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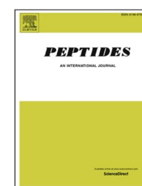

Synthesis of potent antagonists of receptors for growth hormone-releasing hormone with antitumor and anti-inflammatory activity

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ABSTRACT

The syntheses and biological evaluation of GHRH antagonists of AVR series with high anticancer and anti-inflammatory activities are described. Compared to our previously reported GHRH antagonist 602 of MIAMI series, AVR analogs contain additional modifications at positions 0, 6, 8, 10, 11, 12, 20, 21, 29 and 30, which induce greater antitumor activities. Five of nineteen tested AVR analogs presented binding affinities to the membrane GHRH receptors on human pituitary, 2-4-fold better than MIA-602. The antineoplastic properties of these analogs were evaluated *in vitro* using proliferation assays and *in vivo* in nude mice xenografted with various human cancer cell lines including lung (NSCLC-ADC HCC827 and NSCLC H460), gastric (NCI-N87), pancreatic (PANC-1 and CFPAC-1), colorectal (HT-29), breast (MX-1), glioblastoma (U87), ovarian (SK-OV-3 and OVCAR-3) and prostatic (PC3) cancers. *In vitro* AVR analogs showed inhibition of cell viability equal to or greater than MIA-602. After subcutaneous administration at 5 μg/day doses, some AVR antagonists demonstrated better inhibition of tumor growth in nude mice bearing various human cancers, with analog AVR-353 inducing stronger suppression than MIA-602 in lung, gastric, pancreatic and colorectal cancers and AVR-352 in ovarian cancers and glioblastoma. Both antagonists induced greater inhibition of GH release than MIA-602 *in vitro* in cultured rat pituitary cells and *in vivo* in rats. AVR-352 also demonstrated stronger anti-inflammatory effects in lung granulomas from mice with lung inflammation. Our studies demonstrate the merit of further investigation of AVR GHRH antagonists and support their potential use for clinical therapy of human cancers and other diseases.

1. Introduction

Cancer continues to be a major health problem throughout the world [1]. There is a critical medical need for new drugs, that target malignant tumor cells, with few or no side effects. Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide neuro-hormone that regulates the release of growth hormone (GH) from the pituitary gland [2,3]. Although GHRH was initially identified in tumor tissues [2–5], few investigators attempted to explore the possible role of GHRH in carcinogenesis by the late 1990's. At that time, our group, in view of our interest

Abbreviations: 5FPhe, pentafluoro-Phe; 5FPhAc, pentafluoro-phenylacetyl; Abu, α-aminobutanoyl; Ada, 12-aminododecanoyl; Amp, para-amidinophenylalanine; Aoc, 8-amino octanoyl; Cpa, *para*-chloro-Phe; Har, homoArg; Nle, Norleucine; Orn, Ornithine; PhAc, phenylacetyl; Tyr(Me), O-methyl-Tyr; GH, growth hormone; GHRH, GH-releasing hormone; IGF, insulin-like growth factor; LHRH, Luteinizing hormone-releasing hormone.

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in the field, had decided to become involved in the synthesis and evaluation of GHRH antagonists for possible uses in therapy of cancer [6–8].

In the past decades, many antagonists of human GHRH have been synthesized and tested by other investigators [9–13], as well as by us [6–8,14–18]. Our laboratory has developed several classes of GHRH antagonists which show potent inhibitory effects on growth of various tumors [6–8,14–18]. Strategies to improve the bioavailability, short half-life *in vivo*, rapid renal clearance of GHRH antagonists and *in vivo* stability were developed [8,13]. In initial studies performed in our laboratory, early types of GHRH antagonists inhibited the growth of human osteosarcomas (SK-ES-1 and MNNG/HOS) [19] and small cell-and non-small cell lung carcinomas [20] xenografted into nude mice. Subsequent studies demonstrated that antagonists of GHRH also inhibit growth of various other tumors [18].

We and others incorporated pentafluoro-Phe at different positions into several GHRH analogs [14,21]. Acylation of GHRH antagonists with octanoic acid or 12-aminododecanoic acid improved the anti-proliferative effects of these antagonists [8,14]. Our work between 1994 and 2017 resulted in several series of potent GHRH antagonists intended for cancer therapy [6–8,14–18]. Antagonists of MIA series, after subcutaneous administration in microgram doses suppressed tumor growth of diverse human cancer lines xenografted into nude mice [14]. Antagonists MIA-602 and MIA-690 were among the most potent anti-tumor analogs and also displayed anti-inflammatory activities [22–24]. Thus, GHRH analogs of the Miami series powerfully hinder tumor growth and inflammatory activities, but have only a weak endocrine GH inhibitory activity [14]. GHRH antagonists of Miami class, inhibited tumor growth *in vivo* in nude mice of some 16 types of solid human cancers represented by nearly 50 human cancer lines. In this work we describe the design and syntheses of new class of GHRH antagonists [25] with greater tumor inhibitory potency and augmented suppressive activity on the release of GH.

2. Material and methods

2.1. peptide synthesis and purification

AVR GHRH antagonists were prepared by solid-phase methodology using Fmoc synthesis strategy [26]. The antagonists with C-terminal amide such as Har-NH_2 or Ada-NH_2 were synthesized using Rink amide MBHA resin; and the antagonists with C-terminal Har-$NHCH_3$ or Ada-$NHCH_3$ were synthesized on Methyl Indole AM resin.

For the synthesis, the Fmoc group was eluted from the Rink amino MBHA resin with 20% piperidine in dimethyl-formamide for 20 min. The side chains of Fmoc-amino acids were protected with acid labile groups such as β-tert-butyl ester for Asp;tert-butyl (But) for Ser, Thr and Tyr; Nω-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg and D-Arg; Nδ-tert-butoxycarbonyl (Boc) for Orn; Nγ-trityl for Asn and Nδ-trityl for Gln. Dat was unprotected. The coupling of Fmoc amino acid was achieved with 3 equivalents of Fmoc amino acid, HBtU [2- (1H-benzotriazole-1-Yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate] and HOBt [1-Hydroxybenzitriazole] dissolved in DMF, followed by addition of 6 equivalents of *N,N*-diisopropylethylamine (DIPEA) and stirred for 5 min to go into a complete solution. The mixture was immediately added to Fmoc-deblocked resin and shaken for 2 h. After finishing the peptide synthesis, the final de-protection and cleavage of the peptides from the resin were performed by treatment with a mixed reagent and scavengers containing TFA/thioanisole /1,2- ethanedithiol / phenol / H_2O (95 : 1.5 : 1 : 1 : 1.5 by volume) at room temperature for 3 h. All amino acid derivatives, resins, and reagents were obtained from Chem-Impex Int'l INC (Wooddale IL), Sigma-Aldrich, (Billerica, MA), or Novabiochem (Burlington MA). The scale of synthesis was around 0.5 mmol for most of our AVR compounds. The scale for purification was 200 mg for crude products; the average yield was around 30 % with > 95 % purity.

Purification of the crude peptides was performed on a Beckman Gold HPLC system (Beckman Coulter, Inc., Brea, CA) equipped with a 127 P solvent Module, model 166 P UV–vis Detector, using an XBridgeTM BEH C18 OBD Column(10 × 150 mm, 130 Å pore size, 5 μm particle size, Waters Co., Milford, MA). The peptides were eluted with a solvent system consisting of solvent A (0.1 % aqueous TFA) and solvent B (0.1 % TFA in 70 % aqueous acetonitrile [MeCN]) in a linear gradient mode of 30–70 % solvent B for 150 min at a flow rate of 5 ml/min. The eluents were monitored at 220 nm and the fractions were examined by analytical HPLC and pooled to give maximum purity.

The HPLC analyses of crude and purified peptides were carried out on an Agilent 1290 Infinity High Performance Liquid Chromatography unit (Agilent, Santa Clara, CA) equipped with Discovery HS C18 column (2.1 × 50 mm, 120 Å pore size, 3 μm particle size, Supelco Bellefonte, PA). An isocratic and/or gradient elution was used from 40 to 80 % B in 10 min with a solvent system consisting of solvents A and B, described above, with a flow rate of 0.5 ml/min. The peaks were monitored at 220 and 280 nm. The peptides were judged to be substantially (>95 %) pure by analytical HPLC.

Molecular masses were determined by an Agilent 6210 time-of-flight Mass Spectrometer in conjunction with 1200 CapLC (Agilent,Santa Clara, CA). Peptides were eluted on an Agilent Zorbax C18 column (0.5 × 150 mm, 300 Å pore size, 5 μm particle size) with a solvent system consisting of solvent A (0.1 % formic acid) and solvent B (0.1 % formic acid in 90 % aqueous acetonitrile) in a linear gradient mode of 35–85 % solvent A: solvent B, at a flow rate of 15 μl/min in 30 min. TOF settings were as follows: capillary voltage:4000 V, drying gas flow: 7 l/min, drying gas temperature: 300°C, nebulizer gas: 30 psi, fragmentor voltage: 350 V.

2.2. Animals

Male rats (Wistar, obtained from Charles River Laboratory) weighing ~200 g were used for the *in vivo* and *in vitro* endocrine tests; Athymic (Ncr nu/nu) nude mice, 5 to 6-week-old, obtained from Envigo Labs (Tampa, FL) were used in oncologic studies; 6- week-old C57BI/6 male mice (Jackson Laboratory, Bar Harbor, ME) were used in lung granulomatous study. All animals were housed in Laminar airflow cabinets under pathogen-free conditions [27]. The experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals. The protocol of the animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of the Miami VA Medical Center.

2.3. Cell lines

Various human cancer cell lines including HCC827 human lung cancer NSCLC-ADC, H460 human lung cancer LCLC, CFPAC-1 and PANC-1 human pancreatic cancer, NCI-N87 human stomach cancer, HT-29 colon cancer, MX-1 breast cancer OVCAR-3 and SK-OV-3 ovarian, U87 glioblastoma and PC-3 prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture in as instructed by the manufacturer.

2.4. Evaluation of receptor binding affinities in vitro

Preparation of human pituitary membrane fractions and receptor binding assay were performed as previously described [26]. Human pituitaries were purchased from the National Hormone and Peptide Program. In the competitive binding analyses, ^{125}I-labeled [His^1, Nle^{27}]-hGHRH(1-32)-NH_2(0.2 nM) was displaced by GHRH antagonists at 10^{-6}–10^{-12}M. The final binding affinities were expressed as IC_{50} value and were calculated by using the LIGAND PC computerized curve-fitting program of Munson and Rodbard [28] as modified by McPherson [29].

2.5. Cell proliferation assay

Human cancer cells were seeded into 96-wellplates and exposed to 1 μM, 2 μM, and 5 μM of a selected AVR class of GHRH antagonists in quintuplicate wells, each for 70–72 h. The GHRH antagonist MIA-602 was used for comparison of activity. At the end of treatment, cell proliferation was measured using CellTiter 96 aqueous one solution kit (Promega, Madison WI), as described previously [27]. Relative inhibitory potency of AVR-class of GHRH antagonists in comparison to MIA-602 was calculated based on the inhibition of cell viability after cancer cells were treated with the antagonists at a concentration of 5 μM for 72 h.

2.6. Oncologic study in vivo

As previously described [27], athymic (Ncr nu/nu) nude mice were xenografted subcutaneously (s.c) with approximately 3 mm^3 pieces of tumor tissue derived from donor animals. When tumors reached a mean volume of $\approx 40-50$ mm^3, the animals were randomly assigned into groups (6–10 mice per group) and treated s.c. with GHRH antagonists: MIA-602, and selected AVR antagonists at doses of 5 μg/day (prepared in a vehicle solution,10 % propylene glycol containing 0.1 % DMSO) or otherwise indicated. Controls were treated with vehicle solution. Tumor size was measured once a week with a micro-caliper. Tumor volume was calculated as previously described [30]. At the end of the experiment, (after 4–7 weeks of treatment) mice were anesthetized and sacrificed. Tumor growth rate "Vn/V0", tumor volume at the termination of treatment / tumor volume at beginning of the treatment, was calculated.

2.7. In vivo assessment of the antagonistic activity of GHRH analogs on GH release

The potency and duration of inhibitory effects of GHRH antagonists were tested in male rats using 8 rats per group [14]. Twenty min after anesthesia, GHRH antagonists MIA-602, AVR-352 and AVR-353, at dose of 5 μg/kg were injected into the jugular vein of the rats, followed by an iv injection of 3 μg/kg GHRH (1-29)NH_2 5 min later. Controls received vehicle solution (10 % propylene glycol containing 0.1 % DMSO) before GHRH stimulus. Serum GH levels before the administration of the antagonists and 5, 15 and 30 min following the injection of GHRH (1-29) NH_2 were measured by ELISA assay kit (ALPCO Diagnostics, Mill Valley, CA).

2.8. In vitro assessment of the antagonistic activity of GHRH analogs on GH release

Rats were decapitated and pituitaries were collected in prewarmed HBSS (Hanks' Balanced Salt Solution). Tissue was cut into small pieces and digested in HBSS medium containing 3 % BSA, 50 μg/mL gentamycin and 0.5 % collagenase) and seeded onto poly-D-lysine-coated 24-well plates using 7 pituitaries/plate. Cells were allowed to recover for 4 days. Growth medium was then replaced with serum-free DMEM for 4 h and 20 nM of selected GHRH analogs were added in DMEM containing 0.1 % BSA for 30 min. Cells were then incubated in medium containing 20 nM concentration of the analog and 1 nM GHRH(1-29)NH_2 for 30 min. The medium from this step was collected and centrifuged at 800 *g* for 3 min. GH concentration was determined by using Spin Bio Growth hormone (rat) EIA KIT (Cayman chemical, Ann Arbor, MI).

2.9. Mouse model with granulomatous lung reaction

Granulomatous reaction in the mouse lung was developed as previously described [31]. Briefly, 6-week-old C57Bl/6 male mice were challenged intratracheally for 4 times (at day 0, 3, 6, and 9) with microparticles developed from mycobacterium abscess cell wall [31]. Mice were randomly divided in four groups (5 mice per each group) and treated with PBS, MIA-602 (5 μg/day), AVR-352 (5 μg/day) or Methyl prednisolone (138 μg/day) on day 0 through three weeks, respectively. The mice were sacrificed on day 21 days after challenges and the lungs were harvested for protein analyses.

2.10. Protein isolation and Western blot analyses

Lung tissues retrieved from mice with granulomatous lung reaction, or tumors from mice carrying xenografted human lung cancer HCC827 after treatment with antagonists were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitor cocktail (Cell Signaling Technology, Beverly, MA). Samples were sonicated and centrifuged at 10,000 *g* for 5 min 3 times. The supernatant was collected, and protein concentration was determined by BCA protein assay using a kit (Cell Signaling Technology).

Thirty micrograms of total protein were mixed in a reducing sample buffer, and then electrophoresed on a 10–15 % Tris gel as described [27]. Quantification of density of signals was performed using enhanced chemiluminescence (ECL Plus, General Electric Healthcare, Milwaukee, WI). The primary antibodies to IL-2 (26156-1-AP), 2′-5′-Oligoadenylate Synthetase 1 (OAS1, 14955-1-AP), interferon-stimulated gene 15 (ISG15, 15981-1-AP), and actin (20536-1-AP) were from ProteinTech Group, Inc. (Rosemont, IL), to GHRH-R (TA311715) from OriGene (Rockville, MD) to cyclin D1 (#2922S), cyclin D2 (#3741S) and the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit antibody (7074P2) were from Cell Signaling Technology (Danvers, US). Anti-β-actin (A2228) was from Sigma-Aldrich (St. Louis MO) and secondary antibody HRP conjugated anti mouse-IgG (W402B) was from Promega (Madison WI).

2.11. Statistical analysis

Statistical analyses of the results of oncological studies were performed by *t*-test or one-way ANOVA, followed by Tukey's test, using the computer software Sigma Stat (Jandel, San Rafael, CA). Differences were considered significant when $p < 0.05$. GH data was analyzed similarly.

3. Results

3.1. Design and synthesis of new hGHRH antagonists

We have synthesized 115 GHRH antagonists of AVR series using Fmoc-chemistry method instead of Boc chemistry used for GHRH antagonists of MIA series [26]. This AVR series of antagonists contains modification at positions 0, 6, 8, 9, 10, 11, 12, 20, 21, 29 and 30 of the structures of GHRH antagonist MIA-602. Table 1 shows the key amino acid replacements in nineteen AVR antagonists which were selected for testing of antitumor activities in comparison to MIA-602. These AVR compounds have 5FPhAC-Ada at N-terminal; 5FPhe or Cpa at position 6, Asn or Ala at position 8, Arg or Har at position 9, Tyr (Me) or 5FPhe at position 10, Arg or His in position 11 and 20, Lys or Orn at position 12 and 21 and Har-NH_2 or Har-$NHCH_3$ at position 29; or modified C-terminal-NH_2 with Aoc-$NHCH_3$ or Ada-NH_2, Ada-$NHCH_3$ as an additional extension at position 30.

These AVR antagonists were tested for their receptor binding affinities, inhibitory effects in cell viability assays in various human cancer cell lines including lung (HCC827, NSCLC-ADC and H460, NSCLC-LCLC), gastric (NCI-N87), pancreatic (PANC-1 and CFPAC-1), colorectal (HT-29), breast (MX-1), glioblastoma (U87), ovarian (SK-OV-3 and OVCAR-3) and prostatic (PC3) cancers. The compounds which displayed high inhibitory potencies *in vitro*, were further tested *in vivo* in nude mice xenografted with various human tumors.

3.2. Binding affinities of new hGHRH antagonists

The nineteen AVR and two MIA GHRH antagonists MIA-602 and

Table 1
Structures of AVR antagonists tested *in vivo*.[1].

peptides	position of residues[a]																														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
MIA-602	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	5FPhe	Thr	Ala	Har	Tyr(Me)	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
MIA-690	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
JV-1-38	PhAC	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
AVR-104	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Arg	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
AVR-107	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Arg	Tyr	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
AVR-116	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Arg	5FPhe	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
AVR-120	D-Phe-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Arg	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-NH_2	
AVR-201	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Arg	Amp	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-$NHCH_3$	
AVR-234	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Arg	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-$NHCH_3$	
AVR-235	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	5FPhe	Thr	Ala	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-$NHCH_3$	
AVR-321	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Aoc-$NHCH_3$
AVR-322	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Aoc-$NHCH_3$
AVR-332	PhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-333	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-352	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	5FPhe	Thr	Ala	Har	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-353	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-354	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-$NHCH_3$
AVR-542	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har-$NHCH_3$	
AVR-543	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-$NHCH_3$
AVR-552	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Ala	Har	5FPhe	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-553	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	5FPhe	Thr	Ala	Har	Tyr(Me)	His	Orn	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2
AVR-620	5FPhAC-Ada	Tyr	D-Arg	Asp	Ala	Ile	Cpa	Thr	Asn	Arg	Tyr(Me)	Arg	Lys	Val	Leu	Abu	Gln	Leu	Ser	Ala	His	Orn	Leu	Leu	Gln	Asp	Ile	Nle	D-Arg	Har	Ada-NH_2

[1] The original Table 1 is too large for publication. The rest of the data in Table 1 is listed in Table 1S.

[a] Non-coded amino acids and acyl groups used in the peptides are abbreviated as follows: 5FPhe, pentafluoro-Phe; 5FPhAc, pentafluoro-phenylacetyl; Abu, α-aminobutanoyl; Ada, 12-aminododecanoyl; Amp, para-amidinophenylalanine; Aoc, 8-aminooctanoic acid; Cpa, 4-chloro-Phe; Har, homoarginine; Nle, Norleucine; Orn, Ornithine; PhAc, phenylacetyl; Tyr(Me), O-methyl-Tyr.

MIA-690 were tested in receptor binding assay on human pituitary membrane fractions using ^{125}I-labeled [His^1, Nle^{27}]hGH-RH-(1-32)-NH_2. The results of these measurements are listed in Table 2. The IC_{50} values of most analogs were in the range of 0.07−0.61 nM. GHRH analogs AVR- 235, AVR-352, AVR-353, AVR-354 and AVR-553 showed higher binding affinities to the membrane receptors of human pituitary cells. In comparison to the binding affinity of previous best analog MIA-602, the relative affinities of these five AVR analogs were 2.38, 3.44, 4.43, 2.58 and 2.21 times higher, respectively. In addition, the binding affinity of analogs AVR-333 and AVR-552 was also about 1.5 times higher than that of MIA-602. Most AVR GHRH antagonists showed higher binding affinity than MIA-690, one of the top previous MIA class GHRH antagonists with the greatest antitumor activities.

3.3. *Inhibition of cell proliferation in vitro*

The inhibitory activities of several new GHRH antagonists AVR-235, AVR-333, AVR-352, AVR-353, AVR-354, AVR-540, AVR-543 and AVR-553, were tested on the proliferation of various human cancer lines including lung, (HCC827 and H460), pancreatic (CFPAC-1 and PANC-1), stomach (NCI N87), colon (HT-29) and breast (MX-1) were evaluated *in vitro* at concentrations of 1 μM, 2 μM and 5μM, and compared to those of MIA-602. The antagonists suppressed the viability of cancer cells in a concentration-dependent manner in most tests (Fig. 1). Treatment of HCC827 cells with AVR-352, AVR-353, AVR-354 and AVR-553 at concentration of 5μM decreased cell growth by 67.4 %, 58.4 %, 73.7 % and 73.5 % respectively in comparison to 31.7 % exerted by MIA-602 (Fig. 1A). Treatment of N87 cells with 5 μM AVR-235, AVR-352 and AVR-353 reduced cell growth by 52.6 %, 56.0 % and 76.6 % in comparison to 20.5 % shown by MIA-602 (Fig. 1B). Incubation of CFPAC-1 cells with 5 μM AVR-333 or AVR-353 decreased cell growth by 64.5 % and 72.2 % in comparison to 18.8 % inhibition induced by MIA-602 (Fig. 1C $p < 0.01$). In HT29 colon cells (Fig. 1D), treatment with 5 μM AVR-352 produced a greater inhibition of tumors than 5 μM MIA-602. In MX-1 cells (Fig. 1E), treatment with AVR-352, AVR-353 and AVR-354 also significantly reduced cell growth by 77.6 %, 68.7 % and 60.6 % respectively, in comparison to 37.4 % inhibition induced by MIA-602. Table 2 summarizes the inhibitory potency of AVR-compounds relative to MIA-602 in different cell lines, based on the inhibitory effects on cell viability exposed to 5μM of antagonists. Antagonists AVR-235, AVR-333, AVR-352, AVR-353 and AVR-354 showed greater inhibitory effects on the cancer cell lines tested than MIA-602.

Table 2
IC_{50} values of new hGH-RH analogs to membrane receptors on human anterior pituitary cells.

Peptide No.	Peptide code	IC_{50}[a] (nM)
1	MIA-602	0.31 ± 0.04
2	MIA-690	0.59 ± 0.07
3	AVR-104	0.59 ± 0.08
4	AVR-107	0.57 ± 012
5	AVR-116	0.54 ± 0.03
6	AVR-120	0.48 ± 0.10
7	AVR-201	0.61 ± 0.13
8	AVR-234	0.45 ± 0.06
9	AVR-235	0.13 ± 0.02
10	AVR-321	0.23 ± 0.03
11	AVR-322	0.25 ± 0.05
12	AVR-332	0.34 ± 0.08
13	AVR-333	0.20 ± 0.07
14	AVR-352	0.09 ± 0.02
15	AVR-353	0.07 ± 0.01
16	AVR-354	0.12 ± 0.05
17	AVR-542	0.24 ± 0.09
18	AVR-543	0.25 ± 0.11
19	AVR-552	0.20 ± 0.08
20	AVR-553	0.14 ± 0.07
21	AVR-620	0.31 ± 0.05

[a] IC_{50} values represent mean ± SEM of two to three determinations.

3.4. *Oncological studies in vivo*

The compounds which displayed high inhibitory potencies *in vitro*, were further tested *in vivo* for their suppressive effects on the growth of human cancers xenografted into nude mice. Based on the results in Tables 2 and 3, we selected the AVR analogs with higher binding affinity to GHRH receptors on human anterior pituitary cells for further testing for anti-tumor and anti-inflammatory activity *in vivo*.

Table 4 shows a comparison of inhibition of the growth of tumors after therapy with GHRH antagonist MIA-602 and new GHRH antagonists of AVR class. Various human cancers were tested including lung (HCC827 and H460); pancreatic (PANC-1 and CFPAC-1), gastric (NCI-N87), colorectal (HT-29), breast (MX-1), ovarian (SK-OV-3 and OVCAR-3), prostatic (PC-3) cancers and glioblastoma (U87).

As shown in Table 4, AVR- 353 at the dose of 5 μg /day inhibited tumor growth of HCC 827 by 71.0 %, PANC-1 by 52.9 %, CFPAC-1 by 46.2 %, NCI-N87 by 65.0 % and HT-29 by 36.3 %; which is better than MIA-602 at the same doses (49.5 %, 41.1 %, 19.5 %, 52.1 % and 15.5 %, respectively). AVR- 353 also showed superior inhibitory effects to MIA-602 in H460 cancers. Antagonist AVR-352 at 5 μg/day displayed better inhibitory effects in HCC827 and CPFAC-1 cancers than MIA-602, while antagonist AVR-354 was more potent on HT-29 and MX-1. In stomach cancer N87, AVR-235 at the dose of 2 μg /day, AVR-543 and AVR-553 at dose of 5 μg /day also showed greater inhibition than MIA-602 at 5 μg /day (64.9 %, 64.9 %, and 68.3 % *vs* 52.1 % respectively). In addition, we have obtained dose response inhibition of growth of HCC827, PANC-1, CFPAC-1 and N87 cancers with antagonist AVR-353 between 2.5 μg, 5 μg and 10 μg (Table 4 and Fig. 2A–D). These results support the merit of further investigation of GHRH antagonists of AVR class in models of human cancers, particularly in pancreatic and lung cancers. In studies with ovarian cancer (SK-OV-3), AVR-352 at the dose of 2.5 μg or 5 μg /day showed better inhibition (45.0 % and 67.7 % respectively) than MIA-602 at 5 μg /day (38.0 %), while AVR-353 had a weaker antitumor effect. In ovarian cancer OVCAR-3, AVR-352 at dose of 5 μg /day induced better inhibition (58.4 %) than MIA-602 (44.4 %). In prostatic cancer (PC3), AVR-352 and AVR-354 at the dose of 5 μg /day also induced better inhibition (50.0 % and 58.4 % respectively) than MIA-602 (45.5 %). Interestingly, AVR-333 and AVR-352 at 5 μg/day displayed higher antitumor activity in human glioblastomas U87 than MIA-602 (56.7 % and 66.1 % *vs* 48.3 % respectively) while AVR-353, AVR-354 showed similar effects to MIA-602 (Table 4).

3.5. *Inhibition of GH release in vitro and in vivo after administration of GHRH antagonists of AVR class*

The GHRH antagonists which displayed high inhibitory potency in oncologic experiments, were further tested for their effects on GH release *in vitro* using primary cultures of rat pituitaries. Fig. 3A shows that GH-release induced by 1 nM GHRH was partially inhibited by 20 nM MIA-602 by 22 %, AVR-235 by 40 %, AVR-352 by 28 % and AVR-353 by 35 % respectively. The results demonstrated that these AVR GHRH antagonists exhibit inherent inhibitory activity on GH release from the pituitary. Fig. 3B shows that intravenous administration of 5 μg/kg of AVR-352 or AVR-353 into rats caused a significant reduction (28.0 %, 32.0 % respectively) in the serum concentration of GH compared to the GH levels in the animals given vehicle control. The inhibition of GH release induced by AVR compounds was greater than that by MIA-602 (22.7 %) at the same doses.

3.6. *The mechanism mediating the enhanced anticancer effects of GHRH antagonists*

Our previous studies have revealed that antagonist MIA-602 inhibited tumor progression of lung cancer HCC827 by modulating the

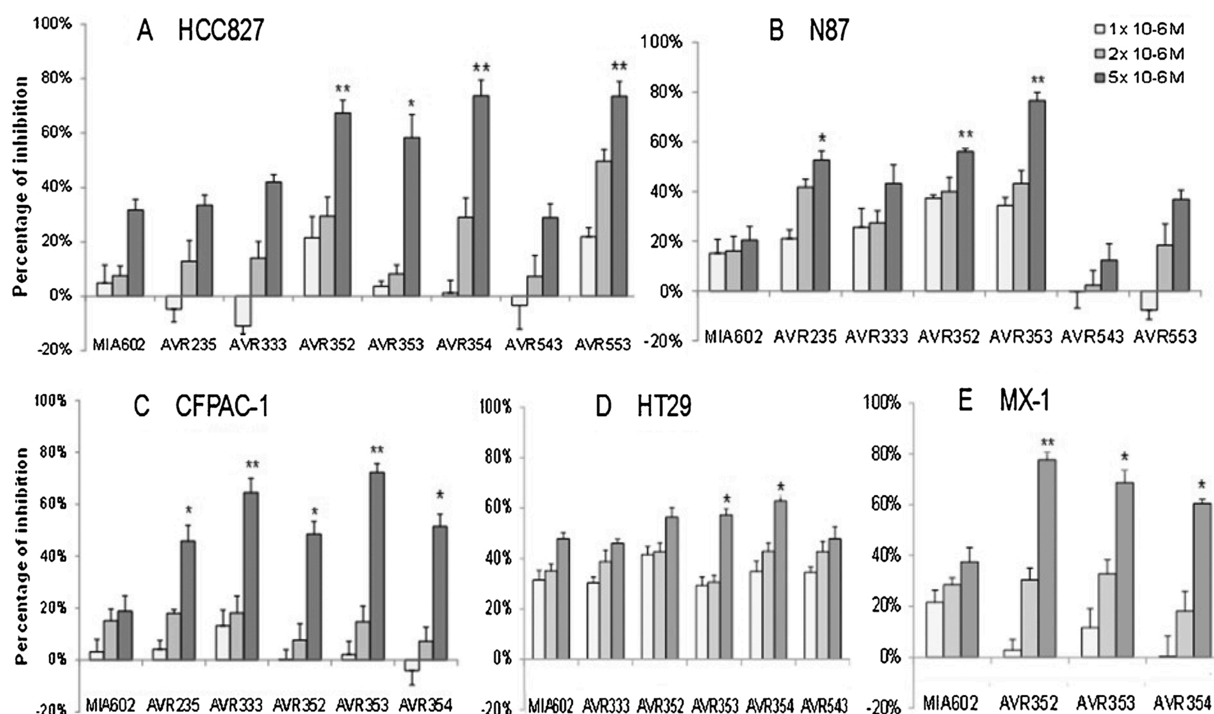

Fig. 1. Evaluation of the inhibitory activity of AVR class of GHRH antagonists *in vitro* in comparison with Miami class GHRH antagonist MIA-602. Human cancer lines including lung HCC827 (A), stomach N87 (B) pancreatic CFPAC-1 (C), colon HT29 (D) and breast MX-1 (E) were treated with GHRH antagonists at concentration of 1 μM, 2 μM and 5μM for 72 h. The percentages of inhibition of cell proliferation were presented. *p < 0.05, **p < 0.01, the differences between GHRH antagonists of AVR class and MIA-602. Experiments were performed with quintuplicates at each concentration, and the experiments were repeated at least twice.

Table 3
In vitro relative inhibitory potency of AVR-class of GHRH antagonists in comparison to MIA-602 assayed in different cancer cell lines.

GHRH Antagonists	HCC827	H460	CFPAC-1	PANC-1	NCI-N87	HT-29	MX-1
MIA-602	1	1	1	1	1	1	1
AVR-235	1.08	1.041	1.497*	1.111	1.671*	0.968	–
AVR-333	1.177	1.12	2.288**	1.061	1.400	–	–
AVR-352	2.010**	1.272	1.576*	0.988	1.809**	1.197	2.793**
AVR-353	1.641*	2.94**	2.922**	1.19	3.404***	1.222*	2.001*
AVR-354	2.595**	1.608*	1.670*	–	–	1.403*	1.594*
AVR-540	1.121	0.537	–	–	0.908	0.999	–
AVR-543	0.961	0.561	–	–	1.260	–	–
AVR-553	2.582**	0.749	–	–	1.158	–	–

The data were calculated based on the inhibition of cell proliferation in cancer cell lines treated with antagonists at concentration of 5 μM for 72H. -, not performed, * p < 0.05, ** p < 0.01, ***p < 0.001.

expression of GHRH receptors, effector proteins in cell cycle G1/S transition check point, and in PAK1-STAT3 and cAMP/CREB signaling pathways [27]. The proteins were extracted from the tumor tissues retrieved from mice xenografted with lung cancer HCC827 treated with MIA-602 or AVR-353 at dose of 5 μg (Table 2 and Fig. 2A) and analyzed by Western blot analysis. As shown in Fig. 4, treatment with AVR-353 significantly down-regulated the expression of GHRH-R, SV1, pSTAT3/STAT3, PAK1, pCREB/CREB; cyclin D1, cyclin D2, cyclin-dependent kinases (CDKs; CDK4, and CDK6); and upregulated the expression of $p27^{kip1}$ (the conditional inhibitor for CDK4/6). GHRH antagonist AVR-353 displayed similar but greater effects in comparison to MIA-602.

3.7. Anti-inflammatory effects of AVR class

GHRH antagonists exhibit inhibitory effects on several inflammatory cytokines in the cancer microenvironment [32]. We aimed to test these effects in an in-mouse lung inflammatory model. To achieve this goal, we developed granulomas in the lungs of mice. Mice were randomly grouped and treated with vehicle solution, MIA-602 (5 μg/day), AVR-352 (5 μg/day) or methyl prednisolone (138 μg/day) for three weeks. Fig. 5 shows the expression of IL2, OAS1 (2′-5′-Oligoadenylate Synthetase 1) and ISG15 (Interferon-stimulated gene 15) in the lung tissues retrieved from the animals, analyzed by Western blots. The levels of all three proteins were decreased in comparison with untreated tissues, however only treatment with AVR-352 resulted in significant reductions (55.8 %, 68.7 % and 63.8 % for IL-2, OAS1 and ISG15 respectively, p < 0.05). The results confirmed that AVR-352 has greater anti-inflammatory activity in comparison with MIA-602 or methyl prednisolone.

3.8. Structures of best GHRH antagonists of AVR class

The structures of analogs, which displayed highest receptor binding affinities, are listed in Table 4. Analogs AVR-352 and 353 appeared to be the most potent GHRH antagonists based on their inhibitory effects on tumor growth in the *in vitro* and *in vivo* tests and had higher antitumor activity than MIA-602.

Table 4
Oncological *in vivo* tests on AVR GHRH antagonists.

Cancer cell lines	Analog	Dose (µg/ Day) weeks	Tumor Growth Rate (Vn/V0)†	% Inhibition
HCC 827 NSCLC-ADC	Control	–	13.12 ± 1.61	–
	MIA-602	(5 µg/day) 7 w	6.62 ± 0.88	49.5*
	AVR-235	(5 µg/day) 7 w	7.71 ± 1.78	41.2
	AVR-333	(5 µg/day) 7 w	4.07 ± 0.60	69.0**
	AVR-352	(5 µg/day) 7 w	3.71 ± 1.02	71.7**
	AVR-353	(5 µg/day) 7 w	3.8 ± 0.86	71.0**
HCC 827 NSCLC-ADC	Control	–	8.41 ± 1.00	–
	MIA-602	(5 µg/day) 7 w	5.23 ± 1.43	37.8*
	AVR-353	(2.5 µg/day) 7 w	5.48 ± 0.75	35.1*
	AVR-353	(5 µg/day) 7 w	4.21 ± 0.55	50.0**
	AVR-353	(10 µg/day) 7 w	3.59 ± 0.73	57.3**
H460 NSCLC	Control	–	33.82 ± 2.36	–
	MIA-602	(5 µg/day) 4 w	16.03 ± 1.64	52.6***
	AVR-352	(5 µg/day) 4 w	17.38 ± 3.07	48.6***
	AVR-353	(5 µg/day) 4 w	13.35 ± 2.52	60.5***
PANC-1 Pancreatic	Control	–	26.71 ± 6.24	–
	MIA-602	(5 µg/day) 7 w	15.73 ± 3.92	41.1
	AVR-333	(5 µg/day) 7 w	13.84 ± 1.69	48.2
	AVR-352	(2.5 µg/day) 7 w	20.73 ± 4.20	22.4
	AVR-352	(5 µg/day) 7w	14.57 ± 2.18	45.4
	AVR-353	(2.5 µg/day) 4 w	14.83 ± 2.16	44.5
	AVR-353	(5 µg/day) 4 w	12.57 ± 2.68	52.9*
	AVR-353	(10 µg/day) 4 w	10.05 ± 2.04	62.4*
CFPAC-1 Pancreatic	Control	–	19.71 ± 3.13	–
	MIA-602	(5 µg/day) 7 w	15.88 ± 3.74	19.5
	AVR-352	(2.5 µg/day) 7 w	16.17 ± 1.66	18
	AVR-352	(5 µg/day) 7 w	14.48 ± 1.93	26.5
	AVR-353	(2.5 µg/day) 7 w	12.94 ± 2.69	34.3
	AVR-353	(5 µg/day) 7 w	10.78 ± 1.61	46.2*
NCI-N87 Stomach	Control	–	8.92 ± 1.93	–
	MIA-602	(5 µg/day) 4 w	4.27 ± 0.72	52.1*
	AVR-235	(2 µg/day) 4 w	3.12 ± 0.41	64.9**
	AVR-353	((2 µg/day) 4 w	4.33 ± 0.32	51.4*
	AVR-353	(5 µg/day) 4 w	3.12 ± 0.50	65.0**
	AVR-543	(2 µg/day) 4 w	4.61 ± 1.00	48.3
	AVR-543	(5 µg/day) 4 w	3.13 ± 0.40	64.9**
	AVR-553	(2 µg/day) 4 w	5.33 ± 0.57	40.3

Table 4 (*continued*)

Cancer cell lines	Analog	Dose (µg/ Day) weeks	Tumor Growth Rate (Vn/V0)†	% Inhibition
	AVR-553	(5 µg/day) 4 w	2.83 ± 0.42	68.3**
HT-29 Colorectal	Control	–	13.25 ± 1.65	–
	MIA-602	(5 µg/day) 6 w	11.2 ± 1.75	15.5
	AVR-353	(2 µg/day) 6 w	8.03 ± 1.08	39.4*
	AVR-353	(5 µg/day) 6 w	8.43 ± 1.46	36.3*
	AVR-354	(5 µg/day) 6 w	8.48 ± 1.59	36.0*
MX-1 Breast	Control	–	11.1 ± 1.65	–
	MIA-602	(5 µg/day) 4 w	6.77 ± 1.19	39.2*
	AVR-352	(5 µg/day) 4 w	6.73 ± 1.08	39.5*
	AVR-353	(5 µg/day) 4 w	7.74 ± 1.10	30.5*
	AVR-354	(5 µg/day) 4 w	4.88 ± 0.45	56.2**
U87 Glioblastoma	Control	–	31.78 ± 7.65	–
	MIA-602	(5 µg/day) 5 w	16.42 ± 3.49	48.3
	AVR-333	(5 µg/day) 5 w	13.76 ± 2.93	56.7*
	AVR-352	(2 µg/day) 5 w	16.94 ± 3.19	46.7
	AVR-352	(5 µg/day) 5 w	10.77 ± 1.67	66.1*
	AVR-353	(2 µg/day) 5 w	17.50 ± 2.60	44.9
	AVR-353	(5 µg/day) 5 w	17.00 ± 4.51	46.5
	AVR-354	(5 µg/day) 5 w	16.36 ± 2.98	48.5
SK-OV-3 Ovarian	Control	–	11.62 ± 1.53	–
	MIA-602	(5 µg/day) 7 w	8.18 ± 0.68	38.0*
	AVR-352	(2.5 µg/day) 7 w	6.23 ± 1.36	45.0**
	AVR-352	(5 µg/day) 7 w	4.59 ± 0.59	67.7**
	AVR-353	(2.5 µg/day) 7 w	9.36 ± 1.04	19.1
	AVR-353	(5 µg/day) 7 w	8.07 ± 1.35	30.9
OVCAR-3 Ovarian	Control	–	7.35 ± 0.44	–
	MIA-602	(5 µg/day) 9 w	4.86 ± 0.27	44.4**
	AVR-352	(2.5 µg/day) 9 w	4.73 ± 0.44	35.9*
	AVR-352	(5 µg/day) 9 w	3.88 ± 0.28	58.4***
	AVR-353	(2.5 µg/day) 9 w	5.44 ± 0.25	25.7*
	AVR-353	(5 µg/day) 9 w	4.87 ± 0.21	46.9**
PC-3 Prostate	Control	–	13.4 ± 0.79	–
	MIA-602	(5 µg/day) 8 w	7.57 ± 0.42	45.5**
	AVR-352	(5 µg/day) 8 w	7.41 ± 0.38	50.0**
	AVR-353	(5 µg/day) 8 w	7.57 ± 0.50	39.9**
	AVR-354	(5 µg/day) 8 w	5.22 ± 0.29	58.4**

ADC, adenocarcinoma. †Vn, mean tumor size in cubic millimeters at the end of treatment; V0, mean tumor size in cubic millimeters at the beginning of treatment. * $p < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Growth hormone-releasing hormone (GHRH) is a hypothalamic peptide neuro-hormone that regulates the release of growth hormone (GH) from the pituitary gland [2,3]. Acting as an autocrine/paracrine growth factor, GHRH also exerts direct effects on various extra-pituitary cells or tissues, mediated by GHRH receptors (GHRH-Rs) [18]. In the past decades, many analogs of GHRH have been synthesized [6–20]. Our laboratory has developed several classes of GHRH antagonists which show potent inhibitory effects on the growth of various tumors [6–8, 14–20]. In our previous publications we reported the design and syntheses of a group of GHRH antagonists of MIA series with greatly increased biological and anticancer activity [14]. Among these antagonists, MIA-602 and MIA-690 showed powerful antitumor activity in human cancers xenografted in nude mice [14]. Antagonist MIA-602 has been tested in a variety of human cancer models including androgen-dependent and castration-resistant prostate cancer [33] glioblastoma [34], experimental ovarian cancers [35], triple negative breast cancers [32], small cell- and non-small cell lung cancers [27], gastric cancer [36], esophagus cancers [37], mesothelioma [38], thyroid cancer [39], melanoma [40] and leukemia [41]. These new AVR GHRH antagonists might complete the armamentarium for therapy of prostate cancer, since they could be used in castration resistant prostate cancer, which no longer responds to androgen deprivation therapy with agonists of LHRH [42]. These AVR GHRH antagonists can obviously be also used for therapy of many types of other cancers such as, lung, bladder, pancreatic and colorectal cancers. Interestingly, MIA-690 exhibited beneficial effects on inhibition of amyloid aggregation and proteotoxicity in a transgenic mouse model of Alzheimer's disease [43]. This effect may be in part due to the anti-inflammatory activity of this class of analogs [44].

The paper describes the syntheses of a new AVR class of GHRH antagonists, in which further modifications of the structures of MIA-602 and MIA-690 were introduced. Our strategy to induce higher activity included the design and synthesis of three groups of modified AVR compounds as follows: Firstly, we synthesized compounds based on the structure of MIA-602 containing $Arg^{11, 20}$ and $Lys^{12,,21}$ to replace $His^{11, 20}$ $Orn^{12, 21}$; because $His^{11, 20}$ $Orn^{12, 21}$ were in the sequence in our previous antagonist JV-1-38 which showed higher inhibitory effects on GH release, but weaker antitumor activity in comparison to MIA-602 or MIA-690 [51]. The analogs with replacements such as AVR-107, AVR-104, AVR-120 displayed weaker antitumor activity in comparison with MIA-602. In the second AVR group we introduced NHCH3 at the C-terminus (analog AVR-201, AVR-234 and AVR-235); interestingly analog AVR-235 containing additional modification with 5FPhAc at the N-terminus and $5FPhe^6$ showed higher affinity in the receptor binding assay and promising antitumor activity in stomach cancer (Tables 3 and 4) [45]. In the third group of AVR compounds, we further modified NHCH3 with fatty amino acid Ada at C-terminus, which resulted in a series of analogs (AVR-352, AVR-353 and AVR-354) with both greater antitumor activity on various cancer cells and higher affinities to the pituitary GHRH receptors, as well as inhibitory effects on GH release (Tables 2–4 and Fig. 3). The molecular masses, of analogs as determined by mass spectrometry, matched very well with their molecular weight as calculated from their structures (Table S1, Figs. S1 and S2).

As shown in Table 2, eleven of nineteen tested GHRH antagonists of AVR class, with different combination of modification in the structures, presented a higher binding affinity to GHRH receptor than MIA-602, and nearly all of the AVR compounds had higher binding affinity than MIA-690, which is a potent GHRH antagonist with important antitumor

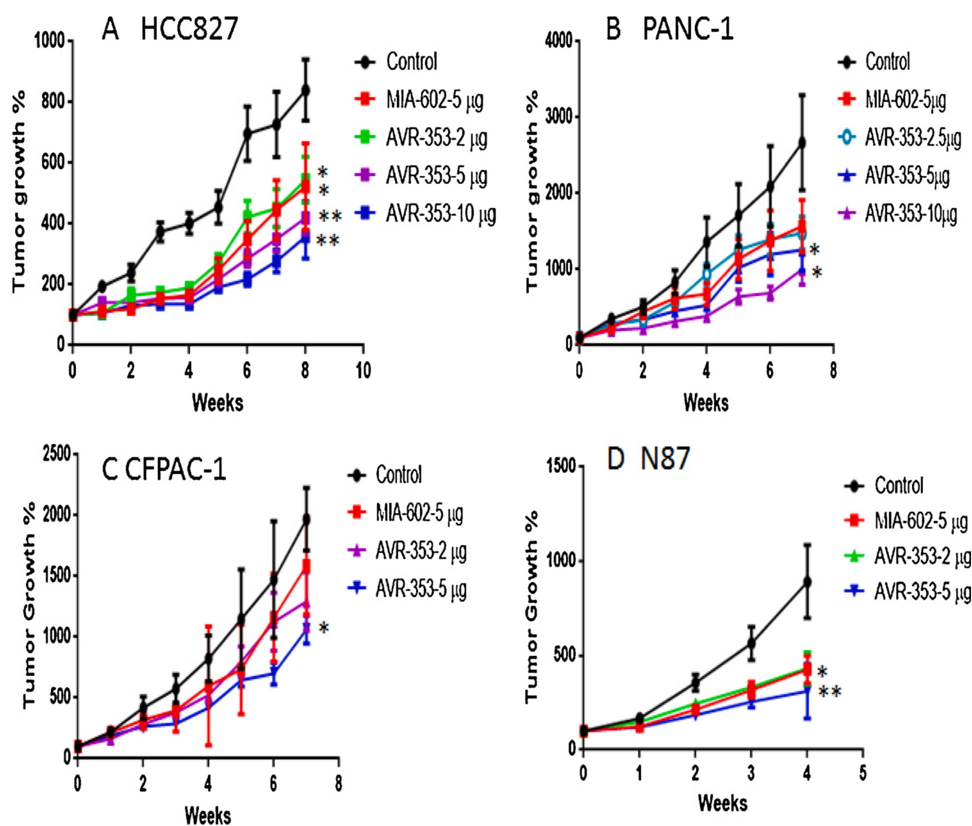

Fig. 2. Inhibition of tumor growth *in vivo* by the treatment of GHRH antagonists AVR compounds or MIA-602. Tumor growth rate (Vn/V0) in nude mice xenografted lung (A), pancreatic (B and C) and stomach (D) cancer cells is presented. The percentage of inhibition of tumor growth at the termination of each experiment was summarized in Table 3. *$p < 0.05$, **$p < 0.01$.

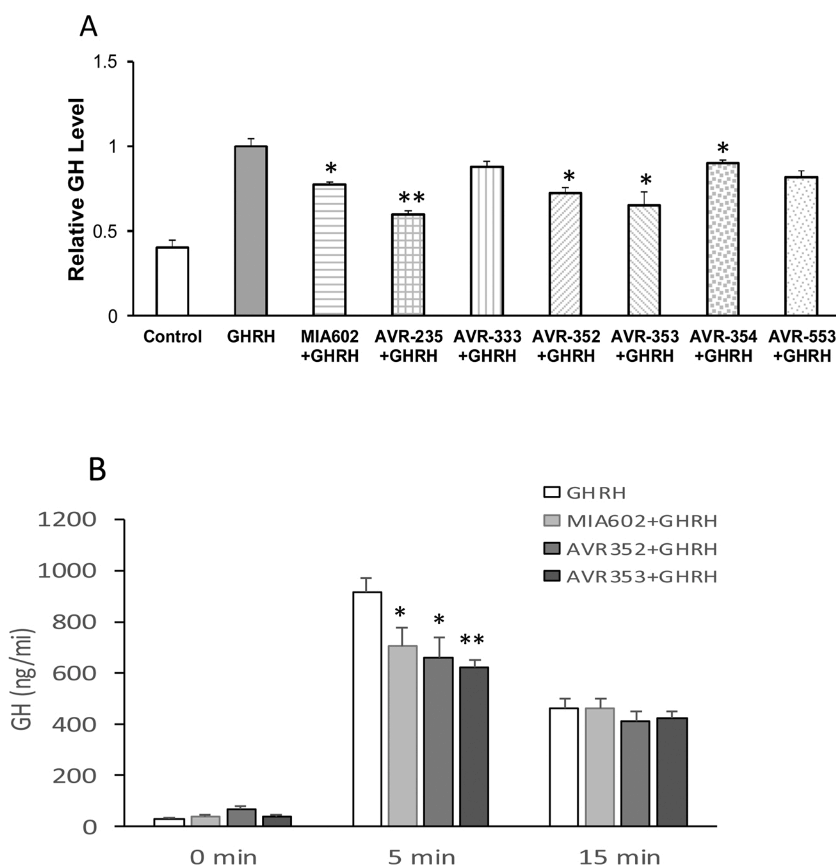

Fig. 3. Inhibition of GHRH-induced GH release by GHRH antagonists *in vitro* and *in vivo*. (A) Inhibitory effects of 20 nM GHRH antagonists MIA-602 and AVR-antagonists on the release of GH from rat pituitary cells *in vitro* induced by GHRH (1 nM). * $p < 0.05$, ** $p < 0.01$. (B) *In vivo* testing was carried out in rats by intravenous (i.v.) administration of GHRH antagonists MIA-602, AVR-352 or AVR-353 (5μg/kg followed by an iv injection GH-RH (1-29)NH_2 3 μg/kg 5 min later). Controls received vehicle solution. Serum GH levels in animals treated with antagonists were compared at 0, 5 and 15 min following the administration of GHRH. *$p < 0.05$, **$p < 0.01$.

effects in experimental tumor models [14,27]. The structures of the five most potent AVR analogs listed in Table 5 showed the advantages of the modifications designed. The antitumor effects of these five antagonists: AVR-235, AVR-333, AVR-352, AVR-353 and AVR-354 were evaluated in various human cancer cell lines and compared directly to MIA-602 in the *in vitro* cell proliferation assay (Fig. 1 and Table 3). In all cancer cell lines tested these AVR-compounds exerted similar or more potent inhibitory effects to MIA-602. Particularly AVR-352 and AVR-353, presented relatively higher inhibitory action than MIA-602 in all tested human cancer cell lines (Table 3). Greater inhibitory effects on tumor growth in the antineoplastic study *in vivo* also indicated a higher potency of AVR-compounds than of MIA-602. However, the relative inhibitory potency varies in different human cancer models with other tested compounds (Table 4). Interestingly, AVR-352 and AVR-353 were more potent, or as potent as MIA-602 in the inhibition of tumor growth in all the tested tumor models including lung HCC827 and H460, pancreatic PANC-1 and CFPAC-1, stomach NCI-N87, colorectal HT-29, breast MX-1, glioblastoma U87, ovarian cancer SK-OV-3 and OVCAR-3. Similar results were observed with AVR-235 in lung cancer HCC827 and stomach cancer NCI-N87, AVR-333 in PANC-1, NCI-N87 and U87; and AVR-354 in HT-29, MX-1 and U87 (Table 4). We believe that the impressive antitumor activity seen with these analogs is due in part to the increased binding affinity to GHRH receptors in comparison to MIA-602 (Table 2). GHRH antagonists also have anti-inflammatory effects in mice with pulmonary granulomatous reaction (Fig. 5). These agents significantly reduced the expression of OAS1, ISG15 and IL2, the cytokine, which plays key functions in regulating cell immunity [46]. AVR-352 showed higher anti-inflammatory property than MIA-602 (Fig. 5).

Considerable evidence already exists and continues to be accumulated in support of the concept that the downregulation of GHRH receptors or their blockade by GHRH antagonists, as well as by GHRH agonists plays a major role in the inhibition of tumor growth, [18,27,30,36]. This downregulation appears to be similar to that exerted by LHRH agonists or antagonists on the pituitary gonadal axis [42,47] and which has been used clinically for the past 30 years for therapy of sex hormone dependent tumors. Studies on different classes of GHRH-R antagonists on cancers have demonstrated their abilities to modulate multiple intracellular pathways involved in cellular proliferation, survival, metastasis, apoptosis and inflammation [16–18,24,27,34–39,48]. The mechanisms of GHRH-R antagonists identified include the modulation of the expression of effector proteins in the cell cycle to inhibit cell proliferation by blocking the G1 to S transition in the cell cycle, enhancing the expression of E-cadherin and β-catenin leading to less invasive cancers [27]. Other signaling pathways such as Raf/Mek/ERK1/2, PI3K/AKT, EGF/HER, PKC [49–51] pathways, and cAMP-CREB pathway [27] were also found to be associated with the inhibitory effects of GHRH antagonists on human cancer cells. These studies extended our knowledge of the possible cross talk between the binding of GHRH antagonists to its receptor and other, not yet explored cell signaling pathways in different cancers. Furthermore, GHRH-R antagonist MIA-602 has been described to modulate multiple inflammation-associated molecules in gastric and lung cancers. In the STAT3/NF-κB signaling pathway, treatment with GHRH antagonist MIA-602 results in downregulation of PAK1, a member of the p21-activated kinase family, and STAT3, a potential effector for G-protein-coupled receptors in regulation of cell progression [36,52]. A recent study [37] also revealed that SV1, splice variant of GHRH-R, is a hypoxia-driven promoter of tumor progression in esophageal squamous cell carcinoma (ESCC). The hypoxia-elevated SV1 activates key glycolytic enzyme, muscle-type phosphofructokinase (PFKM) through nuclear factor-κB (NF-κB) pathway, which enhances glycolytic metabolism and promotes progression of ESCC. The malignant actions induced by SV1-NF-κB-PFKM pathway could be reversed by MIA-602 [37]. Interestingly, the study by Liang et al. [53] revealed that NF-κB subunit p65 transcriptionally activates GHRH-R expression in human ciliary

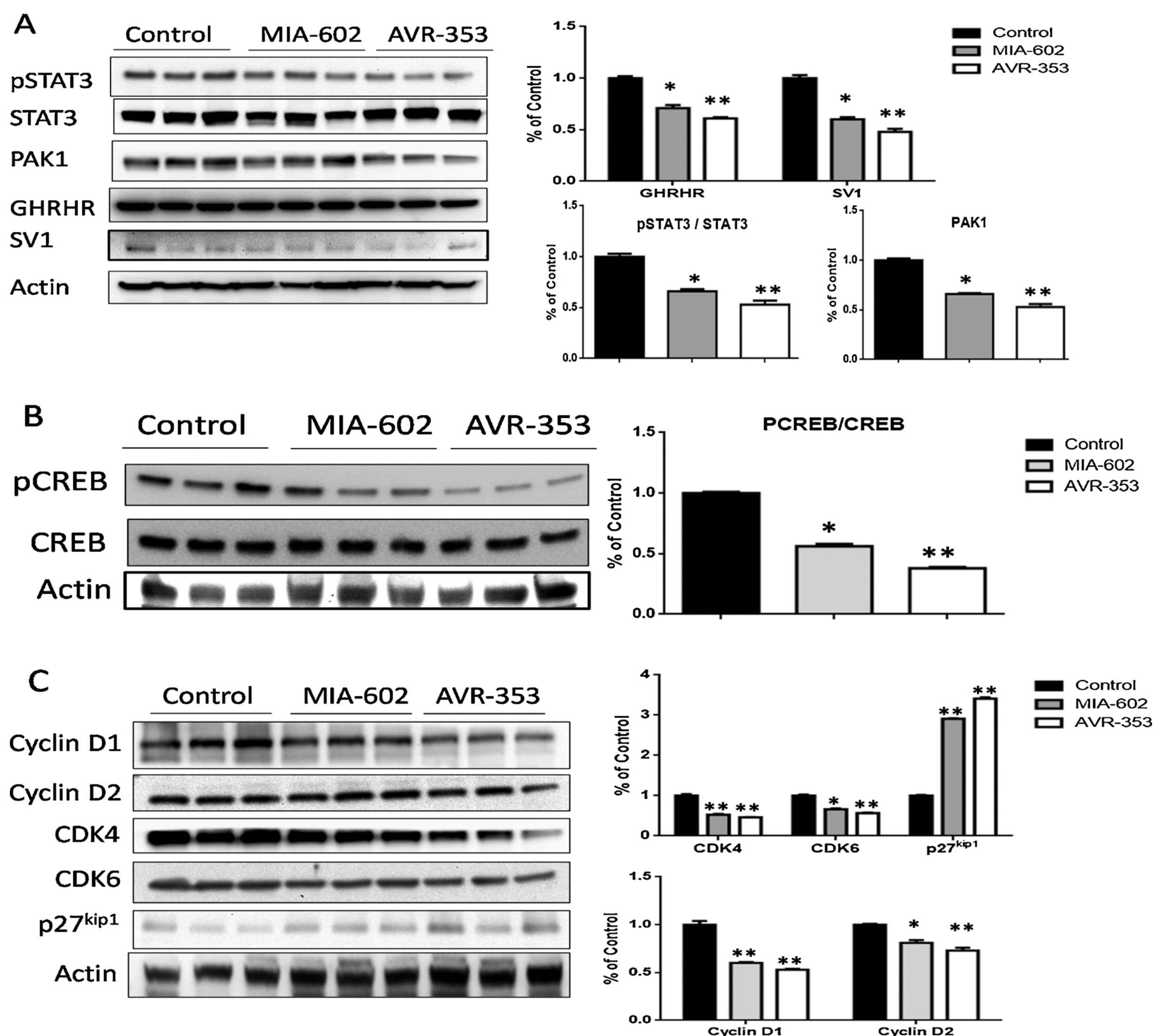

Fig. 4. Expression of GHRH receptor, effector proteins in in PAK1-STAT3 and cAMP/CREB signaling pathways and in cell cycle G1/S transition check point in lung (HCC827) tumor tissues after treatment with MIA-602 and AVR-353, including (A) GHRH-receptors: pituitary-GHRH receptor (GHRHR) and SV1, pSTAT3/STAT3 and PAK1; (B) pCREB/CREB, (C) Cyclin D1, Cyclin D2, CDK4, CDK6 and $p27^{kip1}$. The relative expression of proteins (right panels) is average from tumors retrieved from ▬mice injected with vehicle solution (n = 10); ▭mice treated with 5 μg MIA-602 (n = 12); ▭mice treated with 5 μg AVR-353 (n = 8). Representative images of WB analyses are presented (left panels). *$p < 0.05$, **$p < 0.01$.

epithelial cells, indicating a functional role of GHRH-R/JAK2/STAT3 signaling axis in acute anterior uveitis and GHRH antagonist MIA-602 attenuated expression of proinflammatory factors. In recent studies with Covid-19 pandemics, a very important finding made by D. Kotton's group [54], revealed that soon after air sacs in lung are infected with SARS-Cov-2, NF-κB signaling pathway triggers high levels of inflammation. The discovery of NF-kB's role in this deadly cascade makes promising a new therapy based on GHRH antagonists like MIA-602.

The results shown in Fig. 4 reveal that in correlation with the stronger inhibitory activity on tumor growth, AVR-353 displays greater effects in modulating the expression of effector proteins involved in the signaling pathways described in lung cancer HCC827 after treatment with MIA-602 [27]. The fact that GHRH antagonists of AVR class exert higher binding affinity to its receptor and greater inhibition of GH release and tumor growth suggests that these compounds will also display greater activities in modulating the expression of effector proteins involved in multiple signaling pathways in other cancer lines. Peptide analogs can promote and regulate distinct conformational changes in receptors [14,55], GHRH antagonists of AVR class may affect the conformation of GHRH-Rs. Multiple receptor conformations possessing distinct signaling and regulatory properties have been reported in the activation of G-protein-coupled receptor (GPCR, 56). GHRH-R is one of the members of GPCR receptor superfamily [16,17], thus it is important to reveal the possible effects of GHRH antagonists of AVR class on the conformation of GHRH receptors. It is important to study the possible changes in the mechanism of action of AVR-352 and AVR 353 in comparison with MIA-602 in individual human tumor models.

In addition, immunohistochemistry analyses in specimens of tumor samples from animal models and humans revealed the increased levels of GHRH receptors compared to the surrounding normal tissues [36]. Studies on analysis of multiple cohorts of patients with gastric cancer and ESCC [36,37], demonstrated the association between the expression of GHRH receptors in tumors, malignant properties, and poor survival, and suggested that overexpression of GHRH-receptors as an independent predictive factor for patient prognosis. The finding underscores GHRH-R as promising biomarker and therapeutic target for management of human cancers [36,37].

GHRH and its agonistic analogs have been tested in human subjects and showed little or no toxicity and do not cause any significant side

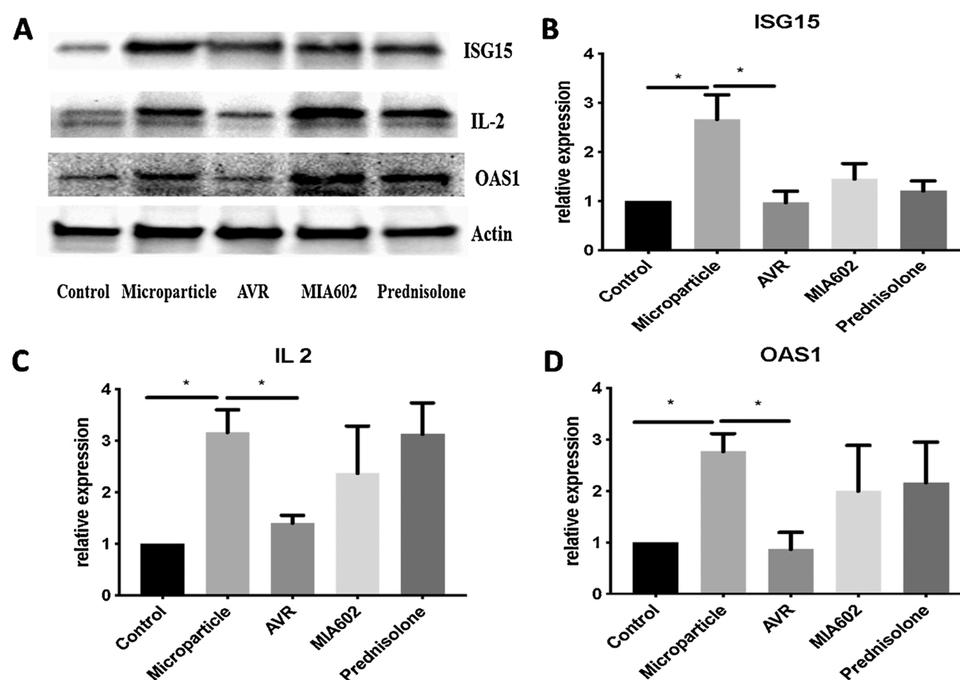

Fig. 5. Expression of IL2, OAS1 and ISG15 in the lung tissues retrieved from the animals. (A) Representative images of Western blot analysis of ISG15 (Interferon-stimulated gene 15), IL2 and OAS1 (2′-5′-Oligoadenylate Synthetase 1), and the relative expression of ISG15 (B), IL2 (C) and OAS1(D). Mean ± SEM were average from 5 animals. Lung tissues from normal (control), treated with PBS (microparticle), AVR-352, (AVR), MIA-602 and prednisolone were analyzed. * $p < 0.05$.

Table 5
Key amino acid replacements in AVR-GHRH Antagonists compared to MIA-602.

Analogs	0	6	8	10	11	12	20	21	29	30
MIA-602	PhAC-Ada	5FPhe	Ala	Tyr(Me)	His	Orn	His	Orn	Har-NH_2	
MIA-690	–	Cpa	–	5FPhe	–	–	–	–	–	
AVR-235	5FPhAC-Ada	5FPhe	Ala	Tyr(Me)	Arg	Lys	Arg	Lys	Har-$NHCH_2$	
AVR-333	–	Cpa	Asn	–	–	–	–	–	Har	Ada-NH_2
AVR-352	–	5FPhe	Ala	–	–	–	–	–	–	–
AVR-353	–	Cpa	–	5FPhe	–	–	–	–	–	–
AVR-354	–	–	–	–	–	–	–	–	–	–

– Indicates the same amino acid as shown on the upper line.

effects [30,57–64]. Khorram et al., described that subcutaneous administration of a synthetic GHRH analog [Norleucine27] GHRH (1-29)-NH2 to elderly subjects for 16 weeks resulted in activation of both immune cells [58] and somatotropic axis [59]; the only adverse side-effect was transient hyperlipidemia, which resolved at end of the study. A long-acting analog of GHRH (1-29)-NH2, CJC-1295, activates the GH/IGF axis while administrated in normal subjects [60]. Tesamorelin, the synthetic analog of GHRH (1-44)-NH2, improves cognitive function in adults with mild cognitive impairment and in healthy older adults [61,62]. In preclinical studies administration of Tesamorelin effectively reduced visceral fat and liver fat and improved liver function in HIV-infected patients [63,64]. Agonist MR-409 synthesized in our laboratory [26] showed feasible and safe in Yorkshire swine model of subacute ischemic cardiomyopathy and significantly reduced infarct size and improved diastolic function [65]. In a recent study the agonist MR-409 ameliorated chronic kidney disease-induced heart failure with preserved ejection fraction in a swine model of chronic kidney disease-induced heart failure [66]. No formal toxicity studies have been performed on AVR antagonists, the investigations have been carried out mainly on antitumor activity of MIA class and AVR class of GHRH antagonists, in those tests, no adverse effects in these antagonists have been recorded in rodents [6,7,14,18,27,67]. The toxicity tests must be done before any clinical studies. However after our extensive studies of GHRH analogs [6–8,14,18,27,30,57,67], the side effect/toxicity profile of GHRH analogs is considered as favorable in contrast to chemotherapeutic agents. We expect that the antagonist of AVR class, like MIA-602, would have little or no toxicity [18,67]. We believe that the impressive antitumor and anti-inflammatory activities of GHRH antagonists of AVR class strongly supports their potential use for clinical therapy of human cancer and other diseases. We believe that the impressive antitumor and anti-inflammatory activities of GHRH antagonists of AVR class strongly supports their potential use for clinical therapy of human cancer and other diseases.

Author contributions

R.C, A.V.S designed new antagonists; R.C, X.Z, A.V.S, MM designed research, X.Z, H.W, T.C, G.H, W.S, J.H, I.V, C.Z performed research. R.C, X.Z, G.H, M.M, A.V.S analyzed data. X.Z, M.M, A.V.S wrote the paper.

Declaration of Competing Interest

R.C, X.Z, A.V.S, H.W, W.S are co-inventors on the patent for on AVR growth hormone-releasing hormone antagonists; R.C, C.Z, M.M, A.V.S are co-inventors for therapeutic role of AVR in sarcoidosis assigned to the University of Miami, Miami, FL, and the Veterans Affairs System. The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.peptides.2021.170716.

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