates to novel compounds having an activity of a member of the Amylin Family of polypeptides. These compounds are useful for treating or preventing conditions such as metabolic disorders, vascular disorders, renal disorders, and/or gastrointestinal disorders. An exemplary condition is one in which the reduction of caloric intake is of value, *e.g.*, obesity, Type II diabetes, eating disorders, metabolic
10 syndrome and insulin-resistance syndrome.

BACKGROUND OF THE INVENTION

Amylin Family Polypeptide-6 (AFP-6) is a member of the Amylin Family, which includes amylin, adrenomedullin (ADM), calcitonin (CT), and calcitonin gene related peptide (CGRP). The human AFP-6 gene, also known as intermedin, encodes a 148 amino
15 acid open reading frame with a 24 amino acid signal peptide for secretion at the N-terminus and a mature amidated peptide having an amino acid sequence of TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:1).

Other AFP-6 polypeptides include:

20 VGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:2);
 PHAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-NH2 (SEQ ID NO:3); and VGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-NH2 (SEQ ID NO:4). Still other Amylin Family polypeptides include:
25 KCNTATCATQRLANFLVHSSNNFGAILSSSTNVGSNTY-NH2 (SEQ ID NO:5);
 CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP-NH2 (SEQ ID NO:6);
 ACDTATCVTHRLAGLLSRSGGWKNFVPTNVGSKAF-NH2 (SEQ ID NO: 7);

ACNTATCVTHRLAGLLSRSGGMVKS NFVPTNVGSKAF-NH₂ (SEQ ID NO:8);
YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-
NH₂ (SEQ ID NO: 9).

Table 1 shows the polypeptide corresponding to the SEQ ID NOs. shown above.

5 SEQ ID NOs: 1-4 relate to forms of human and mouse AFP-6. SEQ ID NOs: 5-9 relate to human Amylin Family polypeptides of amylin, calcitonin, CGRP α , CGRP β , and adrenomedullin.

Table 1

10	SEQ ID NO: 1	Human AFP-6 [1 -47]
	SEQ ID NO:2	Human AFP-6 [8-47]
	SEQ ID NO:3	Mouse AFP-6 [1-47]
	SEQ ID NO:4	Mouse AFP-6 [8-47]
15	SEQ ID NO:5	Human Amylin
	SEQ ID NO:6	Human Calcitonin
	SEQ ID NO:7	Human CGRP α
	SEQ ID NO:8	Human CGRP β
	SEQ ID NO:9	Human Adrenomedullin

It has been reported that the biological actions of these Amylin Family polypeptides are mediated via binding to two closely related type II G protein-coupled
20 receptors (GPCRs), the calcitonin receptor (CTR) and the calcitonin receptor like receptor (CRLR). Cloning and functional studies have shown that CGRP, ADM, and amylin interact with different combinations of CTR or the CRLR and the receptor activity modifying protein (RAMP). Many cells express multiple RAMPs. It is believed that co-expression of RAMPs and either the CTR or CRLR is required to generate functional
25 receptors for calcitonin, CGRP, ADM, and amylin. The RAMP family comprises three members (RAMP1, -2, and -3), which share less than 30% sequence identity, but have a common topological organization. Co-expression of CRLR and RAMP1 leads to the formation of a receptor for CGRP. Co-expression of CRLR and RAMP2 leads to the formation of a receptor for ADM. Co-expression of CRLR and RAMP3 leads to the
30 formation of a receptor for ADM and CGRP. Co-expression of hCTR2 and RAMP1 leads to the formation of a receptor for amylin and CGRP. Co-expression of hCTR2 and RAMP3 leads to the formation of a receptor for amylin.

Amylin regulates gastric emptying and suppresses glucagon secretion and food intake, thus regulating the rate of glucose appearance in the circulation. It appears to
35 complement the actions of insulin, which regulates the rate of glucose disappearance from the circulation and its uptake by peripheral tissues. These actions are supported by

experimental findings in rodents and humans, which indicate that amylin complements the effects of insulin in postprandial glucose control by at least three independent mechanisms, all of which affect the rate of glucose appearance. First, amylin suppresses postprandial glucagon secretion. Compared to healthy adults, patients with type 1 diabetes have no circulating amylin and patients with type 2 diabetes have diminished postprandial amylin concentrations. Furthermore, infusion of an amylin specific monoclonal antibody, which bound circulating amylin, again resulted in greatly elevated glucagon concentrations relative to controls. Both of these results point to a physiological role of endogenous amylin in the regulation of postprandial glucagon secretion. Second, amylin slows gastrointestinal motility and gastric emptying. Finally, intrahypothalamic injections of rat amylin were shown to reduce feeding in rats and alter neurotransmitter metabolism in the hypothalamus. In certain studies, food intake was significantly reduced for up to eight hours following the intrahypothalamic injection of rat amylin and rat CGRP. In human trials, an amylin analog, pramlintide, has been shown to reduce weight or weight gain. Amylin may be beneficial in treating metabolic conditions such as diabetes and obesity. Amylin may also be used to treat pain, bone disorders, gastritis, to modulate lipids, in particular triglycerides, or to affect body composition such as the preferential loss of fat and sparing of lean tissue.

The hormone calcitonin (CT) was named for its secretion in response to induced hypercalcemia and its rapid hypocalcemic effect. It is produced in and secreted from neuroendocrine cells in the thyroid that have since been termed C cells. The best-studied action of CT(1-32) is its effect on the osteoclast. *In vitro* effects of CT include the rapid loss of ruffled borders and decreased release of lysosomal enzymes. Ultimately, the inhibition of osteoclast functions by CT results in a decrease in bone resorption. However, neither a chronic reduction of serum CT in the case of thyroidectomy nor the increased serum CT found in medullary thyroid cancer appears to be associated with changes in serum calcium or bone mass. It is thus most likely that a major function of CT(1-32) is to combat acute hypercalcemia in emergency situations and/or protect the skeleton during periods of "calcium stress" such as growth, pregnancy, and lactation. Reviewed, for example, in Becker (2004) *JCEM* 89(4):1512-1525 and Sexton (1999) *Current Medicinal Chemistry* 6:1067-1093. Consistent with this is recent data from the calcitonin gene knockout mouse, which removes both the calcitonin and the CGRP-I peptides, that revealed that the mouse had normal levels of basal calcium-related values,

but an increased calcemic response (Kurihara *et al.* (2003) *Hypertens Res.* 26 Suppl:S 105-108).

CT has an effect on plasma calcium levels and inhibits osteoclast function and is widely used for the treatment of osteoporosis. Therapeutically, salmon CT appears to
5 increase bone density and decrease fracture rates with minimal adverse effects. CT has also been successfully used over the past 25 years as a therapy for Paget's disease of bone, which is a chronic skeletal disorder that may result in enlarged or deformed bones in one or more regions of the skeleton. CT is also widely used for its analgesic effect on bone pain experienced during osteoporosis, although the mechanism for this effect is not clearly
10 understood.

Calcitonin gene related peptide (CGRP) is a neuropeptide whose receptors are widely distributed in the body, including the nervous system and the cardiovascular system. This peptide seems to modulate sensory neurotransmission and is one of the most potent endogenous vasodilatory peptides discovered to date. Reported biological effects
15 for CGRP include: modulation of substance P in inflammation, nicotinic receptor activity at the neuromuscular junction, stimulation of pancreatic enzyme secretion, a reduction of gastric acid secretion, peripheral vasodilation, cardiac acceleration, neuro-modulation, regulation of calcium metabolism, osteogenic stimulation, insulin secretion, an increase in body temperature and a decrease in food intake. Wimalawansa (1997) *Crit. Rev.*
20 *Neurobiol.* 11(2-3):167-239. An important role of CGRP is to control blood flow to various organs by its potent vasodilatory actions, as evidenced by a decrease of mean arterial pressure following intravenous administration of α -CGRP. The vasodilatory actions are also supported by recent analysis of homozygous knockout CGRP mice, which demonstrated elevated peripheral vascular resistance and high blood pressure caused by
25 increased peripheral sympathetic activity (Kurihara (2003), *Supra.*). Thus, CGRP appears to elicit vasodilatory effects, hypotensive effects and an increase in heart rate among other actions.

Prolonged infusion of CGRP into patients with congestive cardiac failure has shown a sustained beneficial effect on hemodynamic functions without adverse effects,
30 suggesting a use in heart failure. Other indications of CGRP use include renal failure, acute and chronic coronary artery ischemia, treatment of cardiac arrhythmia, other peripheral vascular disease such as Raynaud's phenomenon, subarachnoid hemorrhage, hypertension, and pulmonary hypertension. Preeclamptic toxemia of pregnancy and

preterm labor is also potentially treatable. (Wimalawansa (1997) *Supra*). Recent therapeutic uses include the use of CGRP antagonists for the treatment of migraine headaches.

Adrenomedullin is almost ubiquitously expressed with many more tissues
5 containing the peptide than not. A published review of ADM details its effects on the cardiovascular system, cellular growth, the central nervous system and the endocrine system, with a range of biological actions including vasodilation, cell growth, regulation of hormone secretion, and natriuresis (Hinson *et al.* (2000) *Endocrine Reviews* 21(2):138-167). Studies in rats, cats, sheep, and man confirm that intravenous infusion of ADM
10 results in potent and sustained hypotension that is comparable to that of CGRP. However, the hypotensive effect of ADM on mean arterial pressure in the anesthetized rat is not inhibited by the CGRP antagonist CGRP8-37 suggesting that this effect is not mediated via CGRP receptors. Acute or chronic administration of human ADM in rats, anesthetized, conscious or hypertensive, results in a significant decrease in total peripheral
15 resistance accompanied by a fall in blood pressure, with a concomitant rise in heart rate, cardiac output and stroke volume.

ADM has also been proposed as an important factor in embryogenesis and differentiation and as an apoptosis survival factor for rat endothelial cells. This is supported by recent mouse ADM knockout studies, in which mice homozygous for loss of
20 the ADM gene demonstrated defective vascular formation during embryogenesis and thus died mid-gestation. It was reported that ADM +/- heterozygous mice had high blood pressure along with susceptibility to tissue injury (Kurihara (2003), *Supra*).

ADM affects such endocrine organs as the pituitary, the adrenal gland, reproductive organs and the pancreas. The peptide appears to have a role in inhibiting
25 ACTH release from the pituitary. In the adrenal gland, it appears to affect the secretory activity of the adrenal cortex in both rat and human and it increases adrenal blood flow, acting as a vasodilator in the adrenal vascular bed in intact rats. ADM has been shown to be present throughout the female reproductive tract and plasma levels are elevated in normal pregnancy. Studies in a rat model of preeclampsia show that ADM can reverse
30 hypertension and decrease pup mortality when given to rats during late gestation. Because it did not have a similar effect in animals in early gestation or nonpregnant rats in the preeclampsia model, this suggests that ADM may play an important regulatory role in the utero-placental cardiovascular system. In the pancreas, ADM most likely plays an

inhibitory role since it attenuated and delayed insulin response to an oral glucose challenge, resulting in initial elevated glucose levels. ADM can also affect renal function. A bolus administered peripherally can significantly lower mean arterial pressure and raise renal blood flow, glomerular filtration rate and urine flow. In some cases, there is also an
5 increase in Na⁺ excretion.

ADM also has other peripheral effects on bone and on the lung. For bone, studies have supported a role beyond the cardiovascular system and fluid homeostasis and have demonstrated that ADM acts on fetal and adult rodent osteoblasts to increase cell growth comparable to those of known osteoblast growth factors such as transforming growth
10 factor- β . This is important clinically as one of the major challenges in osteoporosis research is to develop a therapy that increases bone mass via osteoblastic stimulation. In the lung, ADM not only causes pulmonary vasodilation, but also inhibits bronchoconstriction induced by histamine or acetylcholine. Recent studies using aerosolized ADM to treat pulmonary hypertension in a rat model indicate that inhalation
15 treatment of this condition is effective, as evidenced by the fact that mean pulmonary arterial pressure and total pulmonary resistance were markedly lower in rats treated with ADM than in those given saline. This result was achieved without an alteration in systemic arterial pressure or heart rate (Nagaya *et al.* (2003) *Am. J. Physiol. Heart Circ. Physiol.* 285:H2125-2131).

20 A review published by Nicholls *et al.* (*Peptides* (2001) 22:1745-1752) summarizes the effects of infusion of ADM. In healthy volunteers, *i.v.* infusion reduced arterial pressure and stimulated heart rate, cardiac output, plasma levels of cAMP, prolactin, norepinephrine and rennin. In these patients, there was little or no increase in urine volume or sodium excretion observed. In patients with heart failure or chronic renal
25 failure, *i.v.* ADM had similar effects to those seen in normal subjects and also induced diuresis and natriuresis, depending on the dose administered. Experimental ADM treatment has also been shown to be beneficial in arterial and pulmonary hypertension, septic shock and ischemia/reperfusion injury (Beltowski (2004) *Pol. J. Pharmacol.* 56:5-27). Other indications for ADM treatment include: peripheral vascular disease,
30 subarachnoid hemorrhage, hypertension, preeclamptic toxemia of pregnancy and preterm labor, and osteoporosis.

As the newest member of the Amylin Family of peptides, the biological function of AFP-6 is less well characterized than the members discussed above. However, the

expression data obtained from Northern blots of human and mouse tissue is shown in Table 4, in the Example section below, and is consistent with reported data indicating that expression is primarily in the pituitary and gastrointestinal tract. A specific receptor for AFP-6 has not been reported; however, binding studies indicate that AFP-6 binds to all the known receptors of the Amylin Family. AFP-6 has been shown to increase cAMP production in SK-N-MC and L6 cells expressing endogenous CGRP receptors and competes with labeled CGRP for binding to its receptors in these cells. In published *in vivo* studies, AFP-6 administration led to blood pressure reduction in both normal and spontaneously hypertensive rats, most likely via interactions with the CRLR/RAMP receptors. *In vivo* administration in mice led to a suppression of gastric emptying and food intake (Roh *et al* (2004) *J. Biol. Chem.* 279(8):7264-7274).

Studies using mutant mice deficient for a CGRP, ADM, or amylin have indicated that, in different systems, CRLR can be important for cardiovascular morphogenesis, sensory neurotransmission, inflammatory reactions, nociceptive behavior, and glucose homeostasis. Thus, the physiological functions of polypeptides in this family are determined by receptor-binding specificity and the tissue expression profiles of individual ligands. AFP-6 appears to be unique in that it binds to all the receptors of the Amylin Family. For example, while amylin binds to the amylin, calcitonin and CGRP receptors with 0.05 nM to 20 nM affinity, it does not bind with very high affinity to the adrenomedullin receptor (several hundred nM affinity). In bovine studies, AFP-6 was shown to bind the adrenomedullin receptor (around 1-5 nM) in addition to having between 3-30 nM binding at the other 3 receptors, giving it amylin, CT, CGRP and/or ADM properties. See also Table 3 herein for receptor binding data.

Amylin Family proteins in general, and AFP-6 polypeptides in particular, have a variety of biological activities that are of use in the treatment or prevention of a variety of diseases, conditions, and disorders. There remains a need to develop such polypeptides, as well as derivatives and analogs thereof, for use in treating and/or preventing the described diseases, conditions, and disorders.

SUMMARY OF THE INVENTION

In one general aspect, the invention is drawn to novel AFP-6 analogs. It has been discovered that certain regions of the AFP-6 polypeptide are desirable for binding and/or activity. In certain embodiments, novel AFP-6 analogs are compounds comprising

an amino acid sequence of formula (I):

X₁-X₂-X₃-X₄-X₅-X₆-X₇-X₈-QVQNLSHRLWQL-X₂₁-X₂₂-X₂₃-X₂₄-X₂₅-X₂₆-X₂₇-X₂₈-
SAPV-X₃₃-PSSPHSY (SEQ ID NO: 41) wherein

- X₁ is absent, TQAQLLRVG (SEQ ID NO: 42), any of one or more consecutive amino
5 acids of SEQ ID NO:42, N-aryl, or N-acyl with a substituent selected from a C1-C18
alkyl, a substituted alkyl or a heteroaryl moiety;
X₂ is M, S, C, substituted L, K, D or E, where the side chain can be linked via an amide
bond, or any amino acid that can form a bond with X₈, for example a disulfide or an
amide bond;
10 X₃ is V, D, L, G, N, A, or S;
X₄ is V, D, L, G, N, A, S or T;
X₅ is V, D, L, G, N, A, or S;
X₆ is V, D, L, G, N, A, S, or absent;
X₇ is T, S, homoserine, (S)-2-Amino-3-hydroxy-3-methylbutanoic acid (Ab) or (2R,3R)-2-
15 Amino-3-hydroxy-4-methylpentanoic acid (Ap);
X₈ is M, S, C, substituted L, K, D or E, or any amino acid that can form a bond with X₂,
for example a disulfide or an amide bond;
X₂₁ is M, G, P, A, or absent;
X₂₂ is M, G, P, A, or absent;
20 X₂₃ is M, G, P, A, or absent;
X₂₄ is M, G, P, A, or absent;
X₂₅ is M, G, P, A, or absent;
X₂₆ is R or absent, wherein when X₂₆ is absent, X₂₇ is absent;
X₂₇ is Q or absent, wherein when X₂₇ is absent, X₂₆ is absent;
25 X₂₈ is D or E;
X₃₃ is D or E; and
biologically active fragments thereof; however, it is contemplated that the AFP-6 analog
of this genus does not include SEQ ID NOs: 1 or 2.

- In other embodiments, novel AFP-6 analogs comprise, or the active region
30 consists of, an amino acid sequence of formula (II):
X₁-X₂-QNLSHRLWQL-X₁₃-X₁₄-X₁₅-X₁₆-X₁₇-X₁₈-X₁₉-X₂₀-SAPV-X₂₅-PSSPHSY (SEQ
ID NO: 56) wherein
X₁ is Q or absent;
X₂ is V or absent;

- X₁₃ is M, G, P, A, or absent;
 X₁₄ is M, G, P, A, or absent
 X₁₅ is M, G, P, A, or absent;
 X₁₆ is M, G, P, A, or absent;
 5 X₁₇ is M, G, P, A, or absent,
 X₁₈ is R or absent, wherein when X₁₈ is absent, X₁₉ is absent;
 X₁₉ is Q or absent, wherein when X₁₉ is absent, X₁₈ is absent
 X₂₀ is D or E;
 X₂₅ is D or E; and
 10 biologically active fragments thereof, wherein the amino acid sequence is not SEQ ID
 NOs: 13 or 14.

In still other embodiments, AFP-6 analogs comprise the amino acid sequence
 of SEQ ID NO: 10-12, 16-33 and 35-40. In yet other embodiments, AFP-6 analogs
 comprise or the active region consists of the amino acid sequence of SEQ ID NOs: 15 or
 15 34.

In certain embodiments, AFP-6 analogs may be characterized by having no
 more than 25, 30, 35, 40, or 45 amino acids. In other embodiments, AFP-6 analogs may
 have 23 to 49 amino acids. In certain embodiments, AFP-6 analogs comprise at least 80,
 82, 84, 86, 88, 90, 92, 94, 96, 97, or 98% amino acid sequence identity to SEQ ID NOs: 1-
 20 4 and 10-12, 16-33 and 35-40 and having at least one Amylin Family activity, wherein the
 compound is not SEQ ID NOs: 1-4. In other embodiments, AFP-6 analogs comprise, or
 the active region consists of, at least 80, 82, 84, 86, 88, 90, 92, 94, 96, 97, or 98% amino
 acid sequence identity to the sequence of SEQ ID NOs: 15 and 34 and having an
 antagonist activity to an Amylin Family polypeptide, wherein the compound is not SEQ
 25 ID NOs: 13 or 14.

In another general aspect, the invention provides methods of using AFP-6,
 AFP-6 agonists or AFP-6 analogs. In certain embodiments, the invention contemplates
 methods of treating or preventing a condition or disease that can be alleviated by reducing
 caloric or nutrient intake or availability in a subject. Methods of the invention can
 30 include, but are not limited to, conditions and diseases of obesity, insulin resistance,
 metabolic syndrome and diabetes mellitus. In other embodiments, the invention
 contemplates methods of treating or preventing a cardiovascular condition or disease in a

subject. Non-limiting examples of a cardiovascular condition or disease are hypertension, myocardial ischemia, and myocardial reperfusion.

In yet other embodiments, the methods of the invention contemplate use of AFP-6 analogs having antagonist activity. Thus, exemplary uses include treating or preventing anorexia, cachexia, bulimia, and other wasting diseases characterized by loss of appetite, diminished food intake, body weight loss, gastric hypomotility, or hypotension in a subject.

Additional embodiments include administration of the compounds at a dose in the range of from about 0.05 mg/kg to about 2 mg/kg, exemplary modes of delivery such as injection, infusion, absorption (e.g., mucosal, transmucosal, transdermal), and inhalation, nucleotides that encode the amino acid sequence herein described, vectors containing the nucleotides, host cells for propagating nucleotides and/or expressing the polypeptides encoded by the nucleotides, antibodies directed to the AFP-6, AFP-6 agonists and AFP-6 analogs, and their uses in screening or detection/diagnosis in a subject. In another embodiment, the invention provides methods for preparing AFP-6 analogs, including methods comprising culturing host cells containing an expression vector encoding an AFP-6 analog under conditions that provide for expression of the AFP-6 analog and isolating the expressed AFP-6 analog.

All documents described herein are incorporated by reference in their entirety.

20

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the BLAST alignment of adrenomedullin peptide query sequence with the hypothetical protein XP_147916 and alignment of the human adrenomedullin precursor with the full-length hypothetical mouse protein XP_147916, respectively. Like residues are shaded in dark gray while residues with similar chemical nature are shaded in light gray.

Figure 2 shows the sequence of the entire protein XP_147916, now renamed mouse AFP-6, in FASTA format.

Figure 3 shows the alignment of mouse and human AFP-6 precursor proteins performed by AlignX® (Vector NTI®; Invitrogen) and exported to BOXSHADE for shading. Like residues are shaded in dark gray while residues with similar chemical nature are shaded in gray.

Figure 4 shows the alignment of human adrenomedullin and human AFP-6 precursor proteins performed by AlignX® (Vector NTI®; Invitrogen) and exported to BOXSHADE for shading. Like residues are shaded in dark gray while residues with similar chemical nature are shaded in light gray.

5 Figure 5 shows the alignment by AlignX® (Vector NTI®; Invitrogen) of Amylin Family polypeptides: hAFP-6, hAdrenomedullin, hAmylin, hCGRP 1, and hCalcitonin. Like residues are shaded in dark gray while residues with similar chemical nature are shaded in light gray.

10 Figure 6 shows predicted mature bioactive peptides from human and mouse AFP-6 precursors based on homology with Adrenomedullin.

Figure 7 shows the alignment of the 5' UTR of human prostate AFP-6 cDNA aligned with database sequence NM024866.

Figures 8A-8I show selected *in situ* hybridization data from the pancreas, prostate and salivary gland.

15 Figure 9 shows data of elevation of cyclic AMP by AFP-6, hAdrenomedullin and hCGRP 1 at 2 concentrations in HUVEC and CHO cells as measured using the PerkinElmer AlphaScreen™ assay.

20 Figure 10 shows results of an amylin radioligand binding assay (RBA). The graph depicts displacement of radiolabeled amylin binding with calculated inhibitory concentration (IC₅₀) values for AFP-6, hAdrenomedullin, hCGRP 1, hCalcitonin, and rAmylin at the amylin receptor.

Figure 11 shows results of a CGPR radioligand binding assay (RBA). The graph depicts displacement of radiolabeled CGRP binding with calculated IC₅₀ values for AFP-6, hAdrenomedullin, hCGRP 1, hCalcitonin, and rAmylin at the CGRP receptor.

25 Figure 12 shows the effect of AFP-6 on short-term food intake in mice.

Figure 13 shows a dose response of AFP-6 on short-term food intake in mice.

Figures 14A and 14B show the effect of AFP-6 and rAmylin, respectively, on the rate of gastric emptying in rats.

30 Figures 15A and 15B show the effect of AFP-6 and rAmylin, respectively, on plasma ionized calcium levels in rats.

Figures 16A-16C show the effects of rAmylin, AFP-6, and rat adrenomedullin on several cardiac parameters, mean arterial pressure (MAP), heart rate (HR) and dp/dt, in male rats.

Figures 17A and 17B show the effect of 1-week continuous subcutaneous
5 infusion of AFP-6, SEQ ID NO:1, or an exemplary analog of AFP-6, SEQ ID NO:32, on food intake and body weight gain in High Fat-Fed Rats.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the discovery of novel AFP-6 analogs. Through research efforts designed to understand the structure and function of AFP-6, in
10 combination with an understanding of the other Amylin Family polypeptides, novel compounds and their uses are herein described. These compounds, termed AFP-6 analogs, are useful in the treatment and prevention of, among other things, metabolic conditions and disorders. One desirable characteristic of AFP-6 analogs is that they can be designed to favor certain Amylin Family receptors over others (*e.g.*, possess a certain
15 binding profile), which may lead to more effective treatments, fewer side effects, increased stability or solubility, increased activity, increased ease of manufacturing or use of the peptide, for example, making it more economical to produce. Accordingly, peptide analogs are of great interest for clinical use and the development of therapies of conditions involving physiologies which include, but are not limited to, hypertension and
20 maintenance of cardiovascular homeostasis; pulmonary diseases, pregnancy (or infertility) and lactation; gastrointestinal; glucose regulation, obesity, metabolic syndrome and other metabolic disorders.

In certain embodiments, the AFP-6 analogs of the invention may have comparable or higher potency in the treatment and/or prevention of hypertension and
25 cardiovascular conditions; fertility (or infertility) and lactation; pulmonary disorders; and gastrointestinal conditions and conditions associated with hyper- or hypoglycemia, as compared to native AFP-6 polypeptides. In other embodiments, the AFP-6 analogs of the invention may have weaker, though still effective, potency in the treatment and/or prevention of the above described conditions, but may possess other desirable
30 characteristics over native AFP-6, *e.g.*, increased stability or solubility, less side effects, combination of biological activities, and/or ease in manufacturing, formulating, or use. In still other embodiments, AFP-6 analogs of the invention have antagonist activity that

blocks, or reduces, AFP-6 activity. Accordingly, the AFP-6 antagonist analogs of the invention are of use, for example, in the prevention or treatment of wasting diseases or disorders.

Section headings are used herein for organizational purposes only and are not to be construed as in any way limiting the subject matter described.

AFP-6 Analogs

AFP-6 analogs of the invention include, but are not limited to, compounds having at least amino acids 18-27 and 41-47 of SEQ ID NO:1; amino acids 18-27 and 33-47 of SEQ ID NO:1, wherein the amino acid at positions 35 and 40 is either D or E; amino acids 18-27, 36-39 and 41-47 of SEQ ID NO:1; or any of the aforementioned compounds further having a loop at the N terminal end, such as a disulfide or amide loop, provided that the AFP-6 analog is not SEQ ID NOs:1-4 or amino acids 16-47 or 17-47 of SEQ ID NO:1. In certain embodiments, AFP-6 analogs may be characterized by having no more than 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, or 46 amino acids. In other embodiments, AFP-6 analogs may have 47, 48, 49 amino acids or more. In some embodiments, AFP-6 analogs have an Amylin Family activity, such as amylin, calcitonin, CGRP, adrenomedullin, or AFP-6 activity, such as those described herein. In some embodiments, AFP-6 analogs have Amylin Family antagonist activity. Thus, "AFP-6 analog" refers to peptide with either agonist or antagonist activity. Whether an AFP-6 analog is an agonist or an antagonist can be determined by using any of the biological assays described herein.

AFP-6 analogs of the invention may include compounds having at least formula (I)

X₁-X₂-X₃-X₄-X₅-X₆-X₇-X₈-QVQNLSHRLWQL-X₂₁-X₂₂-X₂₃-X₂₄-X₂₅-X₂₆-X₂₇-X₂₈-SAPV-X₃₃-PSSPHSY (SEQ ID NO: 41) wherein
 X₁ is absent, TQAQLLRVG (SEQ ID NO: 42), any of one or more consecutive amino acids of SEQ ID NO: 42, N-aryl, or N-acyl with a substituent selected from a C1-C18 alkyl, a substituted alkyl or a heteroaryl moiety;
 X₂ is M, S, C, substituted L, K, D, or E, where the side chain can be linked via an amide bond, or any amino acid that can form a bond with X₈, for example a disulfide or an amide bond;
 X₃ is V, D, L, G, N, A, or S;
 X₄ is V, D, L, G, N, A, S or T;

- X₅ is V, D, L, G, N, A, or S;
 X₆ is V, D, L, G, N, A, S, or absent;
 X₇ is T, S, homoserine, (S)-2-Amino-3-hydroxy-3-methylbutanoic acid (Ab) or (2R,3R)-2-Amino-3-hydroxy-4-methylpentanoic acid (Ap);
- 5 X₈ is M, S, C, substituted L, K, D, or E, or any amino acid that can form a bond with X₂, for example a disulfide or an amide bond;
 X₂₁ is M, G, P, A, or absent;
 X₂₂ is M, G, P, A, or absent;
 X₂₃ is M, G, P, A, or absent;
- 10 X₂₄ is M, G, P, A, or absent;
 X₂₅ is M, G, P, A, or absent;
 X₂₆ is R or absent, wherein when X₂₆ is absent, X₂₇ is absent;
 X₂₇ is Q or absent, wherein when X₂₇ is absent, X₂₆ is absent;
 X₂₈ is D or E;
- 15 X₃₃ is D or E;
 wherein the AFP-6 analog is not SEQ ID NOs: 1 or 2.

In other embodiments, novel AFP-6 analogs comprise, or the active region consists of, an amino acid sequence of formula (II):

- X₁-X₂-QNLSHRLWQL-X₁₃-X₁₄-X₁₅-X₁₆-X₁₇-X₁₈-X₁₉-X₂₀-SAPV-X₂₅-PSSPHSY (SEQ
- 20 ID NO: 56) wherein
 X₁ is Q or absent;
 X₂ is V or absent;
 X₁₃ is M, G, P, A, or absent;
 X₁₄ is M, G, P, A, or absent
- 25 X₁₅ is M, G, P, A, or absent;
 X₁₆ is M, G, P, A, or absent;
 X₁₇ is M, G, P, A, or absent,
 X₁₈ is R or absent, wherein when X₁₈ is absent, X₁₉ is absent;
 X₁₉ is Q or absent, wherein when X₁₉ is absent, X₁₈ is absent
- 30 X₂₀ is D or E;
 X₂₅ is D or E; and
 biologically active fragments thereof, wherein the amino acid sequence is not SEQ ID NOs: 13 or 14.

The AFP-6 analogs may or may not be amidated at the C-terminal end. AFP-6 analogs encompassed by certain variations of formula (I) are further contemplated. For example, in certain embodiments, X₁ is absent. In other embodiments, X₁ is RVG. In yet other embodiments, X₁ is VG. In still other embodiments, X₁ is Gly. In still other
5 embodiments, X₁ is Val. In certain embodiments, X₂ and X₈ are Cys. In other embodiments, X₂ and X₈ are substituted Leu, Lys, or Asp. In yet other embodiments, X₂ and X₈ are Met. In yet other embodiments, X₂ and/or X₈ are Glu. In still other embodiments, X₂ and X₈ are amino acids having side chains that can be chemically bonded to each other to form an intramolecular linkage. Such side chains for X₂ and X₈
10 include groups derived from alkyl sulfhydryls which may form disulfide bonds; alkyl acids and alkyl amines which may form cyclic lactams; alkyl aldehydes or alkyl halides and alkylamines which may condense and be reduced to form an alkyl amine bridge; or side chains which may be connected to form an alkyl, alkenyl, alkynyl, ether or thioether bond.

15 In certain embodiments of AFP-6 analogs encompassed by formula (I), X₃ is Val. In other embodiments, X₃ is Asn. In still other embodiments, X₃ is Gly. In certain embodiments, X₄ is Leu. In other embodiments, X₄ is Thr. In still other embodiments, X₄ is Asn. In certain embodiments, X₅ is Gly. In other embodiments, X₅ is Ala. In still other embodiments, X₅ is Leu. In certain embodiments, X₆ is absent. In other
20 embodiments, X₆ is Ser. In certain embodiments, X₃ is Val, X₄ is Leu, X₅ is Gly, and X₆ is absent. In other embodiments, X₃ is Asn, X₄ is Thr, X₅ is Ala, and X₆ is absent. In still other embodiments, X₃ is Gly, X₄ is Asn, X₅ is Leu, X₆ is Ser.

In certain embodiments of AFP-6 analogs encompassed by formula (I), X₇ is Thr. In certain embodiments, X₂₁ to X₂₅ are absent. In other embodiments, X₂₁ is Met.
25 In other embodiments, X₂₂ is Gly. In other embodiments, X₂₃ is Pro. In other embodiments, X₂₄ is Ala. In other embodiments, X₂₅ is Gly. In certain embodiments, X₂₆ is R and X₂₇ is Q. In other embodiments, X₂₆ and X₂₇ are absent. In certain embodiments, X₂₈ and/or X₃₃ are Asp. In other embodiments, X₂₈ and/or X₃₃ are Glu.

30 AFP-6 analogs encompassed by certain variations of formula (II) are further contemplated. In certain embodiments, X₁₃ to X₁₇ are absent. In other embodiments, X₁₃ is Met. In other embodiments, X₁₄ is Gly. In other embodiments, X₁₅ is Pro. In other embodiments, X₁₆ is Ala. In other embodiments, X₁₇ is Gly. In certain embodiments, X₁₈

is R and X₁₉ is Q. In other embodiments, X₁₈ and X₁₉ are absent. In certain embodiments, X₂₀ and/or X₂₅ are Asp. In other embodiments, X₂₀ and/or X₂₅ are Glu.

In certain embodiments, the AFP-6 analog is 30 or 31 amino acids in length. In other embodiments, the AFP-6 analog is 32 amino acids in length. In yet other
 5 embodiments, the AFP-6 analog is 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, or 46 amino acids in length. In still other embodiments, the AFP-6 analog is 47 or 48 amino acids or more in length. In certain embodiments, the AFP-6 analog is 23, 24, 25, 26, 27, 28, or 29 amino acids in length.

AFP-6 analogs having antagonist activity may include, for example,
 10 compounds of formula (I) wherein X₁ to X₅ are deleted. Other AFP-6 analogs having antagonist activity may include compounds of formula (II) with the proviso that the AFP-6 analog antagonist is not SEQ ID NO: 13 or 14 (amino acids 16-47 or 17-47 of SEQ ID NO: 1). Other AFP-6 analogs having antagonist activity may include compounds of formula (II) wherein X₁ and/or X₂ are deleted, with the proviso that the AFP-6 analog
 15 antagonist is not SEQ ID NO: 13 or 14. Exemplary AFP-6 analog antagonists include SEQ ID NOs: 13, 14, and 34. Moreover, AFP-6 analog antagonists may include internal substitutions or deletions. For example, the Trp at position 9 of SEQ ID NO: 14 is deleted creating the peptide of SEQ ID NO: 15. An exemplary AFP-6 analog antagonist is SEQ ID NO: 15.

20 Exemplary AFP-6 analogs of the present invention include:
 RVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:10)
 GCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:11)
 CVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:12)
 25 QVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:13)
 VQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:14)
 VQNLSHRL-QLMGPAGRQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:15)
 TQAQLLRVGCVLGTCQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:16)
 30 TQAQLLRVGCVLGTCQVQNLSHRLWQL-----DSAPVDPSSPHSY-NH₂ (SEQ ID NO:17)
 VGCVLGTCQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH₂ (SEQ ID NO:18)
 CVLGTCQVQNLSHRLWQL-----RQESAPVEPSSPHSY-NH₂ (SEQ ID NO:19)

- TQAQLLRVGCSNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
(SEQ ID NO:20)
- TQAQLLRVGCNTATCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
(SEQ ID NO:21)
- 5 RVGCGNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:22)
- TQAQLLRVGCDTATCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
(SEQ ID NO:23)
- TQAQLLRVGCNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
10 (SEQ ID NO:24)
- TQAQLLRVGMVLGT MQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2
(SEQ ID NO:25)
- GMVLGTMQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:26)
- 15 VGMVLGTMQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH2 (SEQ ID NO:27)
- RVGCGNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:28)
- VGCGNLSTCQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH2 (SEQ ID NO:29)
- V-CNTA-TCQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH2 (SEQ ID NO:30)
- 20 GCNTATCQVQNLSHRLWQL-----RQDSAPVDPSSPHSY-NH2 (SEQ ID NO:31)
- TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQESAPVEPSSPHSY-NH2
(SEQ ID NO:32)
- TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVEPSSPHSY-NH2
(SEQ ID NO:33)
- 25 GTMQVQNLSHRLWQL-----RQDSAPVEPSSPHSY-NH2 (SEQ ID NO:34)
- VGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVEPSSPHSY-NH2 (SEQ ID NO:35)
- VGCVLGTCQVQNLSHRLWQL-----RQDSAPVEPSSPHSY-NH2 (SEQ ID NO:36)
- GCNTATCQVQNLSHRLWQL-----RQDSAPVEPSSPHSY-NH2 (SEQ ID NO:37)
- 30 GCSNLSTCQVQNLSHRLWQL-----RQDSAPVEPSSPHSY-NH2 (SEQ ID NO:38)
- GCGNLSTCQVQNLSHRLWQL-----RQDSAPVEPSSPHSY-NH2 (SEQ ID NO:39)
- GCVLGTCQVQNLSHRLWQL-----RQESAPVEPSSPHSY-NH2 (SEQ ID NO:40).

AFP-6 analogs may also include polypeptides having an amino acid sequence with at least 80, 82, 84, 86, 88, 90, 92, 94, 96, 97, or 98% amino acid identity to any

amino acid sequence of SEQ ID NOs: 1-4 and 10 to 40 and having 1) a similar activity to any one of SEQ ID NOs: 1 to 40, or an Amylin Family activity or 2) an antagonist activity of the aforementioned activity, wherein the AFP-6 analog is not SEQ ID NOs: 1-9, or known species variants thereof, or SEQ ID NOs: 13 or 14. Percent identity is determined
5 by analysis with the AlignX® module in Vector NTI® (Invitrogen; Carlsbad CA).

Compounds of the invention may further include additional amino acids, chemicals, or moieties that do not affect the biological activity or function of the peptide but may perform other functions, such as aiding purification (*e.g.*, histidine tag), detection (*e.g.*, biotin), or expression (*e.g.*, expression cassettes/vectors and promoters).

10 The AFP-6 analogs of the invention may also be further derivatized by chemical alterations such as amidation, glycosylation, acylation, sulfation, phosphorylation, acetylation, and cyclization. Such chemical alterations may be obtained through chemical or biochemical methodologies, as well as through *in vivo* processes, or any combination thereof. Derivatives of the analog polypeptides of the invention may
15 also include conjugation to one or more polymers or small molecule substituents. One type of polymer conjugation is linkage or attachment of polyethylene glycol ("PEG") polymers, polyamino acids (*e.g.*, poly-his, poly-arg, poly-lys, etc.) and/or fatty acid chains of various lengths to the N- or C-terminus or amino acid residue side chains of an AFP-6 analog. Small molecule substituents include short alkyls and constrained alkyls (*e.g.*,
20 branched, cyclic, fused, adamantyl), and aromatic groups. In addition, basic residues such as R and K may be replaced with homoR and homoK, citrulline, or ornithine to improve metabolic stability of the peptide. AFP-6 analogs also include acid as well as amide forms of the peptides.

AFP-6 analogs also include biologically active fragments of the larger peptides
25 described herein. Examples of the desired activity include (1) having activity in a food intake, gastric emptying, pancreatic secretion, blood pressure, heart rate or weight loss assay similar to an AFP-6 polypeptide or Amylin Family polypeptide of the present invention, and/or (2) binding in a receptor binding assay for an Amylin Family receptor (*e.g.*, amylin receptor, calcitonin receptor). Exemplary binding assays of Amylin Family
30 receptors are described herein and described in U.S. Patent No. 5,264,372, the entirety of which is incorporated herein by reference.

In one embodiment, AFP-6 analogs will bind in such assays with an affinity of greater than 1 μ M, and, in another embodiment, with an affinity of greater than 1-10 nM.

It is also contemplated that AFP-6 analogs may also possess the desired activity by indirectly interacting with an amylin-family receptor (*e.g.*, amylin, calcitonin, CGRP or adrenomedullin) or bind to other receptor or receptors with which an AFP-6 polypeptide itself may interact to elicit a biological response.

5 The agonist polypeptides of the present invention will generally retain, at least in part, a biological Amylin Family activity, such as an AFP-6 activity. In other words, Amylin Family activity includes amylin activity, calcitonin activity, adrenomedullin activity, CGRP activity or AFP-6 activity. Table 2 provides a summary of Amylin Family biological effects as published in Wimalawansa (1997) *Critical Reviews in Neurobiology*
10 11:167-239.

Table 2: Summary of Biological effect of CGRP, CT, Amylin, and Adrenomedullin

Biological effect	CGRP	Calcitonin	Amylin	ADM
Vasodilation	++++	+/-	++	++
Chronotropic	++	-	++	+
Inotropic	++	-	++	+
Inhibit bone resorption	+	++++	++	+/-
Stimulate bone formation	-	-	+	-
Calcium-lowering effect	-	+++	++	-
Growth factor-like effect	+	-	+	+
Neural regeneration	++	+/-	-	-
Thermoregulation	+	-	+	+/-
Inflammation	++	+/-	-	-
Gastric mucosal protection	++	-	-	-
Effects on β cells	+	+/-	++	-
Skeletal muscle	+	-	+	-
Glucose metabolism	+	-	+	-
Anorectic effect	+	++	++	+
Analgesic effect	-	++	+	-

By a polypeptide having “amylin activity” is meant that the polypeptide demonstrates similar physiological characteristics as amylin, such as those known in the art as well as those described in the instant specification, for example, in reducing food
15 intake. The polypeptides of the present invention may be capable of binding to or otherwise directly or indirectly interacting with an amylin receptor, or other receptor or receptors with which amylin itself may interact to elicit a biological response, *e.g.*, reducing food intake.

By a polypeptide having "calcitonin activity" is meant that the polypeptide demonstrates similar physiological characteristics as calcitonin, such as those known in the art as well as those described in the instant specification, for example, inhibiting osteoclast function. The polypeptides of the present invention may be capable of binding to or otherwise directly or indirectly interacting with a CT receptor, or other receptor or receptors with which calcitonin itself may interact to elicit a biological response, *e.g.*, inhibiting osteoclast function.

By a polypeptide having "CGRP activity" is meant that the polypeptide demonstrates similar physiological characteristics as CGRP, such as those known in the art as well as those described in the instant specification, for example, eliciting a vasodilatory effect. The polypeptides of the present invention may be capable of binding to or otherwise directly or indirectly interacting with a CGRP receptor, or other receptor or receptors with which CGRP itself may interact to elicit a biological response, *e.g.*, eliciting a vasodilatory effect.

By a polypeptide having "adrenomedullin activity" is meant that the polypeptide demonstrates similar physiological characteristics as ADM, such as those known in the art as well as those described in the instant specification, for example, eliciting a hypotensive effect. The polypeptides of the present invention may be capable of binding to or otherwise directly or indirectly interacting with an ADM receptor, or other receptor or receptors with which ADM itself may interact to elicit a biological response, *e.g.*, eliciting a hypotensive effect.

By a polypeptide having "AFP-6 activity" is meant that the polypeptide demonstrates similar physiological characteristics as AFP-6, such as those known in the art as well as those described in the instant specification, for example, eliciting a hypotensive effect. The polypeptides of the present invention are capable of binding to or otherwise directly or indirectly interacting with receptor or receptors with which AFP-6 itself may interact to elicit a biological response, *e.g.*, eliciting a hypotensive effect.

In another aspect of the invention, AFP-6 analogs with antagonist activity will generally inhibit or reduce, at least in part, a biological Amylin Family activity, such as AFP-6 activity. In other words, the amylin, calcitonin, CGRP, adrenomedullin and/or AFP-6 activity is blocked by the antagonist analog.

Table 3 summarizes the results for Amylin Family polypeptides in a food intake assay and various Amylin Family receptor-binding assays. Tables 6 and 7, in the

Example section, summarize the results of some AFP-6 analog Food Intake and binding assay results, respectively.

Table 3. Amylin Family Food Intake (FI) and Receptor Binding data

Compound	FI 30 min ED ₅₀ nmole/kg	FI 60 min ED ₅₀ nmole/kg	IC ₅₀ nM		
			Amylin RBA	rCalcitonin RBA	CGRP RBA
AFP-6	9-14	14-19	13	32	3
r/h Amylin	11-25	25-33	0.16	2.8	12
r/h CGRP	0.9	4.2	0.57	104.8	0.02
r/h Calcitonin	>270	>270	100	1.3-3.4	1000
r/h Adrenomedullin	18	13	123	33-68	0.3

5 Given the biological activity and/or receptor binding activity described herein, the present invention provides AFP-6 analogs and AFP-6 analog compositions for use in a medicament for treating a disease or disorder in a subject in need thereof. The present invention also provides methods for use of AFP-6 analogs and AFP-6 analog compositions in treating a disease or disorder in a subject.

10 As such, in one aspect, the present invention provides AFP-6 agonist analog compositions and methods of using them to reduce weight in a subject; affect body composition; treat diabetes, including type 2 or non-insulin dependent diabetes, type 1 diabetes and gestational diabetes; and to treat eating disorders, pain, insulin-resistance syndrome, metabolic syndrome, cardiovascular disease, pulmonary disease, infertility, or
15 conditions affecting bone.

 In yet another aspect, the present invention provides AFP-6 antagonist analog compositions and methods of using them, such that their ability to block the effects of amylin, calcitonin, CGRP, adrenomedullin, or AFP-6 can be beneficial to the subject in treating, for example, anorexia, cachexia, bulimia, and other wasting diseases
20 characterized by loss of appetite, diminished food intake, body weight loss, or hypotension.

 By "AFP-6" is meant an AFP-6 polypeptide obtained or derived from any species. Thus, the term "AFP-6" includes the human full-length amino acid peptide, and species variations of AFP-6, including *e.g.*, murine, hamster, chicken, bovine, rat, and dog
25 AFP-6 polypeptide. In this sense, "AFP-6 polypeptide" and "wild-type AFP-6 polypeptide" or "native AFP-6 polypeptide," *i.e.*, unmodified AFP-6 polypeptide, are used interchangeably.

By "AFP-6 agonist" is meant any compound, including peptide, peptide-like compounds and small molecules, that elicits similar biological activities as AFP-6.

By "amino acid" and "amino acid residue" is meant natural amino acids, unnatural amino acids, and modified amino acid, all in their D and L stereoisomers if their structure allows such stereoisomeric forms. Natural amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine(Ile), leucine (Leu), Lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), typtophan (Trp), tyrosine (Tyr) and valine (Val). Unnatural amino acids include, but are not limited to azetidinecarboxylic acid, 2-aminoadipic acid, 3-aminoadipic acid, beta-alanine, aminopropionic acid, 2-aminobutyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminopimelic acid, tertiary-butylglycine, 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopimelic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-ethylasparagine, homolysine, homoproline, homoserine, hydroxylysine, allo-hydroxylysine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylalanine, N-methylglycine, N-methylisoleucine, N-methylpentylglycine, N-methylvaline, naphthalanine, norvaline, norleucine, ornithine, pentylglycine, pipercolic acid and thioproline, homolysine, homoarginine, homoserine, citrulline, ornithine, Ne-formyllysine. Modified amino acid include the natural and unnatural amino acids which are chemically blocked, reversibly or irreversibly, or modified on their N-terminal amino group or their side chain groups, as for example, methionine sulfoxide, methionine sulfone, S (carbo amino group or side chain functional group has been chemically codified to another functional group. For example, aspartic acid-(beta-methyl ester) is a modified amino acid of aspartic acid; N-ethylglycine is a modified amino acid of glycine; or alanine carboxamide is a modified amino acid of alanine. Additional residues that can be incorporated are described by Sandberg *et. al.* (1998) *J. Med. Chem.* 41:2481-2491.

In one embodiment, the AFP-6 agonist analogs of the invention retain or affect, by at least about 25%, about 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99%, the biological activity of an AFP-6 polypeptide or another polypeptide of the Amylin Family. In another embodiment, the agonist analog polypeptides of the invention exhibit improved activity over at least one of the other Amylin Family polypeptides. For example, the agonist analog polypeptides of the invention exhibit at least about 110%,

125%, 130%, 140%, 150%, 200%, or more of the biological activity of an AFP polypeptide or another polypeptide of the Amylin Family. An exemplary function of AFP-6 is the reduction of food intake or blood pressure.

Exemplary AFP-6 analogs are those having a potency in one of the assays described herein (for example, receptor binding assays, food intake, and/or weight reduction assays) which is greater than or equal to the potency of human AFP-6 polypeptide in that same assay. For example, the AFP-6 analogs may bind to at least one of the receptors with an affinity of greater than 30 nM, 20 nM, 10 nM, or more, alternatively with an affinity of greater than that of a native adrenomedullin or one of the other Amylin Family polypeptides. However, it is also contemplated that AFP-6 analogs of the invention can have less potency in the assays. AFP-6 analogs may further possess desirable characteristics, such as a specific binding profile, stability, solubility, or ease in manufacturing or formulation.

In one example, the polypeptides of the present invention may demonstrate activity in food intake assays. Such polypeptides demonstrate the ability to reduce cumulative food intake by more than 5% over administration of the vehicle, more than 15%, more than 25%, more than 35%, or more than 50% over the vehicle. In one embodiment, the AFP-6 analog reduces food intake by more than 75 or even 90%.

In another general aspect, the invention includes nucleic acids that can encode the AFP-6 analogs herein described. Such nucleic acids can be determined from the amino acid sequences provided herein using standard coding table well known in the art.

Uses of AFP-6, AFP-6 agonists and AFP-6 analogs

Given the biological activity and/or receptor binding activity described herein, the present invention provides AFP-6 analogs and AFP-6 analog compositions for use in a medicament for treating a disease or disorder in a subject in need thereof. The present invention also provides methods for use of AFP-6 analogs and AFP-6 analog compositions in treating a disease or disorder in a subject.

As used herein, and as well-understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. "Treating" a disease, disorder, or condition means that the extent and/or undesirable clinical manifestations of a condition, disorder, or a disease state are lessened and/or time course of the progression is slowed or lengthened, as compared to not treating the disorder. For example, in treating obesity, a decrease in body weight, *e.g.*, a 5% decrease in body weight, is an example of a

desirable treatment result. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Further, treating does not necessarily occur by administration of one dose, but often occurs upon administration of a series of doses. Thus, a therapeutically effective amount or an amount sufficient to treat a disease, disorder, or condition may be administered in one or more administrations.

As such, in one aspect, the present invention provides AFP-6 agonist analog compositions and methods of using them to reduce weight in a subject; affect body composition; treat diabetes, including type 2 or non-insulin dependent diabetes, type 1 diabetes and gestational diabetes; and to treat eating disorders, pain, insulin-resistance syndrome, metabolic syndrome, cardiovascular disease, pulmonary disease, infertility, and/or conditions affecting bone.

In yet another aspect, the present invention provides AFP-6 antagonist analog compositions and methods of using them, such that their ability to block the effects of amylin, calcitonin, CGRP, adrenomedullin, or AFP-6 can be beneficial to the subject in treating, for example, anorexia, cachexia, bulimia, and/or other wasting diseases characterized by loss of appetite, diminished food intake, body weight loss, or hypotension.

Based upon the pharmacologic activities described herein, AFP-6, AFP-6 agonists and AFP-6 analogs may be useful for the treatment of metabolic diseases (including various manifestations of diabetes mellitus and dysglycemia, insulin resistance and insulin resistance syndrome, obesity, dyslipidemia); the treatment of gastrointestinal disorders, including gastritis, Barrett's and other esophagitis, pancreatitis, and irritable bowel syndrome; the treatment of disorders related to or that can be ameliorated by regulating the rate of gastric emptying (e.g., dumping syndrome, moderating postprandial glucose levels and gastric hypomotility); the treatment of cardiovascular and renal disorders, including hypertension, renal failure, vascular occlusive disorders (including ischemia-reperfusion injury and stroke); the treatment of congestive heart failure and other ventricular dysfunctions and cardiac dysrhythmias and as a cardioprotective and/or

myoprotective agent, especially in association with myocardial infarction; the treatment of pulmonary disorders, including pulmonary hypertension, bronchospasm, chronic obstructive pulmonary disease and disorders of ventilation-perfusion mismatch; the treatment of pain, including bone pain, migraine, and other conditions where opiate-sparing is desired; the treatment of bone disorders, including osteomalacia or disorders of mineralization, for the promotion of osteogenesis and fracture repair; the treatment of ghrelin related disorders, including relative growth-hormone and/or IGF1 excess; the treatment of glucagon-related disorders; and for the release of prolactin, which has effects on the uterus and pregnancy, in growth hormone release, and in ovarian follicle survival and growth.

In particular, AFP-6, AFP-6 agonists and AFP-6 agonist analogs may be used to treat conditions or disorders which can be alleviated by reducing caloric (or nutrient) intake or availability. This would include any condition or disorder in a subject that is either caused by, complicated by, or aggravated by a relatively high nutrient intake or availability, or that can be alleviated by reducing nutrient intake or availability, for example by decreasing food intake. Such conditions or disorders include, but are not limited to, obesity, diabetes mellitus, including type 2 diabetes, eating disorders, and insulin-resistance syndromes.

Obesity and its associated disorders are common and very serious public health problems in the United States and throughout the world. Upper body obesity is the strongest risk factor known for type 2 diabetes mellitus, and is a strong risk factor for cardiovascular disease. Obesity is a recognized risk factor for hypertension, atherosclerosis, congestive heart failure, stroke, gallbladder disease, osteoarthritis, sleep apnea, reproductive disorders such as polycystic ovarian syndrome, cancers of the breast, prostate, and colon, and increased incidence of complications of general anesthesia (see, e.g., Kopelman, *Nature* 404: 635-43 (2000)). It reduces life-span and carries a serious risk of co-morbidities as listed above, as well as disorders such as infections, varicose veins, acanthosis nigricans, eczema, exercise intolerance, insulin resistance, hypertension hypercholesterolemia, cholelithiasis, orthopedic injury, and thromboembolic disease (Rissanen et al, *BMJ* 301: 835-7 (1990)).

Obesity is also a risk factor for the group of conditions called insulin resistance syndrome, or "Syndrome X." Recent estimates for the medical cost of obesity and associated disorders is \$150 billion worldwide. The pathogenesis of obesity is believed to

be multifactorial but the basic problem is that in obese subjects nutrient availability and energy expenditure do not come into balance until there is excess adipose tissue. Obesity is currently a poorly treatable, chronic, essentially intractable metabolic disorder.

It is now disclosed that AFP-6, AFP-6 agonists and AFP-6 agonist analogs
5 may be administered to treat obesity and related conditions or prevent/reduce the risk of other disease states as described herein. Related conditions include, but are not limited to, insulin resistance, diabetes mellitus, hypertension, cardiovascular disease, pseudotumor, cerebri, hyperlipidemia, sleep apnea, cancer, pulmonary hypertension, cardiovascular disease, cholecystitis, and osteoarthritis.

10 AFP-6, AFP-6 agonists, and AFP-6 agonist analogs are useful for regulating gastric emptying. These compounds that delay gastric emptying find use in radiological examinations or magnetic resonance imaging. Alternatively, these compounds may be used to reduce gastric motility in a subject suffering from a gastrointestinal disorder, for example, spasm, which may be associated with acute diverticulitis, a disorder of the
15 biliary tract or a disorder of the Sphincter of Oddi. The compounds may also be used to treat post-prandial dumping syndrome or treat post-prandial hyperglycemia.

AFP-6 antagonists that block activity would be useful in disorders that include, but are not limited to anorexia, cachexia, bulimia, and other wasting diseases characterized by loss of appetite, diminished food intake, or body weight loss in addition
20 to hypotension and gastric hypomotility. Accordingly, AFP-6 analogs having antagonist activity to that of an Amylin Family polypeptide, such as AFP-6, and their uses are also contemplated as being part of the invention.

AFP-6, AFP-6 agonists and AFP-6 analogs may also be used to modify body composition, for example, reducing body fat, but not lean body mass or creating a better
25 (larger) lean body to fat ratio. The change in body composition can be by weight (*e.g.*, loss or gain by grams) or by percent body fat and percent lean body mass or protein. Without wishing to be bound by any particular theory, AFP-6, AFP-6 agonists and AFP-6 analogs may have a metabolic effect leading to the desirable loss of body fat, yet preserving lean body mass or minimizing its loss.

30 While "obesity" is generally defined as a body mass index over 30, for purposes of this disclosure, any subject, including those with a body mass index of less than 30, who needs or wishes to reduce body weight is included in the scope of "obese." In other words, the compounds of the invention are intended to be used in all subjects

without regard to their body mass index as long as the desired effect is achieved. For example, the subjects to whom these methods may be of interest include not only those individuals who are overweight or obese, but also those with lean body composition, *e.g.*, body builders and other athletes. It may be desirable for these individuals to reduce or
5 maintain their body weight, *e.g.*, to stay in a certain weight class range, yet preserve or increase their lean body mass for greater strength, stamina, endurance and/or a more muscular appearance. As such in certain embodiments, methods of the invention include reducing body fat or preventing body fat gain. Other embodiments include maintaining or increasing lean body mass. Still other embodiments include controlling body weight
10 and/or sculpting a body's appearance. Such methods may also be used on any animal for which a greater lean body mass to fat ratio is desired. Examples of such use include, but are not limited to, creating a superior show dog, creating a superior racehorse, or meatier, less fatty livestock. These concepts are set forth in more detail in U.S. Patent Application No. 10/851,574, filed 5/20/2004, incorporated herein by reference in its entirety.

15 Another condition, systemic hypertension, is a disease that, if untreated, strongly predisposes one to atherosclerotic cardiovascular disease. It is estimated that as many as 1 in 4 adult Americans have hypertension. Hypertension is usually diagnosed when the average diastolic blood pressure is 90 mm Hg or greater, or systolic blood pressure is 140 mm Hg or greater. Hypertension is approximately twice as common in
20 persons with diabetes as in those without. Special care is warranted in diagnosing hypertension in persons with diabetes because of greater variability of blood pressure and a much greater likelihood of isolated systolic hypertension. The prevalence of hypertension increases with age. AFP-6, AFP-6 agonists and AFP-6 agonist analogs may be administered to reduce arterial blood pressure and, thereby, also reduce edema and
25 inflammatory exudates volume. Hypertension in the pulmonary circulation is less common, but is a serious condition, resulting in significant mortality. AFP-6, AFP-6 agonists and AFP-6 agonist analogs may be useful in the treatment of pulmonary hypertension.

Local hypoxia in the pulmonary vascular physiologically elicits a
30 vasoconstrictive response, to shunt blood away and towards better-ventilated lung segments. However, this mechanism can be overwhelmed in chronic obstructive pulmonary disease, with the resulting pulmonary hypertension and sub-optimal matching of ventilation and perfusion (so called ventilation/perfusion mismatch), sub-optimal

oxygen loading, and chronic dyspnoea and exercise intolerance. In general, it is important to match gas (ventilation) to blood flow (perfusion) in local lung regions to obtain optimal gas exchange. Blood flow to unventilated regions of the lung is wasted effort, as is ventilation of unperfused regions since no gas exchange between air and blood can occur in such regions. It has been reported that mismatching of ventilation and perfusion can reduce the effectiveness of the lungs as gas exchange organs to as little as one-tenth normal.

Ventilation/perfusion mismatching is a prevalent cause of pulmonary disability, and may also be present in such conditions as asthma, chronic obstructive pulmonary disease, interstitial lung disease, alveolar disease and pulmonary vascular disease. It is theorized that the combination of (1) bronchodilation, leading to increased regional lung ventilation, (2) vasodilation, leading to increased local lung perfusion, and/or (3) activation of carbonic anhydrase, leading to more rapid outgassing of CO₂ (carried in the blood as bicarbonate) would be beneficial in pulmonary disorders where ventilation-perfusion mismatching is a feature.

It is therefore, contemplated that AFP-6, AFP-6 agonists and AFP-6 analogs may be useful in treating conditions such as acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, interstitial lung disease, alveolar disease and pulmonary vascular disease and conditions associated with ventilation/perfusion mismatch. It is further contemplated that these compounds may be delivered by inhalation. The peptides may follow the air-flow to the alveoli. Such delivery of the peptides may include delivery as low or ultra-low density particles, such as "whiffle balls," for example US2004-0170568 and US 6,630,169 (incorporated herein by reference in their entirety) or TECHNOSPHERES™ (Pharmaceutical Discovery Corporation, Elmsford, NY). These peptides may increase perfusion (for example, via pulmonary vasodilation) to comparatively better-ventilated parts of the lung, and may increase ventilation via inhibition of bronchospasm. It is further contemplated that ADM or CGRP, agonists and analogs thereof, may also be used either alone or in combination with AFP-6, AFP-6 agonists or AFP-6 analogs to treat conditions such as acute respiratory distress syndrome, asthma, chronic obstructive pulmonary disease, interstitial lung disease, alveolar disease, pulmonary vascular disease or conditions associated with ventilation/perfusion mismatch. In such situations, the teachings in the Pharmaceutical

Compositions section of the instant application also apply to ADM and CGRP, their agonists and analogs.

AFP-6, AFP-6 agonists and AFP-6 analogs may stimulate Na/K-ATPase, restoring the ionic milieu in excitable tissues (muscle, including myocardium, and nerves) following ischemic insult and depolarization, and reducing the frequency and severity of abnormal electrical events, such as dysrhythmias.

AFP-6, AFP-6 agonists and AFP-6 analogs may be used as cardioprotective agents, for example to ameliorate ischemic injury, reduce myocardial infarct size consequent to myocardial ischemia or to otherwise limit the extent of myocardial injury. Interest in therapies capable of providing additional myocardial protection which could be administered in conjunction with thrombolytic therapy, or alone, since retrospective epidemiological studies have shown that mortality during the first few years following infarction appears to be related to original infarct size.

Myocardial ischemia is the result of myocardial oxygen supply and demand and includes exertional and vasospastic myocardial dysfunction. Exertional ischemia is generally ascribed to the presence of critical atherosclerotic stenosis involving large coronary arteries resulting in a reduction in subendocardial flow. Vasospastic ischemia is associated with a spasm of focal variety, whose onset is not associated with exertion or stress. The spasm is better defined as an abrupt increase in vascular tone.

Congestive heart failure describes a pump function that is insufficient to deliver enough oxygen to peripheral tissues. This can sometimes be a result of decreased oxygen carrying capacity in the blood (*e.g.*, in severe anemia). It is most commonly a result in reduced cardiac performance (*e.g.*, after myocardial damage) or as a result of increased myocardial work demand (*e.g.*, in the face of increased peripheral resistance with systemic hypertension). Toxic hypervolemia can exacerbate failure by increased filling pressures resulting in cardiac dilatation and suboptimal contractility. AFP-6, AFP-6 agonists and AFP-6 analogs may be useful in the treatment of congestive heart failure through a variety of mechanisms that include reduction of cardiac afterload (vasodilation, shown herein), an increase in cardiac contractility (exemplified by an increase in dp/dt , shown herein), an amelioration of toxic hypervolemia, an amelioration of ionic disequilibrium.

AFP-6, AFP-6 agonists and AFP-6 analogs may find use in the reduction of edema, for example in rheumatoid arthritis, edema secondary to brain tumors or

irradiation for cancer, edema resulting from stroke, head trauma or spinal cord injury, post-surgical edema, asthma and other respiratory diseases and cystoid macular edema of the eye.

AFP-6, AFP-6 agonists and AFP-6 analogs may further be useful in the
5 treatment of renal failure, nephropathy and may be useful as a trophic factor in kidney.

AFP-6, AFP-6 agonists and AFP-6 analogs may be useful in the treatment of pain, and may decrease the need for opiates. It may especially be useful in pain that is associated with vasomotor disturbance, including migraine headache, and in the treatment of inflammation. AFP-6, AFP-6 agonists and AFP-6 analogs may be useful in the
10 treatment of certain gastrointestinal disorders, especially those that can be ameliorated by inhibition of secretions. Such disorders include gastritis, esophagitis (including Barrett's with combined bile and acid reflux) and pancreatitis. It may additionally be useful in the treatment of pain associated with those disorders.

AFP-6, AFP-6 agonists and AFP-6 analogs may be useful in the treatment of
15 demineralization disorders of bone, including osteoporosis, diabetic and other osteopenia. It may additionally promote osteogenesis and be useful in accelerating fracture repair.

AFP-6, AFP-agonist and AFP-6 analog administration may also result in the release of prolactin and regulation of growth hormone release. Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary
20 gland, the lactotrophs. Prolactin serves multiple roles in reproduction, but it also plays multiple homeostatic roles in the organism. Synthesis and secretion of prolactin is not restricted to the anterior pituitary gland, other organs and tissues in the body have this capability. The varied effects of prolactin on the mammary gland include growth and development of the mammary gland, synthesis of milk, and maintenance of milk
25 secretion. Lactogenesis clearly requires pituitary prolactin, since hypophysectomy during pregnancy prevents subsequent lactation.

Actions of prolactin on luteal function depend on species and the stage of the estrous cycle. Prolactin acts as a luteotrophic hormone by maintaining the structural and functional integrity of the corpus luteum. This "luteotrophic" action of prolactin is
30 characterized by enhanced progesterone secretion. Prolactin enhances progesterone secretion in two ways: prolactin potentiates the steroidogenic effects of luteinizing hormone (LH) in granulosa-luteal cells and inhibits the 20 α hydroxysteroid dehydrogenase enzyme, which inactivates progesterone. In humans, high levels of

prolactin inhibit granulosa cell luteinization and steroidogenesis. Prolactin is essential for progesterone biosynthesis and luteal cell hypertrophy during pregnancy. In addition to luteal function, the prolactin-R mediates numerous functions in granulosa cells and oocytes as well.

5 Aside from its action on reproductive processes, prolactin plays a role in maintaining the constancy of the internal environment by regulation of the immune system, osmotic balance, and angiogenesis. Prolactin is a common mediator of the immunoneuroendocrine network, where nervous, endocrine, and immune systems communicate with each other. Its main feature is cooperation with cytokines and
10 hemopoietins, and it has been implicated as a “stress hormone,” functioning to restore hemopoietic homeostasis under conditions of dysregulation. Prolactin plays a significant role in regulation of the humoral and cellular immune responses in physiological as well as pathological states, such as autoimmune diseases. Immune responses in vivo are enhanced by prolactin, including T cell proliferation and mitogenesis; maturation of
15 dendritic cells, etc. Circulating prolactin is elevated in a number of autoimmune diseases, and about 20% of systemic lupus erythematosus (SLE) patients are hyperprolactinemic. Prolactin also regulates solute and water transport across mammalian cell membranes. For example, prolactin exerts a host of activities on transport of solute across mammary epithelial cell membranes. Prolactin decreases the transport of sodium and increases the
20 transport of potassium across mammary epithelial cells. Prolactin also affects chloride, and calcium transport across intestinal epithelial membranes. Prolactin has angiogenic activities; however, angiogenesis is inhibited by proteolytic fragments of native prolactin. Understanding the physiological role of prolactin, as described above, and methods of regulating it, such as by AFP-6, AFP-6 agonists and AFP-6 analogs, can lead to new
25 therapies in regulating imbalances as contemplated herein.

AFP-6, AFP-6 agonists and AFP-6 analogs may secondarily mediate other hormonal responses, including the suppression of ghrelin secretion. It may be useful in the treatment of ghrelin-related disorders, and disorders of the ghrelin-growth hormone-IGF axis, including the treatment of retinopathy.

30 Moreover, while the mechanism of islet neogenesis remains poorly understood (for review see Bouwen (2004) *Cell Biochem Biophys.* 40(3 Suppl):81-88), it has been hypothesized that de-differentiated or metaplastic exocrine cells (acinar and ductal), acquire a multipotential state and can serve as islet precursors. Since AFP-6 mRNA is in

acinar cells and SAGE analysis found the RNA in pancreas epithelium ductal adenocarcinoma, it is possible that AFP-6, AFP-6 agonists, and AFP-6 analogs could be useful for islet neogenesis.

Further, given the expression of AFP-6 mRNA in the secretory epithelium,
5 AFP-6 may be involved in normal prostate function and, thus, it, its agonists and its analogs could be useful in treating various prostate disorders.

AFP-6, AFP-6 agonists and AFP-6 analogs may further be used for screening other compounds having a property of the AFP-6 analogs described herein. Exemplary screening methods are described in PCT application WO 2004/048547, the contents of
10 which are incorporated by reference in its entirety. The present invention provides for antibodies specific for an AFP-6, AFP-6 agonist or AFP-6 analog. Moreover, AFP-6, AFP-6 agonists and AFP-6 analogs and/or their antibodies can also be used in diagnostic applications for determining, or propensity for developing, conditions or disorders as described herein. It is also contemplated that the antibodies may be used as antagonists.

15 Making AFP-6 analogs

The AFP-6 analogs described herein may be prepared using standard recombinant techniques or chemical peptide synthesis techniques known in the art, *e.g.*, using an automated or semi-automated peptide synthesizer, or both. Likewise, the derivatives of the polypeptides of the invention may be produced using standard chemical,
20 biochemical, or *in vivo* methodologies.

The AFP-6 analogs of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, *e.g.*, Stewart and Young, *Solid Phase Peptide Synthesis*, 2d. ed., Pierce Chemical Co.
25 (1984); Tarn et al, *J. Am. Chem. Soc.* 105: 6442 (1983); Merrifield, *Science* 232: 341-7 (1986); and Barany and Merrifield, *The Peptides*, Gross and Meienhofer, eds., Academic Press, New York, 1-284(1979).

The AFP-6 analogs of the present invention may alternatively be produced by recombinant techniques well known in the art. See, *e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor (1989). These polypeptides
30 produced by recombinant technologies may be expressed from a polynucleotide, *e.g.*, a DNA or RNA molecule. These polynucleotide sequences may incorporate codons

facilitating transcription and translation of mRNA in host cells. Such manufacturing sequences may readily be constructed according to the methods well known in the art. See, *e.g.*, WO 83/04053. A variety of expression vector/host systems may be utilized to contain and express an AFP-6 analog coding sequence.

5 As such, the amino acid sequences of the AFP-6 analogs determine the polynucleotide sequences that are useful in generating new and useful viral and plasmid DNA vectors, new and useful transformed and transfected prokaryotic and eukaryotic host cells (including bacterial, yeast, algae, plant, insect, avian, and mammalian cells grown in culture), and new and useful methods for cultured growth of such host cells capable of
10 expression of the present polypeptides. The polynucleotide sequences encoding the AFP-6 analogs, either agonist or antagonist, may also be useful for gene therapy.

 DNA sequences encoding the AFP-6 analog may be created using well known molecular biology (or recombinant) techniques such as amplification by PCR or site directed mutagenesis and cloning into an appropriate vector, for example, pGEX-3X
15 (Pharmacia, Piscataway, New Jersey).

 The present invention also provides for processes for the production of the present AFP-6 analogs. Provided is a process for producing the polypeptides from a host cell containing nucleic acids encoding such AFP-6 analogs comprising: (a) culturing said host cell containing polynucleotides encoding AFP-6 analogs under conditions facilitating
20 the expression of such DNA molecules; and (b) obtaining such AFP-6 analogs. Host cells may be prokaryotic or eukaryotic, such as bacterial, yeast, algae, plant, insect, avian, and mammalian cells. Mammalian host cells include, for example, human cells cultured *in vitro*. Also contemplated are processes of producing polypeptides using a cell free system. An example of a cell free protein expression system is the Rapid Translation
25 System (RTS) by Roche Diagnostics Corp.

 A variety of expression vector/host systems may be utilized to contain and express an AFP-6 analog coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect
30 cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transfected with virus expression vectors (*e.g.*, cauliflower mosaic virus, tobacco mosaic virus) or transformed with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmid); or mammalian cell systems. Mammalian cells that are useful in recombinant protein

productions include, but are not limited to, VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), WI 38, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and 293 cells. Exemplary protocols for the recombinant expression of the protein in any of these expression/host systems, as well as other
5 expression/host systems, are well known in the art.

It may be desirable to purify the AFP-6 analogs generated by the present invention. Peptide purification techniques are well known to those of skill in the art. These techniques may involve the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. The polypeptides of interest may be further purified using
10 chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography, polyacrylamide gel electrophoresis, and isoelectric focusing. A particularly efficient and preferred method of purifying peptides is reverse phase HPLC,
15 followed by characterization of purified product by liquid chromatography/mass spectrometry (LC/MS) and Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry. Additional confirmation of purity is obtained by determining amino acid analysis.

The term "purified peptide" as used herein, is intended to refer to a
20 composition, isolatable from other components, wherein the peptide is purified to any degree relative to its naturally obtainable state. A purified peptide therefore refers to a peptide, free from the environment in which it may naturally occur. The term "substantially purified" is used to refer to a composition in which the peptide forms the major component of the composition, such as constituting at least about 50%, about 60%,
25 about 70%, about 80%, about 90%, about 95% or more of the peptides in the composition. Methods for purifying a polypeptide can be found, for example, in U.S. Patent No. 5,849,883, incorporated by reference in its entirety.

Various techniques suitable for use in peptide purification are well known in the art. These include, for example, precipitation with ammonium sulfate, PEG,
30 antibodies, and the like; heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of

conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the peptides always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed, utilizing an HPLC apparatus, will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein. Also it is contemplated that a combination of anion exchange and immunoaffinity chromatography may be employed to produce purified peptide compositions of the present invention.

Pharmaceutical Compositions

The present invention also relates to pharmaceutical compositions comprising a therapeutically or prophylactically effective amount of at least AFP-6, AFP-6 agonist or AFP-6 analog of the invention, or a pharmaceutically acceptable salt thereof, together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in the delivery of AFP-6, AFP-6 agonist or AFP-6 analog. Such compositions may include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength; additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimersol, benzyl alcohol), and bulking substances (e.g., lactose, mannitol); incorporation of the material into particulate preparations of polymeric compounds, such as polylactic acid, polyglycolic acid, etc., or in association with liposomes. Such compositions will influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present AFP-6 analog. See, e.g., Remington's Pharmaceutical Sciences 1435-712, 18th ed., Mack Publishing Co., Easton, Pennsylvania (1990). Exemplary methods for formulating pharmaceutical compositions can be found in WO 2004/048547, the entire contents of which are incorporated by reference.

As used herein, the phrase "pharmaceutically acceptable" refers to an agent that does not interfere with the effectiveness of the biological activity of an active ingredient, and which may be approved by a regulatory agency of the Federal government or a state government, or is listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly for use in humans. Accordingly, 5 suitable pharmaceutically acceptable carriers include agents that do not interfere with the effectiveness of a pharmaceutical composition or produce an adverse, allergic or other untoward reaction when administered to an animal or a human.

As used herein, the phrase "pharmaceutically acceptable salts" refers to salts 10 prepared from pharmaceutically acceptable, preferably nontoxic, acids and bases, including inorganic and organic acids and bases, including but not limited to, sulfuric, citric, maleic, acetic, oxalic, hydrochloride, hydro bromide, hydro iodide, nitrate, sulfate, bisulfite, phosphate, acid phosphate, isonicotinate, acetate, lactate, salicylate, citrate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, 15 gentisinate, fumarate, gluconate, glucaronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate and pamoate (i.e., 1,1'-methylene-bis-(2-hydroxy-3-naphthoate)) salts. Pharmaceutically acceptable salts include those formed with free amino groups such as, but not limited to, those derived from hydrochloric, phosphoric, acetic, oxalic, and tartaric acids. 20 Pharmaceutically acceptable salts also include those formed with free carboxyl groups such as, but not limited to, those derived from sodium, potassium, ammonium, sodium lithium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, and procaine.

The present AFP-6, AFP-6 agonist or AFP-6 analog may be formulated for 25 peripheral administration, including formulation for injection, oral administration, nasal administration, pulmonary administration, topical administration, or other types of administration as one skilled in the art will recognize. More particularly, administration of the pharmaceutical compositions according to the present invention may be via any common route so long as the target tissue is available via that route. In a preferred 30 embodiment, the pharmaceutical compositions may be introduced into the subject by any conventional peripheral method, e.g., by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, retrobulbar, intrapulmonary (e.g., term release); by oral, sublingual, nasal, anal, vaginal, or transdermal delivery, or by surgical

implantation at a particular site. Examples include, intravenous or subcutaneous injection; nasal, oral or muscosal administration; and pulmonary inhalation by nose or mouth. The treatment may consist of a single dose or a plurality of doses over a period of time.

Controlled continual release of the compositions of the present invention is also

5 contemplated.

The pharmaceutical compositions suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form should be sterile and should be fluid to the extent that is easily syringable. It is also desirable for the

10 polypeptide of the invention to be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, sorbitol, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), dimethylacetamide, cremophor EL, suitable mixtures

15 thereof, and oils (*e.g.*, soybean, sesame, castor, cottonseed, ethyl oleate, isopropyl myristate, glycofurol, corn). The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, meta-cresol, benzyl alcohol, parabens (methyl, propyl, butyl), chlorobutanol,

20 phenol, phenylmercuric salts (acetate, borate, nitrate), sorbic acid, thimerosal, and the like. In many cases, tonicity agents (for example, sugars, sodium chloride) will be included in the compositions. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption (for example,

25 aluminum monostearate and gelatin).

In one embodiment, the pharmaceutical compositions of the present invention are formulated so as to be suitable for parenteral administration, *e.g.*, via injection or infusion. In one embodiment, the AFP-6 analog is suspended in an aqueous carrier, for example, in an isotonic buffer solution at a pH of about 3.0 to about 8.0, in another

30 embodiment at a pH of about 3.5 to about 7.4, 3.5 to 6.0, or 3.5 to about 5.0. Useful buffers include sodium citrate-citric acid and sodium phosphate-phosphoric acid, and sodium acetate/acetic acid buffers. A form of repository or "depot" slow release preparation may be used so that therapeutically effective amounts of the preparation are

delivered into the bloodstream over many hours or days following transdermal injection or delivery.

Generally, a therapeutically or prophylactically effective amount of the present AFP-6, AFP-6 agonist or AFP-6 analog will be determined by the age, weight, and condition or severity of the diseases or metabolic conditions or disorders of the recipient. See, e.g., Remington's Pharmaceutical Sciences 697-773. See also Wang and Hanson, Parenteral Formulations of Proteins and Peptides: Stability and Stabilizers, Journal of Parenteral Science and Technology, Technical Report No. 10, Supp. 42:2S (1988). Typically, a dosage of between about 0.001 $\mu\text{g/kg}$ body weight to about 1000 $\mu\text{g/kg}$ body weight, may be used, but more or less, as a skilled practitioner will recognize, may be used. Dosing may be one or more times daily, or less frequently, and may be in conjunction with other compositions as described herein. It should be noted that the present invention is not limited to the dosages recited herein.

Appropriate dosages may be ascertained through the use of established assays for determining level of metabolic conditions or disorders in conjunction with relevant dose-response data. The final dosage regimen will be determined by the attending physician, considering factors that modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions.

An effective dose will typically be in the range of about 1 to 30 μg to about 5 mg/day, alternatively about 10 to 30 μg to about 2 mg/day and in additional embodiments from about 5 to 100 μg to about 1 mg/day, or about 5 μg to about 500 $\mu\text{g/day}$, for a 50 kg patient, administered in a single or divided doses or controlled continued release. Exemplary dosages are between about 0.01 to about 100 $\mu\text{g/kg/dose}$. Administration should begin whenever the suppression of nutrient availability, food intake, weight, blood glucose or plasma lipid lowering or blood pressure lowering or increasing is desired, for example, at the first sign of symptoms or shortly after diagnosis of obesity, diabetes mellitus, insulin-resistance syndrome, hypertension or hypotension. Administration may be by any route, e.g., injection (including subcutaneous or intramuscular), oral, nasal,

transdermal, etc. Dosages for certain routes, for example oral administration, may be increased to account for decreased bioavailability, for example, by about 5-100 fold.

In one embodiment where the pharmaceutical formulation is to be administered parenterally, the composition may be formulated so as to deliver a dose of AFP-6, AFP-6 agonist, or AFP-6 analog ranging from 0.01 µg/kg to 100 mg/kg body weight/day, or at a range of about 0.01 µg/kg to about 500 µg/kg per dose, about 0.05 µg/kg to about 250 µg/kg or below about 50 µg/kg. Another exemplary dose range is from 0.1 mg/kg to about 50 mg/kg body weight/day. Dosages in these ranges will vary with the potency of each analog or derivative, of course, and may be determined by one of skill in the art. Exemplary body weights contemplated in for the dosing regimen can be about 40, 50, 60, 70, 80, 90, or 100 kg or more. Parenteral administration may be carried out with an initial bolus followed by continuous infusion to maintain therapeutic circulating levels of drug product. Those of ordinary skill in the art can readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient.

In the methods of the present invention, the polypeptides may be administered separately or together with one or more other compounds and compositions that exhibit a long term or short-term action or complementary action, i.e., combination therapy. For example, an additional compound may be added to AFP-6, AFP-6 agonist or AFP-6 analog that also reduces nutrient availability, such compounds include, but are not limited to an amylin or amylin analog agonist, salmon calcitonin, a cholecystokinin (CCK) or CCK agonist, a leptin (OB protein) or leptin agonist, an exendin or exendin agonist or analog agonist, or a GLP-1 or GLP-1 agonist or analog agonist or a PYY or PYY agonist or analog, or a PYY related polypeptide. Suitable amylin agonists include, for example, [^{25,28,29}Pro]-human amylin (also known as "pramlintide," and described in U.S. Pat. Nos. 5,686,511 and 5,998,367). The CCK used is, for example, CCK octopeptide (CCK-8). Leptin is discussed, for example, in Pelleymounter et al. (1995) *Science* 269:540-543, Halaas et al. (1995) *Science* 269:543-546, and Campfield et al. (1995) *Science* 269:546-549. Suitable exendins include exendin-3 and exendin-4, and exendin agonist compounds including, for example, those described in PCT Publications WO 99/07404, WO 99/25727, and WO 99/25728. Suitable PYY polypeptides and analogs include those described in U.S. Application Nos. 60/543,406 and 60/543,407, PCT publications WO 03/026591 and WO 03/057235.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in fields of human medicine and veterinary medicine. Thus, the subject to be treated may be a mammal, for example a human or other animals. For veterinary purposes, subjects include for example, farm animals
5 including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, laboratory animals including mice, rats, rabbits, guinea pigs and hamsters; and poultry such as chickens, turkeys, ducks and geese.

In addition, the present invention contemplates a kit comprising AFP-6, AFP-6 agonist or AFP-6 analog, components suitable for preparing said compounds for
10 pharmaceutical application, instructions for using said compounds and components for pharmaceutical application.

To assist in understanding the present invention, the following Examples are included. The experiments relating to this invention should not be construed as specifically limiting the invention and such variations of the invention, now known or
15 later developed, which would be within the purview of one skilled in the art are considered to fall within the scope of the invention as described herein and hereinafter claimed.

EXAMPLES

The present invention is described in more detail with reference to the
20 following non-limiting examples, which are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof. The examples illustrate the preparation of the AFP-6 analogs (which includes derivatives) and the testing of these AFP-6 analogs *in vitro* and/or *in vivo*. Those of skill in the art will understand that the techniques described in these examples are only representative techniques described by
25 the inventors that function in the practice of the invention. It should be appreciated that those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific methods that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1. AFP-6 Nucleic Acid Molecules and Polypeptides

30 1A. Identification of AFP-6 in Mouse and Human

A BlastP was performed with human adrenomedullin (SwissProt Accession Number P35318), restricting the search to mouse proteins and all other parameters set to default, to find the mouse homolog to human adrenomedullin. The top two sequences returned by the BlastP search were mouse adrenomedullin from Genbank and from
5 SwissProt, the fourth sequence was CGRP2 (CGRP1 was #8 on the hits list). Unexpectedly, the third sequence on the list was hypothetical protein XP_147916. The match to the query sequence adrenomedullin (SEQ ID NO:43) spanned almost exactly the region of the adrenomedullin mature peptide (SEQ ID NO:44) and is shown in Figure 1A.

The homology in the matched region is 44% with 32% identity as shown in
10 Figure 1B. Alignment of the mouse sequence (SEQ ID NO:45) with human adrenomedullin precursor (SEQ ID NO:46) shows that the homology across the entire protein is 25% (15% identity; all alignments performed with the AlignX® program (VectorNTI® Advance suite, purchased from Invitrogen Inc., Carlsbad, CA).

Two signal sequence detection programs, SignalP and PSort, were used to
15 determine if this putative protein contained a cleavable signal. Both programs predicted a signal peptide cleaving between residues 25 and 26. The EST used to support the Refseq entity XP_147916 was BG918210.1 and had been cloned from mouse virgin mammary gland, infiltrating ductal carcinoma. The sequence of the entire protein XP_147916 (hereinafter referred to as mouse Amylin Family Polypeptide-6 or mAFP-6 precursor) in
20 FASTA format is shown in Figure 2 and is designated SEQ ID NO:45.

NCBI Blink is an automatically generated Blast available for most Genbank entries that indicates likely relationships to other proteins. The mAFP-6 precursor showed no matches at all. A BlastP search (limited to metazoa), however, performed with mAFP-6 precursor showed homology to mAFP-6 precursor itself, and, with much lower scores,
25 to adrenomedullin from a variety of species (best expect score for this group was $e = 0.59$). This BlastP, therefore, did not show any hits to a human ortholog although the apparent paralog, adrenomedullin itself, was detected in a variety of species.

To investigate if the XM147916 transcript might encode another ORF with a closer homology to another protein, a BlastX was performed. BlastX translates a query
30 sequence in 6 frames and compares it to a protein database. A match was detected to a human hypothetical protein FLJ21135, but not in the frame of the mouse transcript encoding the Amylin Family Polypeptide-6 sequence.

However, in silico translation of the transcripts AK024788 and NM_024866 encoding the hypothetical protein FLJ21135 showed that they also contain an upstream ORF that does match XP_147916. A longer transcript, AK090635, also contains an upstream ORF that matches XP_147916. The sequence surrounding the start codon of the upstream ORF is a better match to the Kozak consensus than is the sequence around the annotated start Met, compare: CCG CCC GCC ATG G (SEQ ID NO: 47) to CTC TCC GGG ATG G (SEQ ID NO: 48) (Classic Kozak is GCC GCC PuCC ATG G (SEQ ID NO: 49) (Kozak (1987) *J. Mol. Biol.* 196:947 and Kozak (1987) *Nucl. Acids Res.* 15:8125). This adrenomedullin-like ORF of AK024788 shows 67% similarity and 62% identity overall to hAFP-6 precursor. Hereinafter, the human predicted protein will be referred to as hAFP-6 precursor. The in silico translations of transcripts AK024788 and NM_024866 are designated herein hAFP-6 short precursor (SEQ ID NO:51) and the in silico translation of AK090635, which contains an insertion is designated hAFP-6 long (SEQ ID NO:50). An alignment of mouse and human AFP-6 precursor is shown in Figure 3.

Alignment of human adrenomedullin precursor with the hAFP-6, NM_024866 ORF, shows that they have 26% similarity and 16% identity overall. However, they have greater homology over the region of the AFP-6 predicted peptide: 39% similarity and 32% identity. Alignment between human adrenomedullin precursor (SEQ ID NO:46) and hAFP-6 short form (NM24866/FLJ21135) (SEQ ID NO:51) is shown in Figure 4.

BLAT (BLAT - The BLAST-Like Alignment Tool. Kent (2002) *Genome Res.* 12:656-664) analysis of these sequences against the human genome as assembled by the International Human Genome Sequencing Consortium (<http://genome.ucsc.edu/>), the UCSC genome bioinformatics website, indicates that hAFP-6 precursor is located on human chromosome 22, at 22q13. A Unigene entry exists matching hAFP-6 precursor and interestingly the SAGE data from the Unigene Contig Hs. 196985 shows the highest virtual score is for SAGE Pane 91 16113 pancreas epithelium ductal adenocarcinoma non-normalized bulk EST.

Adrenomedullin is part of the Amylin/Calcitonin/CGRP peptide family. Comparison to other members of this extended peptide family is shown in Figure 5. Human AFP-6 has 27% identity to human amylin peptide, 25% identity to human CGRP peptide and 24% identity to human calcitonin peptide.

AFP-6 precursor is likely to encode a bona fide peptide hormone, with homologs at least in mouse and man. Based on the expected use of dibasic and monobasic sites for cleavage of mature peptides, and with guidance from the homology with adrenomedullin, the likely mature bioactive peptides are shown in Figure 6.

5 IB. Cloning of mouse and human AFP-6 cDNAs

A 646 bp portion of the mouse AFP-6 cDNA containing the relevant open reading frame, was cloned by designing oligonucleotides to the database sequence XM147916 described above. These oligonucleotides (forward oligo 5'- AGC TTT GCC AGC TGT CTC CAG AT -3' (SEQ ID NO:52) and reverse oligo 5'- GGT ATC CAA
10 AGC CAC GAG GAA TG -3' (SEQ ID NO:53)) were used to amplify this sequence from both mouse lung cDNA and 7-day embryo cDNA reverse transcribed at 42°C using oligo dT as the primer from RNA purchased from BD Biosciences Clontech. The sequence was confirmed using ABI Big Dye Terminator cycle sequencing run on the ABI PRISM 310 Genetic Analyzer, demonstrating the existence of this transcript in the mouse
15 embryo and lung. This sequence was then used as a probe to determine which other mouse tissues contain this transcript via northern analysis (described below).

An 886 bp portion of the human AFP-6 cDNA, containing only the 3' end of the relevant open reading frame, was cloned by designing oligos (forward oligo 5'- CCG ACC TGT GGT CTG GAA GCT T -3' (SEQ ID NO:54) and reverse oligo 5'- ATC CAG
20 GTG GAG TCT CCA TGG C -3' (SEQ ID NO:55)) to the database sequence NM024866 described above. This sequence was amplified from human pancreas cDNA made from RNA obtained from BD Biosciences Clontech, which was reverse transcribed at 65°C using Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo dT as the primer. The hAFP-6 sequence was confirmed using ABI Big Dye Terminator cycle
25 sequencing run on the ABI PRISM 310 Genetic Analyzer. Although clones obtained from this experiment did not contain the entire reading frame of interest, this sequence was used as a probe for northern blots to determine which other human tissues contain this transcript. It was also discovered that all four clones obtained from pancreas cDNA (which came from 15 male/female donors) corresponded to sequence that translates to the
30 AFP-6 long form of the protein (shown in the alignment in Figure 3).

An approximately 1.6kb portion of the human AFP-6 cDNA containing the entire open reading frame of interest plus a large amount of 3' untranslated sequence was

cloned from human prostate cDNA made with a high temperature reverse transcription enzyme (Thermoscript from Invitrogen). This was necessary due to the CpG island at the 5' end of this gene. The amplification was performed using the Advantage-GC kit (Clontech) to enhance recovery of GC rich sequences that tend to be difficult to amplify due to secondary structure. In the 5' untranslated regions (UTR) of three clones recovered from this experiment, there is a 72 bp sequence deletion relative to NM_024866 (between nucleotides 209 and 281 in Figure 7) that may represent an alternatively spliced intron, or an intron inappropriately retained in the NM_024866 clone. A potential 5' donor site, GT, and 3' acceptor site, AG, are shown in gray in Figure 7.

10 1C. Expression Analysis

Northern analysis from mouse and human: The 646 bp mouse insert and the 886 partial human cDNA clone insert were isolated, labeled with 32PdCTP and used to probe northern blots to determine which tissues express AFP-6 mRNA. Data is summarized in Table 4.

15 Table 4

Tissue/Transcript	Mouse 1.6 kb	Mouse 1.2 kb	Human 4.7 kb	Human 1.8 kb
Adrenal Cortex	n.d.	n.d.	-	-
Adrenal Medulla	n.d.	n.d.	-	-
Brain	-	-	+	-
Cervix	n.d.	n.d.	-	-
Colon	n.d.	n.d.	++	-
Heart	-	-	n.d.	n.d.
Ileum	n.d.	n.d.	+++	-
Jejunum	n.d.	n.d.	+++	+
Kidney	+++	+++	++	+
Large Intestine	-	-	n.d.	n.d.
Liver	-	+	n.d.	n.d.
Lung**	-	-	++	-
Pancreas	n.d.	n.d.	++++	-
Placenta	n.d.	n.d.	+	-
Prostate	-	+++	++++	-
Rectum	n.d.	n.d.	++	-
Salivary Gland	-	++++	++++	-
Skeletal Muscle	-	-	n.d.	n.d.
Small Intestine*	n.d.	n.d.	-/+	-/+
Spleen	-	-	-	-
Stomach	-	++	++++/++	+/-
Testis	-	-	-	-
Thyroid	-	++	++++	+
Thymus	-	-	-	-
Uterus	-	-	-	-

7-day embryo	++++	-	n.d.	n.d.
11-day embryo	-	-	n.d.	n.d.
15-day embryo	-	-	n.d.	n.d.
17-day embryo	-	+	n.d.	n.d.

n.d.= not determined

+ = very weak expression (after more than 3 week exposure)

++ = weak expression (best seen after 3-5 day exposure)

+++ = mid level expression

5 ++++ = strong expression

10 *No signal was seen in small intestine on the Clontech blot (BD Biosciences/Clontech, San Jose, CA), while the sections of the small intestine (ileum and jejunum) came up positive on the BioChain blot (BioChain, Hayward, CA). This may be due to amounts of poly A+ RNA on the blot (3 µg for Biochain vs 2 µg for Clontech) ** Although there was no signal for AFP-6 in mouse lung, it is possible to RT-PCR the cDNA from this tissue.

15 In human tissues, the major band expressed in the prostate, salivary gland, pancreas, thyroid, stomach, jejunum, ileum, and colon is larger than expected (about 4.7 kb rather than 2.6 kb for AK090635, which is the largest cDNA in the public database).

20 The smaller band seen in the thyroid lane on the Clontech blot was calculated to be approximately 1.8 kb, similar in size to NM_024866, which is about 1.7kb. Thus, there appears to be two forms of the AFP-6 transcript. Tissues that express AFP-6 weakly, requiring long exposures, were brain, lung, rectum, placenta and kidney. Tissues that did not express AFP-6 from this analysis were spleen, cervix, uterus, adrenal medulla, adrenal cortex, testis, and thymus. There was no signal on the Clontech endocrine blot from small intestine, but there is a signal on the BioChain blot for both jejunum and ileum. This is most likely due to different amounts of RNA on the blots, as previously discussed.

25 In tissues obtained from mice, the embryonic northern (from Clontech) revealed a 1.64kb transcript expressed at 7 days, whereas a 1.28 kb transcript appeared at 17 days. Neither form was present at day 11 or 13. The 1.28 kb band was a little larger than expected as the Genbank sequence (XM_147916) is 765 bp. The difference in size may be due to several factors such as an expanded 5' or 3' UTR or a very long poly A tail. The hybridization conditions used reveal at least two different-sized transcripts that were identified with this probe. The larger transcript may represent a possible alternative splice.

30 Tissues with the strongest expression on the mouse adult blot were prostate and salivary gland, while from the human blots, prostate, salivary gland, pancreas, thyroid, stomach and intestine were the strongest. Weak expression on the mouse adult blot was thyroid and stomach. The pancreas was not analyzed by northern in mouse tissue; however, a dot blot in mouse tissue showed a positive signal for AFP-6, but this

does not indicate which form is present in the pancreas. Tissues that did not express AFP-6 on this adult mouse northern blot were large intestine, thymus and uterus.

A second mouse northern was hybridized with the mouse AFP-6 probe. AFP-6 appeared to be present in kidney and perhaps at a very low level in liver. It was not
5 detected in heart, brain, spleen, lung, skeletal muscle, or testis on this blot. Kidney expressed both forms of AFP-6 identified in the mouse (above). Previously, the larger form was only found in early embryogenesis (day 7 embryo). Size analysis confirms that the two bands found in the kidney corresponded to the larger embryonic form and the smaller adult form discussed above. There also appeared to be two forms of AFP-6 in the
10 human, but the size difference between the two forms is much greater than is found in the mouse.

In situ analysis of mouse tissues. The same mouse AFP-6 probe described for the Northern analysis was used for *in situ* hybridization of mouse tissue sections to determine which cells in these tissues express the AFP-6 mRNA. Tissues were fixed in
15 4% paraformaldehyde in phosphate buffered saline (PBS) overnight, dehydrated and infiltrated with paraffin. 5 to 7 micron serial sections were mounted on gelatinized slides. One to three sections were mounted per slide, deparaffinized in xylene, re-hydrated, and post-fixed. The sections were digested with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed and dehydrated. The cRNA transcripts were
20 synthesized according to manufacturer's conditions (Maxiscript Kit (T7) Ambion Inc., Austin, TX) and labelled with ³⁵S-UTP (>1000 Ci/mmol; Amersham Biosciences, Piscataway, NJ). cRNA transcripts larger than 200 nucleotides were subjected to alkali hydrolysis to give a mean size of 70 bases for efficient hybridization. Sections were hybridized overnight at 52°C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl
25 pH 7.4, 5 mM EDTA, 10 mM NaPO₄, 10% dextran sulfate, IX Denhardt's, 50 µg/ml total yeast RNA, and 50-75,000 cpm/µl ³⁵S-labelled cRNA probe. The tissue was subjected to stringent washing at 65°C in 50% formamide, 2X SSC, 10 mM DTT and washed in PBS before treatment with 20 µg/ml RNase A at 37°C for 30 minutes. Following washes in 2X SSC and 0.1X SSC for 10 minutes at 37°C, the slides were dehydrated and dipped in
30 Kodak NTB-2 nuclear track emulsion and exposed for one week in light-tight boxes with dessicant at 4°C. Photographic development was carried out in Kodak D-19. Slides were counterstained lightly with toluidine blue and analyzed using both light- and darkfield

optics of a Zeiss Axiophot microscope. Sense control cRNA probes (identical to the mRNAs) always gave background levels of hybridization signal.

Results indicate that in the mouse embryo at stages E9.5 and E12.5, days of embryonic development, the mRNA was widespread with uniform expression in all structures. At E17.5, the mRNA was still expressed widely, but expression was slightly higher in salivary gland, kidney and intestine. In the adult, AFP-6 mRNA was detected in Paneth cells in the crypts of Lieberkuhn of the small intestine, the gastric glands of the stomach epithelium, the acinar cells of the pancreas, the adenomeres of a salivary gland, the epithelium of the prostate, and the convoluted tubules of the kidney cortex. All of these cell types are secretory in nature. Representative pictures from pancreas, prostate and salivary gland are shown in Figures 8A-8I. The specific AFP-6 mRNA signal is seen in the Darkfield anti-sense images in the middle column.

Tissues analyzed in these experiments indicate that the AFP-6 mRNA for the peptide precursor has a tissue distribution pattern consistent with a secretory or exocrine function.

ID. Synthesis of AFP-6 Polypeptides

The following polypeptides can be synthesized using standard polypeptide synthesis methods. Such methods are described below and in U.S. Patent No. 6,610,824 and U.S. Patent No. 5,686,411 and in patent application Serial No. 454,533 (filed December 6, 1999), the entireties of which are incorporated herein by reference.

Polypeptides were synthesized on a Pioneer continuous flow peptide synthesizer (Applied Biosystems) using PAL-PEG-PS resin (Applied Biosystems) with a loading of 0.2 mmol/g (0.25 mmole scale). Fmoc amino acid (4.0 eq, 1.0 mmol) residues were activated using 4.0 eq HBTU, 4.0 eq of HOBT, 8.0 eq DIEA and coupled to the resin for 1 hour. The Fmoc group was removed by treatment with 20% (v/v) piperidine in dimethylformamide. Final deprotection and cleavage of the peptide from the solid support was performed by treatment of the resin with reagent B (93% TFA, 3% phenol, 3% water and 1% triisopropylsilane) for 2-3 hours. The cleaved peptide was precipitated using tert-butyl methyl ether, pelleted by centrifugation and lyophilized. The pellet was re-dissolved in water (10-15 mL), filtered and purified via reverse phase HPLC using a C-18 column and an acetonitrile/water gradient containing 0.1% TFA. The purified product was

lyophilized and analyzed by ESI-LC/MS and analytical HPLC and were demonstrated to be pure (>98%). Mass results all agreed with calculated values.

Alternatively, peptides were assembled on a Symphony® peptide synthesizer (Protein Technologies, Inc., Woburn, MA) using Rink amide resin (Novabiochem, San Diego, CA) with a loading of 0.43-0.49 mmol/g at 0.050-0.100 mmol. Fmoc amino acid (Applied Biosystems, Inc. 5.0 eq, 0.250-.500 mmol) residues were dissolved at a concentration of 0.10 M in 1-methyl-2-pyrrolidinone. All other reagents (HBTU, HOBT and N,N-diisopropylethylamine) were prepared as 0.55 M dimethylformamide solutions. The Fmoc protected amino acids were then coupled to the resin-bound amino acid using, HBTU (2.0 eq, 0.100-0.200 mmol), HOBT (1.8eq, 0.090-0.18 mmol), N,N-diisopropylethylamine (2.4 eq, 0.120-0.240 mmol) for 2 hours. Following the last amino acid coupling, the peptide was deprotected using 20% (v/v) piperidine in dimethylformamide for 1 hour. Once peptide sequence is completed, the Symphony® peptide synthesizer is programmed to cleave the resin. Trifluoroacetic acid (TFA) cleavage of the peptide from resin was carried out using a reagent mixture composed of 93% TFA, 3% phenol, 3% water and 1% triisopropylsilane. The cleaved peptide was precipitated using tert-butyl methyl ether, pelleted by centrifugation and lyophilized. The pellet was dissolved in acetic acid, lyophilized and then dissolved in water, filtered and purified via reverse phase HPLC using a C18 column and an acetonitrile/water gradient containing 0.1% TFA. Analytical HPLC was used to assess purity of peptide and identity was confirmed by LC/MS and MALDI-MS.

Example 2: Receptor Bioactivity Analysis

2A. Adrenomedullin-like activity of AFP-6 as determined by cAMP production in human umbilical vein endothelial cells (HUVECs).

Since AFP-6 has the closest homology to adrenomedullin, AFP-6 was tested for bioactivity in HUVECs that contain the adrenomedullin receptor (Kato et.al. (1995) *Eur. J. Pharmacol.* 289:383-385) using the Perkin Elmer AlphaScreen™ assay for cyclic AMP using an optimum of 25-30,000 cells per well. The peptides tested were adrenomedullin, CGRP alpha, and AFP-6. Elevation of cAMP levels was not large for HUVEC compared to CHO cells. CHO cells were chosen as a negative control since they do not express the adrenomedullin receptor.

The results of the screen, shown in Figure 9, demonstrate that AFP-6 was active in increasing cAMP levels at concentrations of 1 μ M and 10 nM, but was less potent than adrenomedullin on HUVECs. Surprisingly, AFP-6 was more active on CHO cells, which do not contain an adrenomedullin receptor, than on HUVEC cells, which do contain the adrenomedullin receptor. The rank order of potency in HUVECs was as follows: adrenomedullin > AFP-6 » CGRP alpha. The rank order of potency in CHO cells was: AFP-6 > adrenomedullin = CGRP alpha.

Thus, AFP-6 interacts with the adrenomedullin receptor but with lower affinity than adrenomedullin itself. Since AFP-6 appears more potent than adrenomedullin at increasing cAMP levels in CHO cells, this interaction is most likely through another receptor, for example the calcitonin receptor (D'Santos *et.al.* (1992) *Mol. Pharmacol.* 41:894-899).

2B. AFP-6 peptide functionally interacts with the calcitonin receptor as determined by cAMP production in CHO cells.

To test whether AFP-6 interacts with the calcitonin receptor, a panel of related peptides was retested in CHO cells and also in T47D cells, which also express the calcitonin receptor (Muff *et.al.* (1992) *Ann. NY Acad. Sci.* 657:106-116 and Kuestner *et.al.* (1994) *Mol. Pharmacol.* 46:246-255). In the CHO cell experiments, AFP-6 was similar in potency to amylin, and more potent than adrenomedullin with a rank order of potency of: calcitonin > AFP-6 > amylin > hCGRP alpha > adrenomedullin. The EC₅₀ values for the compounds on CHO cells are shown in Table 7A below. The table summarizes EC₅₀ values calculated from dose response curves ranging from 1 pM to 1 μ M of Amylin Family peptides on cell lines containing the calcitonin receptor, CHO cells (A) and T47D cells (B), respectively. Data from three exemplary independent alpha screen assays are summarized with EC₅₀ values calculated in PRISM.

Table 7A.

CHO (30,000 cells/well) + Compound	Experiment 1 EC ₅₀ nM	Experiment 2 EC ₅₀ nM	Experiment 3 EC ₅₀ nM	Average EC ₅₀ nM	SD
rAmylin	21.6	30.9	19.6	24.03	6.03
(hCalcitonin)	0.077	0.14	0.075	0.097	0.03
(Adrenomedullin)	999	3600	622	1740.33	1621.51

AFP-6	11.3	10.6	7	9.63	2.30
(hCGRP)	70.3	81.2	89.7	80.4	9.72

Table 7B.

T47D (7500 cells/well) + Compound	Experiment 1 EC ₅₀ nM	Experiment 2 EC ₅₀ nM	Experiment 3 EC ₅₀ nM	Average EC ₅₀ nM	SD
rAmylin	0.72	5	2.3	2.67	2.16
(hCalcitonin)	0.001	0.029	0.014	0.014	0.01
(Adrenomedullin)	37.7	124	69.8	77.16	43.61
AFP-6	10.4	20.7	9.5	13.53	6.22
(hCGRP)	11.9	66.7	27.8	35.46	28.19

AFP-6 also increased cAMP production in T47D cells. The EC₅₀ values for the compounds on T47D cells are shown in Table 7B above and establish a rank order of potency similar to that seen in CHO-K1 cells: calcitonin > amylin > AFP-6 = hCGRP
 5 alpha > adrenomedullin. These data demonstrate that AFP-6 interacts with the calcitonin receptor, not as well as calcitonin itself, but with an affinity equivalent to that of amylin and much better than that of adrenomedullin.

2C: AFP-6 interacts with the amylin and CGRP receptors as determined by
 10 radioligand binding.

Evaluation of the binding of some exemplary compounds of the invention to amylin receptors was carried out as follows in nucleus accumbens membranes prepared from rat brain. ¹²⁵I-rat amylin was Bolton-Hunter labeled from Amersham Corporation (Arlington Heights, IL). Unlabeled peptides were obtained from BACHEM Inc. (Torrance,
 15 CA).

Male Sprague-Dawley® rats (200-250) grams were sacrificed by decapitation. Brains were removed and placed in cold phosphate-buffered saline (PBS). From the ventral surface, cuts were made rostral to the hypothalamus, bounded laterally by the olfactory tracts and extending at a 45° angle medially from these tracts. This basal forebrain tissue,
 20 containing the nucleus accumbens and surrounding regions, was weighed and homogenized in ice-cold 20 mM HEPES buffer (20 mM HEPES acid, pH adjusted to 7.4 with NaOH at 23°C). Membranes were washed three times in fresh buffer by

centrifugation for 15 minutes at 48,000.times.g. The final membrane pellet was resuspended in 20 mM HEPES buffer containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF).

To measure ^{125}I -amylin binding (see, Beaumont *et al.* (1995) *Can. J. Physiol. Pharmacol.* 73(7):1025-1029), membranes from 4 mg original wet weight of tissue were incubated with ^{125}I -amylin at 12-16 pM in 20 mM HEPES buffer containing 0.5 mg/ml bacitracin, 0.5 mg/ml bovine serum albumin, and 0.2 mM PMSF. Solutions were incubated for 60 minutes at 2C. Incubations were terminated by filtration through GF/B glass fiber filters (Whatman Inc., Clifton, NJ.) that had been presoaked for 4 hours in 0.3% polyethyleneimine in order to reduce nonspecific binding of radiolabeled peptides. Filters were washed immediately before filtration with 5 ml cold PBS, and immediately after filtration with 15 ml cold PBS. Filters were removed and radioactivity assessed in a gamma-counter at a counting efficiency of 77%. Competition curves were generated by measuring binding in the presence of 10^{-12} to 10^{-6} M unlabeled test compound and were analyzed by nonlinear regression using a 4-parameter logistic equation (INPLOT program; GRAPHPAD Software, San Diego, CA).

Evaluation of the binding of compounds of the invention to CGRP receptors was essentially as described for amylin except using membranes prepared from SK-N-MC cells, known to express CGRP receptors (Muff, R. *et.al.* Ann NY Acad. Sci. 1992: 657, 106-16). Binding assays were performed as described for amylin except using 13,500 cpm 1251-hCGRP /well or 21.7 pM/well (Amersham).

IC₅₀ values for the displacement of bound amylin shown in Figure 10 establish a rank order of potency of: amylin > CGRP > AFP-6 » adrenomedullin > calcitonin, showing that AFP-6 is a potent ligand at the amylin receptor, much more so than adrenomedullin, but not as potent as amylin itself.

Similarly, Figure 11 shows IC₅₀ values for the displacement of CGRP, with a rank order of: CGRP > adrenomedullin > AFP-6 > amylin » calcitonin, showing that AFP-6 is a potent ligand at the CGRP receptor, but not as potent as CGRP itself or adrenomedullin.

Relative binding potencies of the Amylin Family of polypeptides are shown in Table 4. AFP-6 is a promiscuous ligand, having high potency at multiple receptors.

Example 3. Effects on Caloric Intake

The effect of AFP-6 on food intake was investigated using an acute food intake assay. This assay measured food consumption in lean, group-housed, overnight-fasted NIH/Swiss mice. 12 mice were housed 3 to a cage. The sample compound, in this case
 5 AFP-6, was injected IP in mice and food intake was measured during the next 2-hours. Decreased, as well as increased, food intake can be measured. Testing various doses of the compound generated ED₅₀'s. The assay was performed as follows: 1. Remove food (water ad libitum) at 5:00 pm the evening before the study. 2. Weigh food pellets (3 pellets / cage). 3. Inject animals intraperitoneally with 200 ul of appropriate solution, t=0.
 10 4. Immediately add pre-weighed food to each cage (away from water bottle spout) and allow animals to feed undisturbed. 5. At 30, 60, and 120 minutes, collect and weigh food. 6. Analyze data. Data points represent mean $\pm$ sd of n=12.

In Figure 12, AFP-6, at 1 mg/kg, is shown to inhibit food intake by 91%. Rat calcitonin at 0.63 mg/kg did not inhibit food intake significantly (16%). In contrast, rat
 15 amylin, with as little as 0.1mg/kg, inhibited food intake by 30-40%. Adrenomedullin inhibited food intake up to 71%, with an ED₅₀ of 13 nmol/kg for this effect. Adrenomedullin, when administered intracerebroventricularly, does inhibit food intake in rats, but purportedly through activation of CGRP receptors (Taylor *et.al.* (1996) *Endocrinology* 137:3260-3264).

20 Thus, it appears that while AFP-6 can interact with the calcitonin receptor, it is not this pharmacological interaction which mediates the effects of AFP-6 on food intake, since calcitonin itself does not have an effect on food intake. Relative potencies of the Amylin Family of polypeptides are shown in Table 4. Whilst calcitonin is not effective at reducing food intake, AFP-6 is as effective as amylin, CGRP and adrenomedullin.

25 Following the above procedure, Figure 13 shows a dose response of AFP-6 on short-term food intake. Twelve mice were injected IP at t=0 for each dose, 0.3, 1, 3, 10, 30, 100, or 300 nmol/kg of AFP-6 (Amylin Pharmaceuticals, Inc.) (n=12/group). Food was introduced immediately after injection and amount consumed measured at t=30, 60, and 120 min. From non-linear regression: ED₅₀ = 19nmol/kg at the 60 min time point.

30 Example 4: Activity of AFP-6 on Gastric Emptying Rate

Male Harlan Sprague Dawley® rats, 237-317 g, were housed with a 12:12 hour light:dark cycle. The experiment was carried out during the light cycle. At time=0

min, test peptide (amylin or AFP-6) at 6.3, 20, 63, 200, 632, and 1122 µg/kg or vehicle was injected (subcutaneous) into conscious rats (n=6/group). At t=5 min, a solution of 1 mL sterile water containing 5 µCi D-[3-³H] glucose was gavaged by oropharyngeal tube to conscious rats. Blood samples were collected 30 min after gavage and assayed for counts per minute (CPM) in plasma. 20% benzocaine liquid topical anesthetic was used to eliminate pain during sampling. (Gedulin *et al.* (1995) *Gastroenterology* 108:A604). The appearance of counts in plasma is a reflection of the rate of gastric emptying. Significance of difference (p<0.05) was determined by ANOVA. As can be seen in Figure 14A, AFP-6 inhibits gastric emptying with an ED₅₀ of 49 µg/kg. Figure 14B shows that rat amylin also inhibits gastric emptying, decreasing the appearance of counts in plasma, with an ED₅₀ of 4 µg/kg.

Example 5: Activity of AFP-6 on total plasma calcium

Using the samples obtained from Example 4, ionized calcium was measured in plasma samples obtained from gastric emptying experiments described above, using an ion selective electrode, Ciba/Corning 634 Ca/pH analyzer (Ciba/Corning, Inc., Medfield, MA). Figures 15A and B show that, like amylin, AFP-6 also decreases plasma ionized calcium with an ED₅₀ of 25 µg/kg.

Example 6: Chemical Characterization and Activity of Some AFP-6 Analogs.

6A: Chemical Characterization of Some Exemplary AFP-6 Analogs.

The purified analogs, synthesized according to the procedures described in Example ID were analyzed by ESI-LC/MS and analytical HPLC and were demonstrated to be pure (>98%). Mass results all agreed with calculated values and are shown in Table 5.

6B: Effects of Some AFP-6 analogs on Receptor Binding Activity and Food Intake.

Some exemplary AFP-6 analogs were tested for receptor bioactivity and food intake, essentially as described in Examples 2 and 3. Results are shown in Tables 5 and 6, food intake and receptor binding, respectively.

Table 5. AFP-6 Polypeptide analogs: Food Intake data and analytical data

SEQ ID NO:	Sequence	FI Inhibitor (%)	MS [M+H] ⁺ Calcd	MS [M+H] ⁺ Found
1	TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-42.5	5100.78	5100.9
2	VGCVLGTCTQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-47	4289.83	4290.2
10	RVGCVLGTCTQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-35	4446.02	4447.4
11	GCVLGTCTQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-40	4190.1	4190.9
12	CVLGTCTQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-41	4133.65	4134.5
16	TQAQLLRVGCVLGTCQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-35	4687.28	4686.4
17	TQAQLLRVGCVLGTCQVQNLSHRLWQLDSAPVDPSSPHSY -NH ₂	-17	4402.97	4402.4
18	VGCVLGTCTQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-33	3876.34	3878.65
19	CVLGTCTQVQNLSHRLWQLRQESAPVEPSSPHSY -NH ₂	-27	3748.21	3750.0
20	TQAQLLRVGCSNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -	-23	5232.86	5234.9
21	TQAQLLRVGCNTATCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-41	5117.8	5118.25
22	RVGCGNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-37	4550.08	4550.4
27	VGMVLGTMQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-12	3934.46	3934.5
28	RVGCGNLSTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY -NH ₂	-37	4550.08	4550.4
29	VGCGNLSTCQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-13	3978.38	3979.05
30	VCNTATCQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-26.5	3836.23	3837.05
31	GCNTATCQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH ₂	-18	3794.15	3794.05
32	TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQESAPVEPSSPHSY -NH ₂	-33	5128.83	5130.25
35	VGCVLGTCTQVQNLSHRLWQLMGPAGRQDSAPVEPSSPHSY -NH ₂	-35.5	4303.86	4305.4
36	VGCVLGTCTQVQNLSHRLWQLRQDSAPVEPSSPHSY -NH ₂	-17	3890.36	3809.75
37	GCNTATCQVQNLSHRLWQLRQDSAPVEPSSPHSY -NH ₂	-12	3808.17	3809.75
39	GCGNLSTCQVQNLSHRLWQLRQDSAPVEPSSPHSY -NH ₂	-10	3893.28	3893.7
40	GCVLGTCTQVQNLSHRLWQLRQESAPVEPSSPHSY -NH ₂	-12	3805.26	3806.7

Table 6. AFP-6 Polypeptide analogs: Receptor Binding data

SEQ ID	Sequence	Amylin (nM)	CGRP (nM)	CT (nM)	ADM (nM)
1	TQAQLLRVGCVLGTCQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	13	3	32	19.9
2	VGCVLGTCQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	12	1	24	6.4
10	RVGCVLGTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	5.1	1.4	55	7.1
11	GCVLGTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	7.7	0.93	27	11
12	CVLGTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	9.8	2.2	198	18
14	VQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	>1000	>1000	>1000	59
16	TQAQLLRVGCVLGTCQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	21	1.5	22	1000
17	TQAQLLRVGCVLGTCQVQNLSHRLWQLDSAPVDPSSPHSY -NH2	209	10.5	>1000	304
18	VGCVLGTCTQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	18	3.6	24	1000
19	CVLGTCTQVQNLSHRLWQLRQESAPVDPSSPHSY -NH2	29	12.7	247	1000
20	TQAQLLRVGCSNLSTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	1.9	25	16	36
21	TQAQLLRVGCNTATCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	8.3	8.5	17	47
22	RVGCGNLSTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	2.4	35	7.3	19
25	TQAQLLRVGMVLGTMQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	139	80	>1000	120
26	GMVLGTMQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	48	26	>1000	37
27	VGMVLGTMQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	>1000	>1000	>1000	1000
28	RVGCGNLSTCTQVQNLSHRLWQLMGAPAGRQDSAPVDPSSPHSY -NH2	2.4	35	7.3	19
29	VGCGNLSTCTQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	7.5	111	9	1000
30	VCNTATCTQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	19	37	128	1000
31	GCNTATCTQVQNLSHRLWQLRQDSAPVDPSSPHSY -NH2	12	18	51	1000
32	TQAQLLRVGCVLGTCQVQNLSHRLWQLMGAPAGRQESAPVEPSSPHSY -NH2	16	2.3	1000	7.1
35	VGCVLGTCTQVQNLSHRLWQLMGAPAGRQDSAPVEPSSPHSY -NH2	17	4.7	153	37
36	VGCVLGTCTQVQNLSHRLWQLRQDSAPVEPSSPHSY -NH2	39	14	198	1000

37	GCNTATCQVQNLSHRLWQLRQDS APVEPSSPHSY -NH ₂	39	130	121	1000
38	GCSNLSTCQVQNLSHRLWQLRQDS APVEPSSPHSY -NH ₂	9.9	208	17	n.d.
39	GCGNLSTCQVQNLSHRLWQLRQDS APVEPSSPHSY -NH ₂	14	>1000	31	1000
40	GCVLGTCQVQNLSHRLWQLRQESA PVEPSSPHSY -NH ₂	>1000	>1000	>1000	1000

Example 7: Effects of AFP-6 on the Cardiovascular System

Telemetry allows for real-time hemodynamic readings including arterial pressure, heart rate, arterial dP/dt, ECG (with waveform intervals) and core temperature via an implanted radio transmitter in conscious, non-anesthetized, unrestrained rats. The rats were injected with either saline, 10 nmol/kg rat amylin, 10 nmol/kg rat adrenomedullin, or 10 nmol/kg AFP-6 by remote intravenous dosing. Remote intravenous dosing is achieved through in-dwelling vascular access ports.

As shown in Figures 16 A-C, amylin increases mean arterial pressure (MAP) acutely and then may lower MAP in the first 30 minutes, while adrenomedullin decreases MAP throughout the 1-hour time period. For heart rate, all three peptides increase the number of beats per minute over the hour. Finally, amylin again acutely increases the dp/dt, but the effect tapers off over time, while adrenomedullin has a sustained increase in dp/dt over the hour. It appears that for all three cardiovascular parameters, AFP-6 has a profile more amylin-like than adrenomedullin like.

Example 8. Effects of AFP-6 on Food Intake and Body Weight in Rats

Individually housed male Sprague-Dawley® rats (beginning weight= 310g; 12-h light/dark cycle) were maintained on a high fat (HF) diet (Research Diet 58% kcal from fat). Rats were fattened for 34 days. At the end of the fattening period, 7-day osmotic pumps (Durect Corp.) were implanted interscapularly under anesthesia (body weight at time of implant = 403g). The rats received pumps delivering compound or vehicle (50% DMSO). The following compounds were examined at a 75 nmol/kg/day dose: AFP-6, and an AFP-6 analog SEQ ID NO:32. Food intake and body weight measurements were obtained on day 2 and day 7. Group differences were analyzed using analysis of variance (ANOVA) and Fisher's Least-Significant Difference test.

Figures 17A and 17B show that both AFP-6 and the exemplary AFP-6 analog significantly reduced food intake compared to the Vehicle group after 2 days of treatment.

By Day 7, food intake in both groups was equivalent to the Vehicle-treated group. Both peptides showed significantly less body weight gain than Vehicle on Day 7; the percent body weight loss on Day 7 for AFP-6 and the AFP-6 analog (vehicle-corrected) was 3.2% and 2.1%, respectively.

- 5 While the present invention has been described in terms of preferred examples and embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

CLAIMS

What is claimed is:

1. An Amylin Family Polypeptide-6 (AFP-6) analog having the amino acid sequence:
 $X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-QVQNLSHRLWQL-X_{21}-X_{22}-X_{23}-X_{24}-X_{25}-X_{26}-$
5 $X_{27}-X_{28}-SAPV-X_{33}-PSSPHSY$ (SEQ ID NO: 41) wherein
 X_1 is absent, TQAQLLRVG (SEQ ID NO: 42), any of one or more
consecutive amino acids of SEQ ID NO:42, N-aryl, or N-acyl with a
substituent selected from a C1-C18 alkyl, a substituted alkyl or a heteroaryl
moiety;
10 X_2 is M, S, C, substituted L, K, D or E, where the side chain can be linked
via an amide bond, or any amino acid that can form a bond with X_8 ;
 X_3 is V, D, L, G, N, A, or S;
 X_4 is V, D, L, G, N, A, S or T;
 X_5 is V, D, L, G, N, A, or S;
15 X_6 is V, D, L, G, N, A, S, or absent;
 X_7 is T, S, homoserine, (S)-2-Amino-3-hydroxy-3-methylbutanoic acid (Ab)
or (2R,3R)-2-Amino-3-hydroxy-4-methylpentanoic acid (Ap);
 X_8 is M, S, C, substituted L, K, D or E, or any amino acid that can form a
bond with X_2 ;
20 X_{21} is M, G, P, A, or absent;
 X_{22} is M, G, P, A, or absent;
 X_{23} is M, G, P, A, or absent;
 X_{24} is M, G, P, A, or absent;
 X_{25} is M, G, P, A, or absent;
25 X_{26} is R or absent, wherein when X_{26} is absent, X_{27} is absent;
 X_{27} is Q or absent, wherein when X_{27} is absent, X_{26} is absent;
 X_{28} is D or E;
 X_{33} is D or E; and
biologically active fragments thereof; wherein the amino acid sequence is
30 not SEQ ID NO: 1 or SEQ ID NO: 2.
2. The AFP-6 analog according to claim 1 wherein the amino acid sequence is
selected from the group consisting of SEQ ID NOs: 10-12, 16-33 and 35-40.

3. An AFP-6 analog having the amino acid sequence:

X₁-X₂-QNLSHRLWQL-X₁₃-X₁₄-X₁₅-X₁₆-X₁₇-X₁₈-X₁₉-X₂₀-SAPV-X₂₅-
PSSPHSY (SEQ ID NO: 56) wherein

X₁ is Q or absent;

5 X₂ is V or absent;

X₁₃ is M, G, P, A, or absent;

X₁₄ is M, G, P, A, or absent

X₁₅ is M, G, P, A, or absent;

X₁₆ is M, G, P, A, or absent;

10 X₁₇ is M, G, P, A, or absent,

X₁₈ is R or absent, wherein when X₁₈ is absent, X₁₉ is absent;

X₁₉ is Q or absent, wherein when X₁₉ is absent, X₁₈ is absent

X₂₀ is D or E;

X₂₅ is D or E; and

15 biologically active fragments thereof, wherein the amino acid sequence is
not SEQ ID NOs: 13 or 14.

4. The AFP-6 analog according to any of claims 1-3 wherein the C-terminus is
amidated.

5. The AFP-6 analog according to any of claims 1-4 wherein the analog has AFP-6
20 agonist activity.

6. The AFP-6 analog according to any of claims 1-4 wherein the analog has AFP-6
antagonist activity.

7. An AFP-6 analog comprising the amino acid sequence SEQ ID NO:15.

8. A compound comprising an AFP-6 analog according to claims 1-3.

25 9. A polypeptide comprising an AFP-6 analog according to claims 1-3.

10. A composition comprising at least one AFP-6 analog any of claims 1-4 in a
pharmaceutically acceptable carrier.

11. A method of treating or preventing a condition or disease that can be alleviated
by reducing caloric or nutrient intake or availability in a subject in need

thereof comprising administering to the subject an amount of an AFP-6 analog according to claim 5 therapeutically effective to reduce caloric or nutrient intake or availability.

- 5 12. A method according to claim 11 wherein the condition or disease is obesity, insulin resistance, metabolic syndrome or diabetes mellitus.
13. The method according to claim 11 wherein the condition or disease is a cardiovascular condition or disease.
- 10 14. A method of treating or preventing a wasting condition or disease in a subject in need thereof comprising administering to the subject an amount of an AFP-6 analog according to claim 6 therapeutically effective to treat or prevent a wasting condition or disease.
15. The method according to claim 14 wherein the wasting condition or disease is anorexia, cachexia or bulimia.
- 15 16. The method according to claim 14 wherein the wasting condition or disease is characterized by loss of appetite, diminished food intake, body weight loss, gastric hypomotility, or hypotension.
17. An isolated nucleic acid encoding an AFP-6 analog according to claim 1 or claim 3.
- 20 18. The isolated nucleic acid according to claim 17 wherein the nucleic acid is a DNA molecule.
19. A vector comprising the DNA molecule according to claim 18.
20. An expression vector comprising the DNA molecule according to claim 18 operatively associated with an expression control sequence.
21. A host cell comprising the vector according to claim 20.
- 25 22. The host cell according to claim 21, wherein the host cell is selected from the group consisting of bacteria, yeast, algae, avian cells, mammalian cells, plant cells, and insect cells.

23. The host cell according to claim 21, wherein the host cell produces the AFP-6 analog for use in treating metabolic disorders.

24. A method for preparing an AFP-6 analog comprising culturing the host cell according to claim 21 under conditions that provide for expression of the AFP-6 analog and isolating the expressed AFP-6 analog.

5

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Figure 1A

>gi|20903165|ref|XPM_147916) hypothetical protein XP_147916 [Mus musculus] Length = 150

Score = 32.3 bits (72), Expect = 0.23, Identities = 24/74 (32%), Positives = 33/74 (44%), Gaps = 11/74 (14%)

```

Query: 75  GASRSPEDSSPDAARIRVKRYRQSMNMFQGLRSFGCRFGTCTVQKLAHQIYQFT-DKDKD 133
          G SR P   P  +R   + + Q       L   GC  GTC VQ L+H+++Q       +
Sbjct: 87  GGSRRHPGPRPTGSR---RPHAQ-----LLRVGCVLGTCQVQNLSHRLWQLVRPAGRR 136

Query: 134 NVAPRSKISPQGYG 147
          + AP   SP  YG
Sbjct: 137 DSAPVDPSSPHSYG 150

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Figure 1B

```

hAdm P35318  1  MKLVSVALMYLGSLAFLGADTARLDVASEFRKKWNKVALSRGKRELRMSSSYPTGLADVK
XP_147916    1  MAQLLMVTVTILGCISLLYLLPGTSLGSLGKGLRHSRPREPPAKIPSSNLQPGHPSLQPVV

hAdm P35318  61  AGPAQTLIRPQDMKGASRSPEDSSPDAARIR..VKRYRQSMNMFQGLRSFGCRFGTCTVQ
XP_147916    61  WKSRRHAPQPQGRGNRALAMVHLPQGGGSRHPGPQPTGSRPHQAQLLRVGCVLGTCQVQ

hAdm P35318  119 KLAHQIYQFT.DKDKDNVAPRSKISPQGYGRRRRRSLPEAGPGRTLVSSKPAHGAPAPP
XP_147916    121 NLSHRLWQLVRPAGRRDSAPVDPSSPHSYG.....

hAdm P35318  178  SGSAPHFL
XP_147916    .....

```

Figure 2

>gi|20903165|ref|XP_147916.11 hypothetical protein XP_147916 [Mus musculus]

```

MAQLLMVTVTILGCISLLYLLPGTSLGSLGKGLRHSRPREPPAKIPSSNLQPGHPSLQPVWKSRRHAPQP
QGRGNRALAMVHLPQGGGSRHPGPQPTGSRPHQAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRRDS
APVDPSSPHSYG

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Figure 3

Mouse AFP-6 1 MAQLLMVTVTLGCTISLLYL.LPGTLSGSLGKGLRHSRPREPPAKIPSSNLQPGHPSLQPV
 Human AFP-6 long 1 ..MARIPATAALGCISLLCLQLPGSLSRSLGGDPRPVKPREPPARSPSSSLQPRHPAPRPV
 Human AFP-6 short 1 ..MARIPATAALGCISLLCLQLPGSLSRSLGGDPRPVKPREPPARSPSSSLQPRHPAPRPV

 Mouse AFP-6 60 VWKSRRHAPQPOQGRGNRALAMVHLPOGGGSRHPGPQRPTGSRRPHAQLLRVGCVLGTCQV
 Human AFP-6 long 59 VWKLHRALQAQRGAG.LAPVMGQPLRDGGRQHSGPRRHSGPRRTQAQLLRVGCVLGTCQV
 Human AFP-6 short 59 VWKLHRALQAQRGAG.LAPVMGQPLRDGGRQHSGP.....RRTQAQLLRVGCVLGTCQV

 Mouse AFP-6 120 QNLSHRLWQLVRPAGRRDSAPVDPSSPHSYG
 Human AFP-6 long 118 QNLSHRLWQLMGPAGRQDSAPVDPSSPHSYG
 Human AFP-6 short 112 QNLSHRLWQLMGPAGRQDSAPVDPSSPHSYG

Figure 4

hAdm P35318 1 .MKLVSVALMYLGSLAFLGADTARLDVASEFRKKWNKWALSRGKRELRMSSSYPTGLADV
 Human AFP-6 short 1 MARIPATAALGCISLLCLQLPGSLSRSLGGDPRPVKPREPPARSP.....SSSLQPRHPAP

 hAdm P35318 60 KAGPAQTLIRPODMKGASRSPEDSSPDAAIRVKRYRQSMNNFQGLRSFQGRFGTCTVQK
 Human AFP-6 short 56 RPVVWKLHRALQAQRGAGLAP..VMGQPLRDGGRQHSGPRRTQAQLLRVGCVLGTCQVQV

 hAdm P35318 120 LAHQIYQFT.DKDKDNVAPRSKISPOGYGRRRRRSLPEAGPGRTLVSCKPQAHGAPAPS
 Human AFP-6 short 114 LSHRLWQLMGPAGRQDSAPVDPSSPHSYG.....

 hAdm P35318 179 GSAPHFL
 Human AFP-6 short

Figure 5

hs_AFP6 1TQAQLLRVGC.VLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSYG
 hs_adrenomedullin 1 YRQSMNNFQGLRSFGC.RFGTCTVQKLAHQIYQFT.DKDKDNVAPRSKISPOGYG
 hs_Amylin 1KC.NTATCATORLANFLVHSSN.NFGAILSSSTN.VGSNTYG
 hs_CGRP1 1AC.DTATCVTHRLAGLLSRSG.GVVKNNFVPTN.VGSKAFG
 hs_Calcitonin 1CGNLSTCM...LGTYYTQDFN....KFHTFEQTAIGVGAPG

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Figure 6

Human
TQAQLLRVGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:1)
VGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY-NH2 (SEQ ID NO:2)

Mouse
PHAQLLRVGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-NH2 (SEQ ID NO:3)
VGCVLGTCQVQNLSHRLWQLVRPAGRRDSAPVDPSSPHSY-NH2 (SEQ ID NO:4)

Figure 7

AFP-6_5'200bp 1
NM_024866 1 ATTCAGCCCTGGAGGTGCCATCCCGGGCCGCGACTCCGCTCCAGGCAGGACCCCCAACCC

AFP-6_5'200bp 1
NM_024866 61 GCCCAGCCCGCTCCGCCTTGCGCCCCGACCCGCGGCCGACCCAGACCCGCTGCCCGCT

AFP-6_5'200bp 1GACGGACGCCCCGTGCCAGCTTGCCACGCCACGCCCG
NM_024866 121 TCGCGCCCGAGGCCTGCGCCCCGACGGACGCCCCGTGCCAGCTTGCCACGCCACGCCCG

AFP-6_5'200bp 39 GCGCCCCGACCGCGGAGGACTCCCCGAG.....
NM_024866 181 GCGCCCCGACCGCGGAGGACTCCCCGAGGTGCCGGCGAGGGGGTGGCTCGCGGCTCAGG

AFP-6_5'200bp 67CCCCGCCCGCCATGGCCCG
NM_024866 241 CTGCCCCCGACGTGCCCGGCTCACCGCCCCCTCCCTGCAGCCCCGCCCGCCATGGCCCG

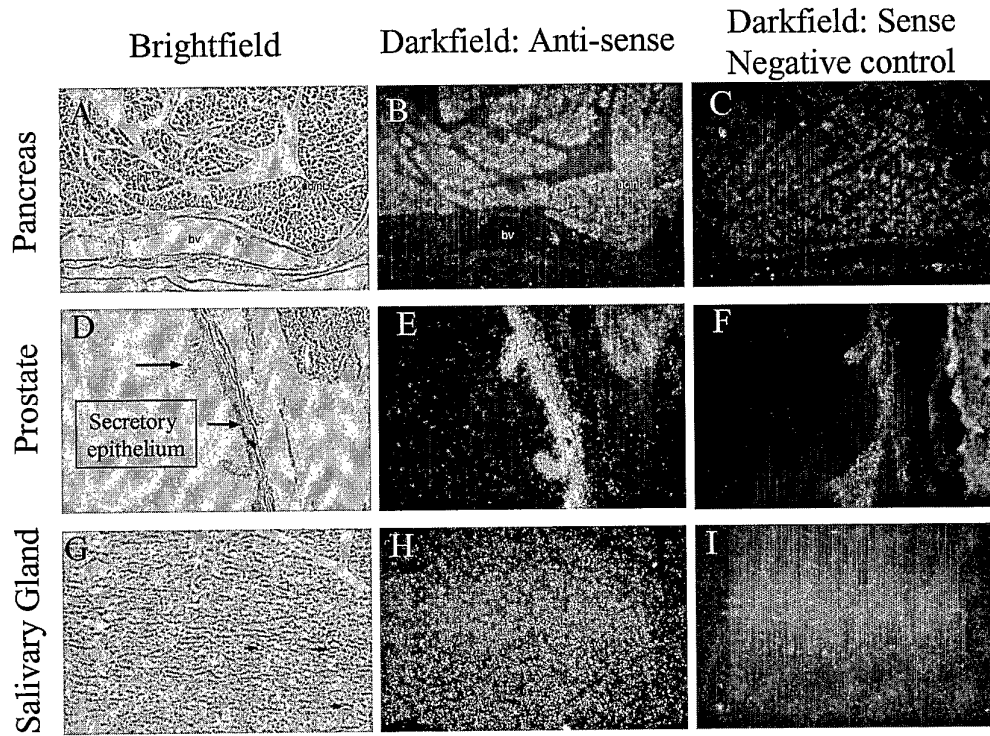
AFP-6_5'200bp 86 GATCCCGACGGCCGCCCTGGGTTGCATCAGCCTCCTCTGCCTGCAGCTCCCTGGCTCGCT
NM_024866 301 GATCCCGACGGCCGCCCTGGGTTGCATCAGCCTCCTCTGCCTGCAGCTCCCTGGCTCGCT

AFP-6_5'200bp 146 GTCCCGCAGCCTGGGCGGGGACCCGCGACCCGTCAAACCCAGGGAGCCCCAGCCCG...
NM_024866 361 GTCCCGCAGCCTGGGCGGGGACCCGCGACCCGTCAAACCCAGGGAGCCCCAGCCCGGAG

AFP-6_5'200bp
NM_024866 421 CCCTTCCAGCAGCCTGCAGCCAGGCACCCCGACCCCGACCTGTGGTCTGGAAGCTTCA

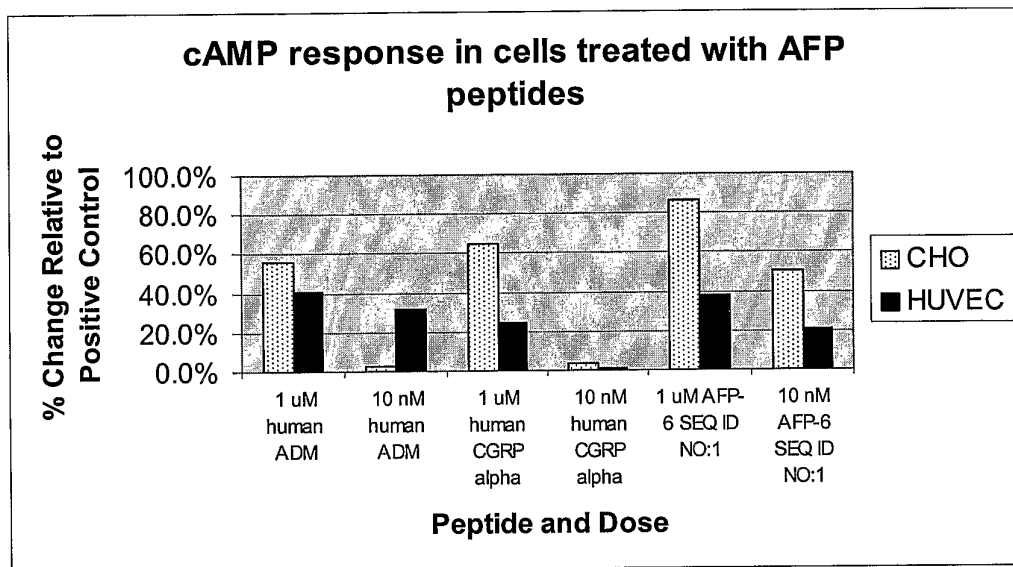
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Figures 8A-8I



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Figure 9



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Figure 10

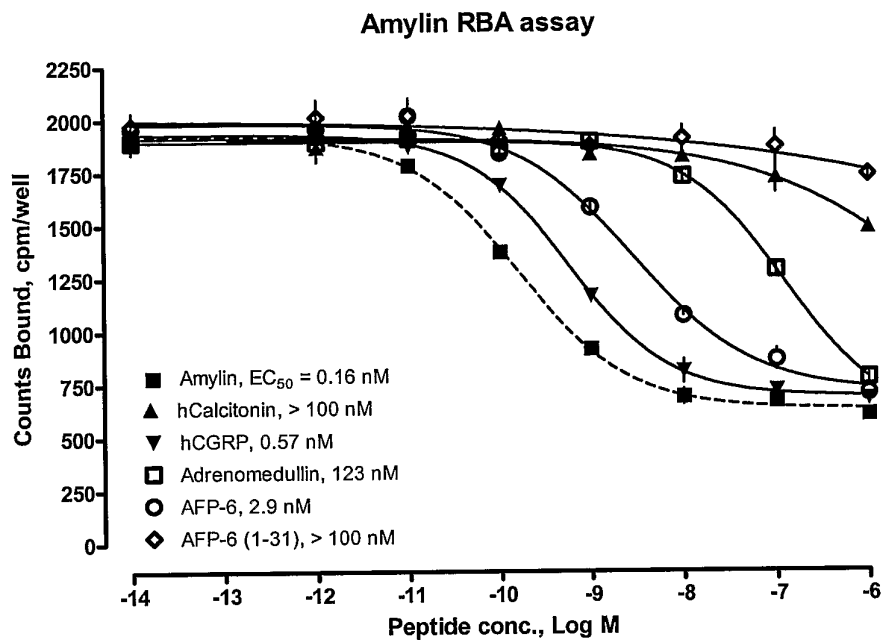
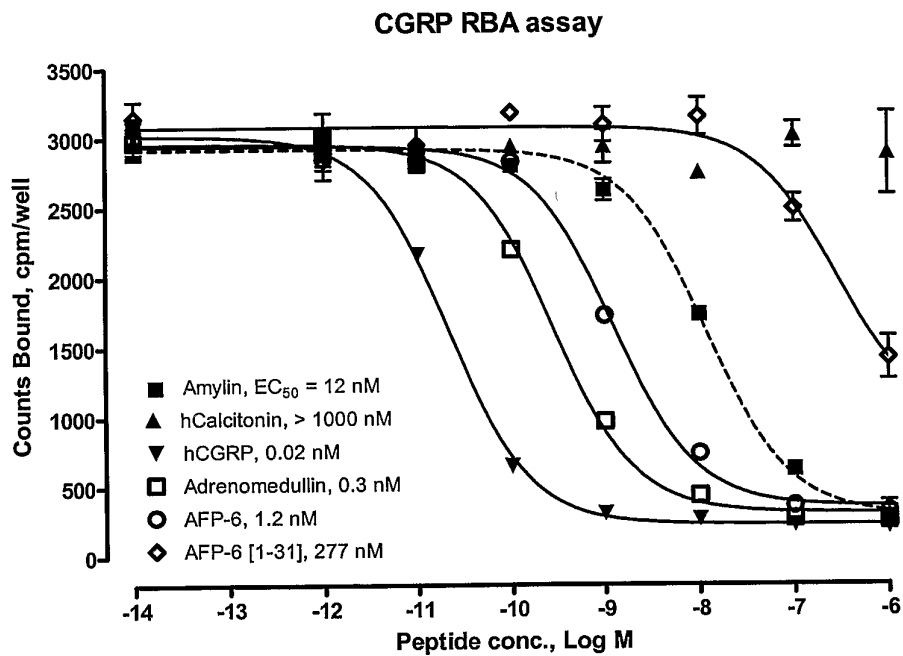
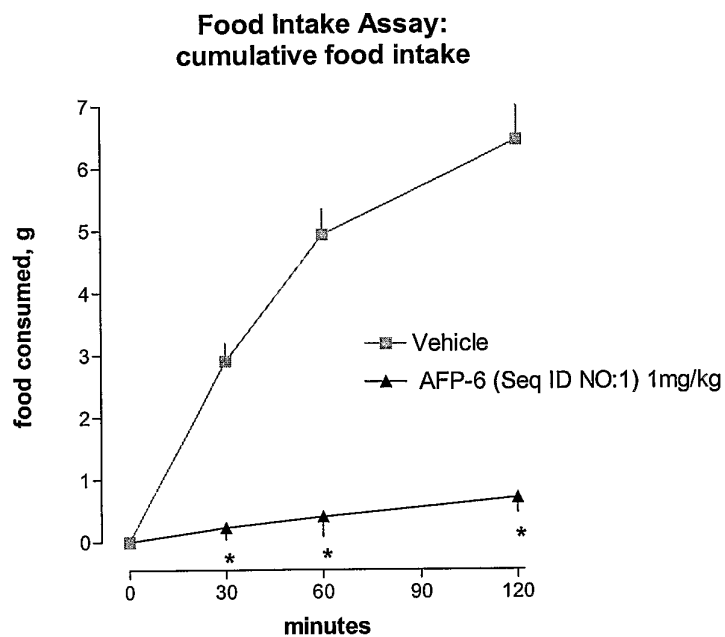


Figure 11



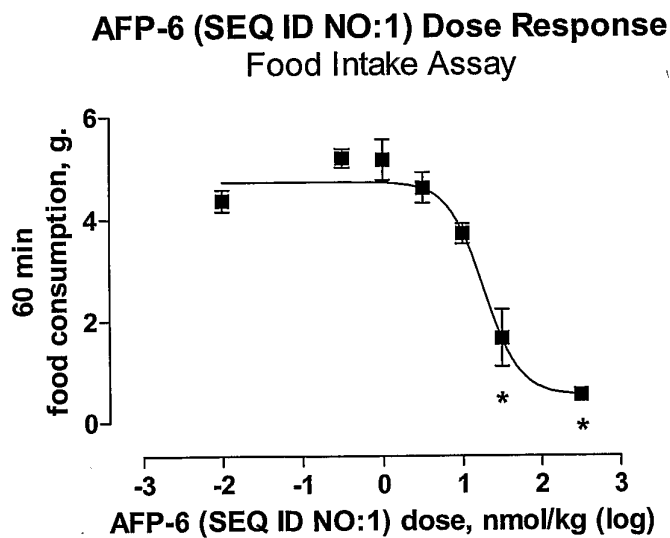
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Figure 12



- $p < 0.05$ vs. vehicle control; ANOVA, Dunnett's test

Figure 13



- $p < 0.05$ vs. vehicle control; ANOVA, Dunnett's test

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Figure 14A

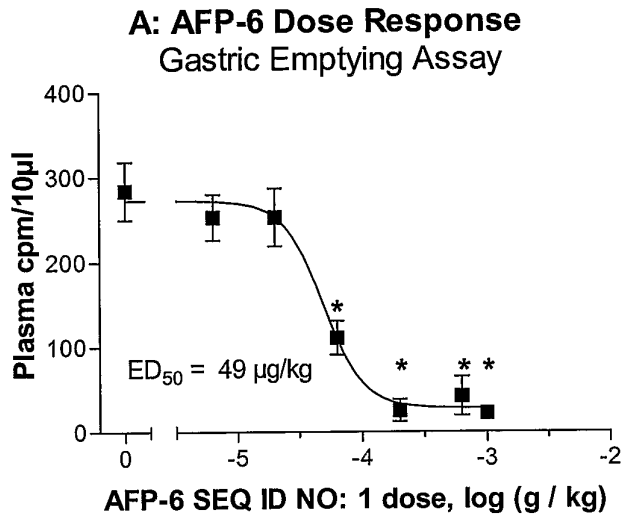
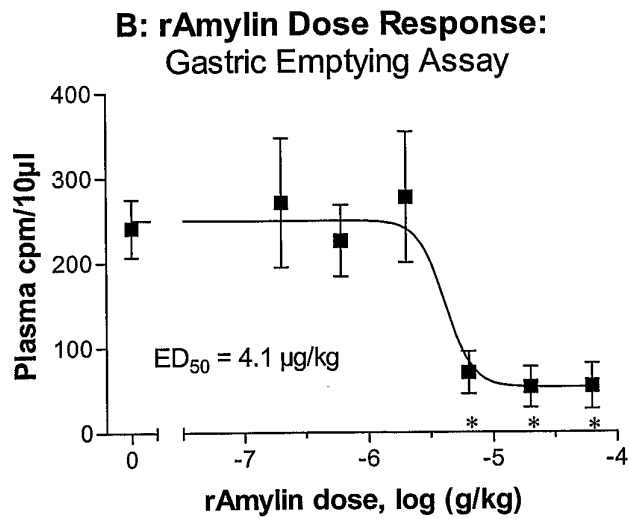


Figure 14B



p<0.05 vs. vehicle control; ANOVA, Dunnett's test

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Figure 15A

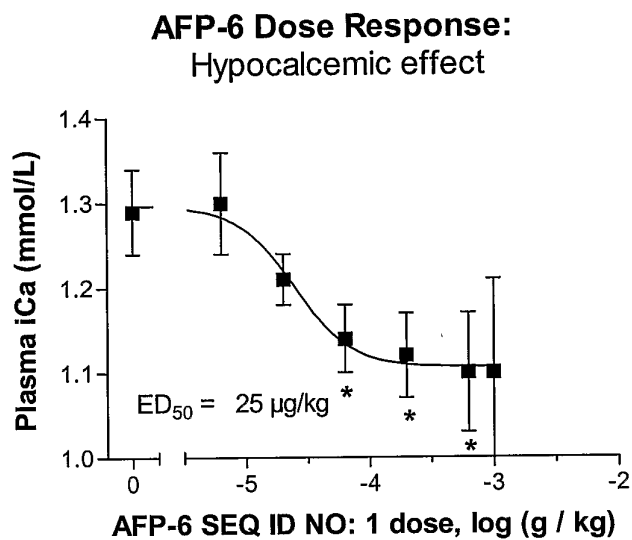
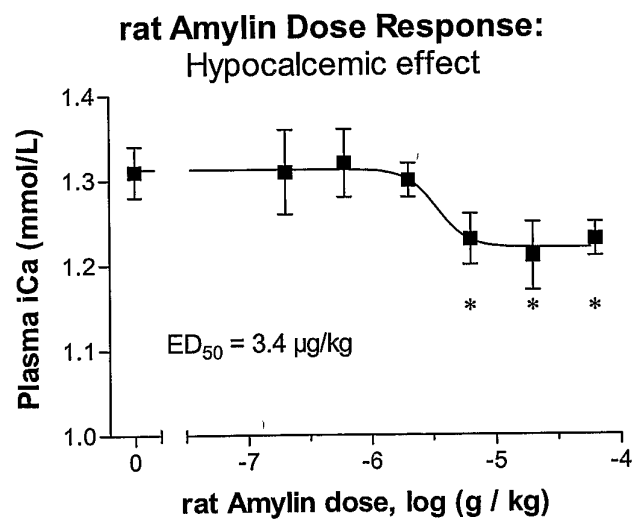


Figure 15B



p<0.05 vs. vehicle control; ANOVA, Dunnett's test

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Figure 16A

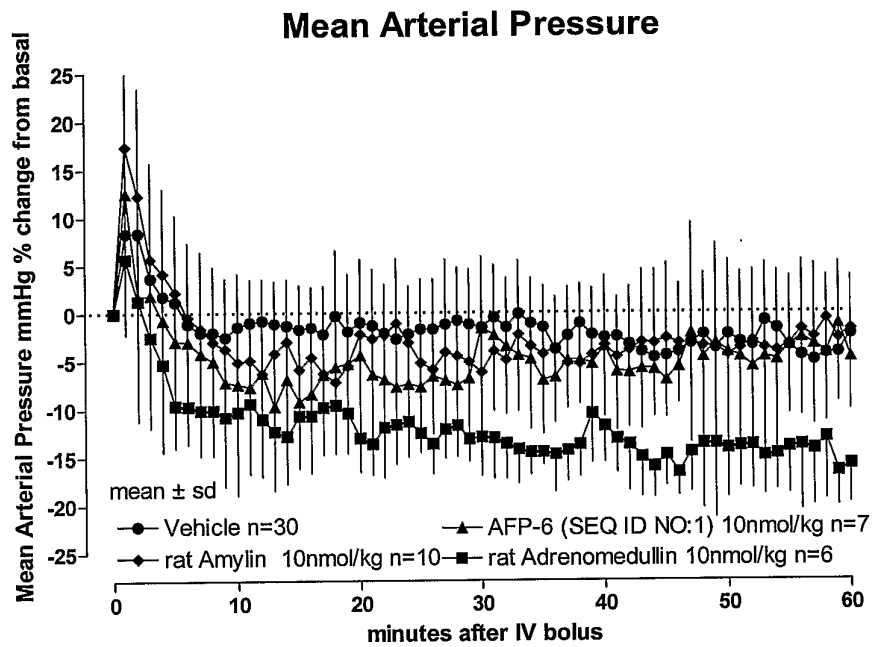
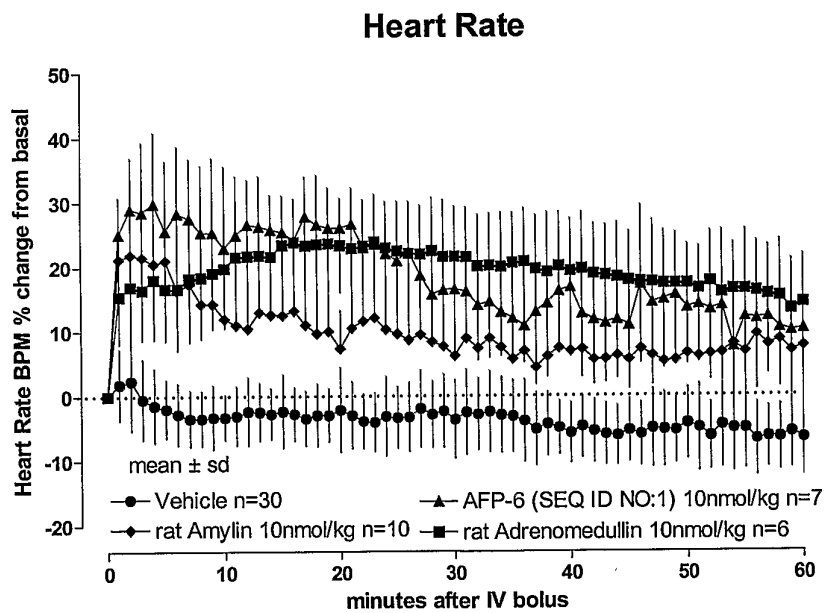
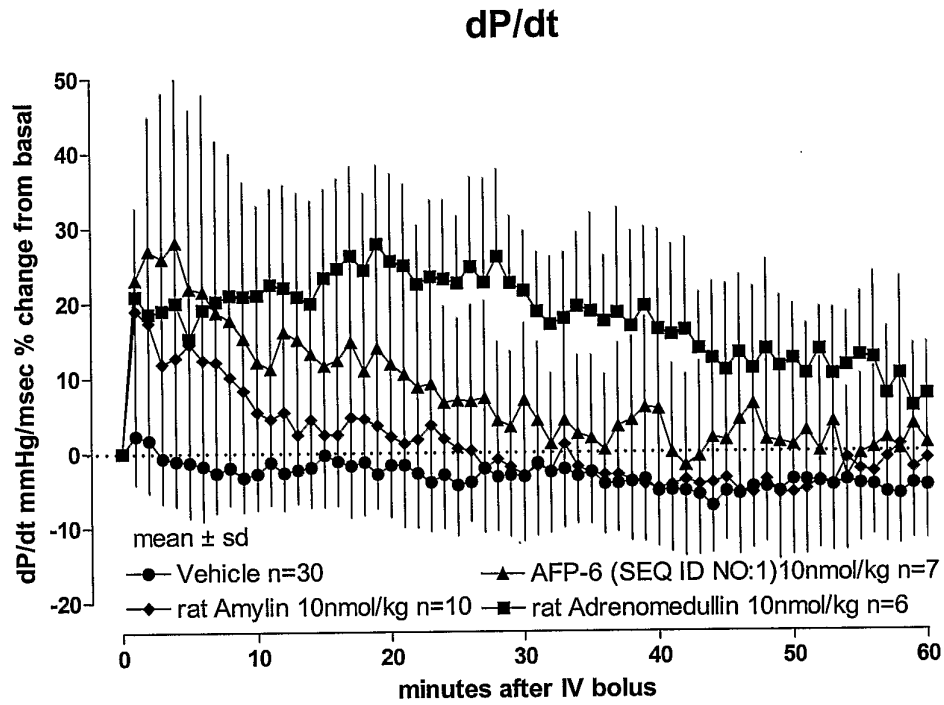


Figure 16B



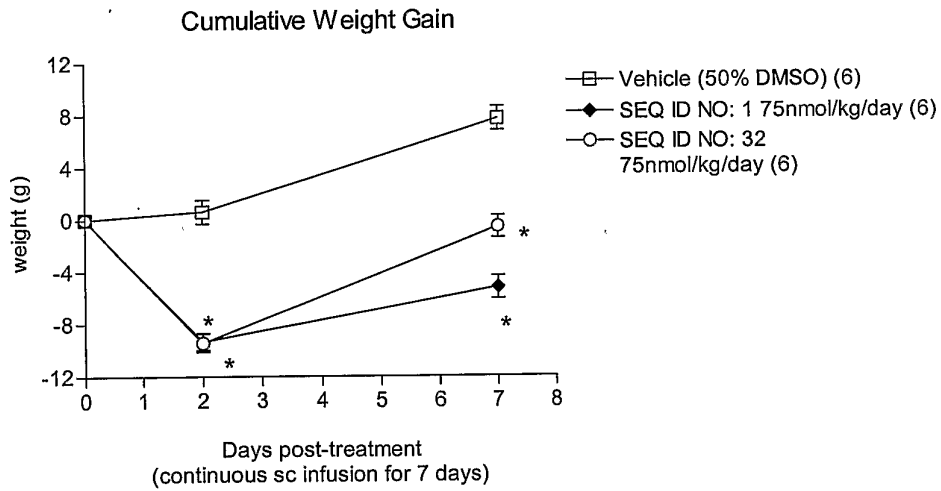
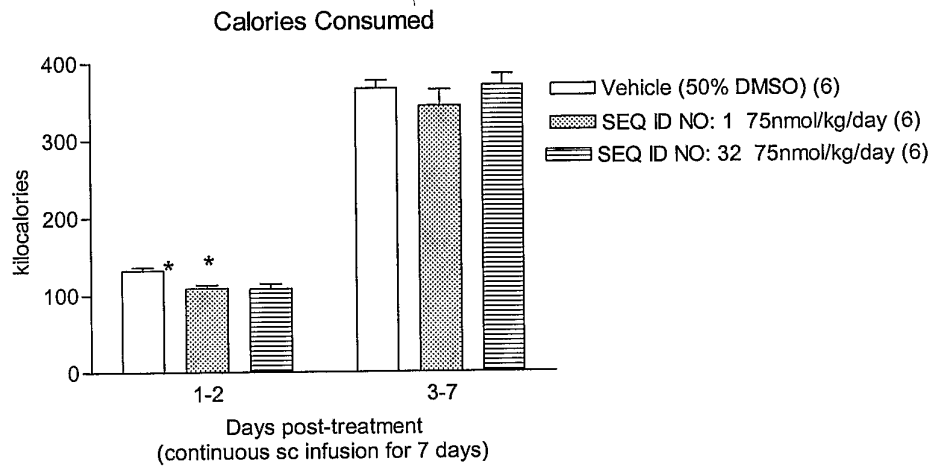
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Figure 16C



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Figures 17A and 17B:



*P<0.05 compared to Vehicle group