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Synthesis and structure-activity studies on novel analogs of human growth hormone releasing hormone (GHRH) with enhanced inhibitory activities on tumor growth

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ABSTRACT

The syntheses and biological evaluations of new GHRH analogs of Miami (MIA) series with greatly increased anticancer activity are described. In the design and synthesis of these analogs, the following previous substitutions were conserved: D-Arg^2, Har^9, Abu^{15}, and Nle^{27}. Most new analogs had Ala at position 8. Since replacements of both Lys^{12} and Lys^{21} with Orn increased resistance against enzymatic degradation, these modifications were kept. The substitutions of Arg at both positions 11 and 20 by His were also conserved. We kept D-Arg^{28}, Har^{29} –NH_2 at the C-terminus or inserted Agm or 12-amino dodecanoic acid amide at position 30. We incorporated pentafluoro-Phe (Fpa_5), instead of Cpa, at position 6 and Tyr(Me) at position 10 and ω-amino acids at N-terminus of some analogs. These GHRH analogs were prepared by solid-phase methodology and purified by HPLC. The evaluation of the activity of the analogs on GH release was carried out *in vitro* on rat pituitaries and *in vivo* in male rats. Receptor binding affinities were measured *in vitro* by the competitive binding analysis. The inhibitory activity of the analogs on tumor proliferation *in vitro* was tested in several human cancer cell lines such as HEC-1A endometrial adenocarcinoma, HCT-15 colorectal adenocarcinoma, and LNCaP prostatic carcinoma. For *in vivo* tests, various cell lines including PC-3 prostate cancer, HEC-1A endometrial adenocarcinoma, HT diffuse mixed β cell lymphoma, and ACHN renal cell carcinoma cell lines were xenografted into nude mice and treated subcutaneously with GHRH antagonists at doses of 1–5 μg/day. Analogs MIA-602, MIA-604, MIA-610, and MIA-640 showed the highest binding affinities, 30, 58, 48, and 73 times higher respectively, than GHRH (1-29) NH_2. Treatment of LNCaP and HCT-15 cells with 5 μM MIA-602 or MIA-690 decreased proliferation by 40%–80%. In accord with previous tests in various human cancer lines, analog MIA-602 showed high inhibitory activity *in vivo* on growth of PC-3 prostate cancer, HT-mixed β cell lymphoma, HEC-1A endometrial adenocarcinoma and ACHN renal cell carcinoma. Thus, GHRH analogs of the Miami

Abbreviations: Abu, α-aminobutanoyl; Ada, 12-aminododecanoyl; Agm, agmatine; Aoc, 8-amino octanoyl; Cpa, *para*-chloro-Phe; Fpa_5, pentafluoro-Phe; GH, growth hormone; GHRH, growth hormone releasing hormone; h, human; Har, homoArg; IGF, insulin-like growth factor; LHRH, luteinizing hormone releasing hormone; Me-Ala, N-methyl Ala; MeCN, acetonitrile; PhAc, phenylacetyl; s.c., subcutaneous; TOF, time-of-flight; Tyr(Me), Tyr methyl ether.

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series powerfully suppress tumor growth, but have only a weak endocrine GH inhibitory activity. The suppression of tumor growth could be induced in part by the downregulation of GHRH receptors levels.
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1. Introduction

Cancer continues to be a major health problem in the world. In spite of the impressive progress in diagnosis, surgery, and chemotherapy, the overall cancer mortality is still high. There is a critical medical need for new drugs, that target malignant tumor cells, with few or no side effects. Therefore, synthetic analogs of natural peptides must continue to be developed for cancer therapy. Analogs of LHRH already have important applications in oncology. Somatostatin analogs on the other hand, do not adequately suppress growth hormone (GH) and the insulin-like growth factor (IGF-I) levels and somatostatin receptors are absent in many tumors [69] In contrast, various previous classes of antagonists of growth hormone releasing hormone (GHRH) suppress GH secretion, decrease the synthesis of IGF-I by the liver and other tissues, and reduce serum IGF-I levels [71,72]. IGF-I and II are involved in malignant transformation of cells, tumor progression, and metastases of various cancers [35]. GHRH antagonists also inhibit the autocrine/paracrine production of IGF-I and/or IGF-II by various tumors and lead to decreased growth of IGF-I dependent tumors [71,72]. Thus, GHRH antagonists could be used for suppressing the growth of tumors, such as osteosarcomas, that lack receptors for somatostatin [54]. Therefore, GHRH was first isolated and identified from human pancreatic tumors that caused acromegaly [19,62]. The tumoral origin of GHRH was an early hint of potential oncologic applications for GHRH analogs; an interest in developing GHRH analogs, for possible uses in cancer therapy led to our synthesis and investigation of antagonists of GHRH [71].

During the past years, multiple antagonists of human growth hormone-releasing hormone (hGHRH) have been synthesized and tested by other investigators [11,63,65–67], as well as by us [38–40,81–83,88–90]. Studies performed in our laboratory showed, that GHRH antagonists inhibit the growth of human osteosarcomas (SK-ES-1 and MNNG/HOS) [52] and small cell-and non-small cell lung carcinomas [53] xenografted into nude mice. Subsequent studies demonstrated that antagonists of GHRH also inhibit growth of many other tumors and do so by multiple mechanisms [72].

Heretofore therapeutic usages of synthetic peptides have been limited by their bioavailability, short half-life, rapid renal clearance, and lack of *in vivo* stability due to proteolytic degradation. Recently however, unique strategies have been developed to increase the therapeutic utility of peptides [67]. Strategies to protect bioactive peptides from proteolytic degradation by serum have included incorporation of non-natural amino acids, conformational constraints, large polymeric tags, or other synthetic manipulations, such as amide bond replacements. Previously, we synthesized a series of antagonists of GHRH(1-29)NH_2 acylated at the N-terminus with monocarboxylic or α, ω-dicarboxylic acids containing six to sixteen carbon atoms, in order to enhance their lipophilicity and their anticancer activities [90]. Acylation of GHRH antagonists with octanoic acid or 12-dodecanedicarboxylic acid improved the anti-proliferative effects of earlier antagonists *in vitro* [90]. These acylations combined with other substitutions in the molecule resulted in analogs that suppressed the growth of various human tumors xenografted into nude mice at doses lower than those used previously. These tumors included H460 and A-540 non-SCLC [31], PC-3 and DU-145 androgen-independent prostate cancer [23,60,75,77], MDA-PCa-2b and LuCaP-35 prostate cancer [76], human non-Hodgkin's lymphomas (NHL) [33], HEC-1A human endometrial cancer [13], HT29, HCT-116 and HCT-15 colon cancer [24,61], ES-2, and UCI-107 ovarian cancer [47], MX-1 breast cancer [7], and MDA-MB-231 human triple-negative breast cancer [73].

Fluorine substitutions have become one of the standard strategies in the pharmaceutical industry for usefully modulating the properties of compounds [20]. More than 20% of pharmaceuticals now contain one or more fluorine atoms [15,57]. It has been recognized that the presence of fluorine in compounds results in metabolic stability [48] leading to improved bioactivity and bioavailability [1,2,6,8,9]. This is due to the unique interactions provided by the fluorine atoms [91] such as, increase in the covalent radii, changed polarities and hydrogen bond acceptor ability, and altered water solubility [44]. Additionally, introduction of fluorine into small molecules can be used to reduce toxicity by blocking the formation of toxic metabolites [48] and to increase membrane permeability [16,22], binding efficacy and selectivity to receptor sites [46]. Aromatic fluorine substitution can also enhance the affinity of a molecule for a macromolecular recognition site through non-covalent interactions [12,86].

Combination of the unique physical and chemical properties of fluorine with proteinogenic amino acids represents a new approach to the design of biologically active peptides with improved pharmacological properties [74]. Due to the unique electronic properties, induced, the fluorination of amino acids has huge effects on protein stability, protein–protein as well as ligand-receptor interactions, and the physical properties of protein- or peptide-based materials [5,9,10,42,79]. It was shown that fluorinated aromatic amino acids may act as cytostatic compounds, irreversibly suppressing cell proliferation and effectively inhibiting the growth of cells, such as MCF-7, with IC_{50} values in the low micromolar range [17]. Since many peptides have shown great potential as highly active and nontoxic pharmaceuticals, incorporation of fluorinated amino acids would be of even further use to beneficially modulate their properties [64] and increase their stability in those cases where proteolytic degradation limits their medical value [43]. Although the impact of fluorination may be position dependent, and the complexity of the process does not allow any stability prediction, it was empirically demonstrated that the presence of fluorinated amino acids does have a significant influence on the proteolytic stability of peptides [1,44]. For example, a linear increase in binding affinity was observed with progressive fluorination of the aromatic ring in phenylalanine (Phe) [80], and the perfluoro-substitution of the aromatic ring in Phe may enhance useful interactions between fluorinated and non-fluorinated phenyl rings [64].

Based on these favorable physiological properties of fluorinated peptides, the proven efficacy of increasing numbers of fluorinated antitumor agents [26], and the fact that fluorinated aromatic amino acid analogs show potential for use in combination with classical cancer therapies [17], we incorporated pentafluoro-Phe at different positions into several of our GHRH analogs. These analogs proved to have anticancer activities even greater than those of our previous compounds [7,13,23,24,31,33,47,60,61,73,75–77]. In addition, to further increase anticancer activity, ω-amino acids were introduced either at the N- or at the C-terminus.

In this work, we focused on new designs of analogs with the aim of improving bioavailability, reducing renal clearance and biodegradation, and increasing the affinity to the receptors. This