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The Alpha-Fetoprotein–Derived Growth Inhibitory Peptide 8-Mer Fragment: Review of a Novel Anticancer Agent

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ABSTRACT

This review describes the antigrowth and anticancer activities of the alpha-fetoprotein (AFP)-derived growth inhibitory peptide (GIP) 8-mer fragment. The 8-amino acid peptide (GIP-8) comprises the carboxy-terminal portion of a 34-amino acid peptide (GIP-34) previously identified as an occult epitopic segment of the full-length human AFP molecule. The GIP-8 segment has been chemically synthesized, purified, characterized, and bioassayed. The purified 8-mer segment was characterized as a random coil (disordered) structure extending from a C-terminal β-hairpin that forms a horseshoe-shaped partially cyclic octapeptide; this structure can be formulated into a fully cyclic form by the addition of asparagine or glutamine residues. The pharmacophore of the octo- and nanopeptide forms is largely composed of a PXXP motif known to interact with Src-3 (SH3) domains of serine/theronine kinases. The GIP-8 has been shown to be growth-suppressive largely in estradiol (E2)-dependent neonatal and tumor-cell proliferation models and to inhibit tumor-cell adhesion to extracellular matrices. The 8-mer GIP displays antigrowth properties in immature mouse uterine cells and anticancer cell proliferation traits in estrogen receptor positive (ER^+), but not (ER^-) negative breast tumor cells. Even though its mechanism of action has not been fully elucidated, GIP-8 has been shown by computer modeling to dock with the extracellular loops of G-coupled seven transmembrane helical-like receptors, which could possibly interfere with signal transduction through MAP kinase pathways. It was apparent that the GIP-8 derived from the 34-mer GIP fragment of HAFP represented an E2-sensitive growth inhibitory motif, which allows the participation in cellular events, such as receptor binding, contact inhibition, extracellular matrix adhesion, angiogenesis, and T-cell activation. Thus, it was proposed that the 8-mer fragment derived from GIP could potentially serve as a lead compound for targeted cancer therapeutic agents of the biologic-response modifier type.

Key words: alpha-fetoprotein, growth inhibitory peptide, tumor growth, 8-mer fragment, cell adhesion, breast cancer

INTRODUCTION

High- and low abundant serum blood proteins represent circulating reserves of potentially active peptides as degradation products. Mass spectrometric analysis of the serum/plasma proteome has revealed the existence of large numbers of previously unknown peptides and protein proteolytic fragments derived from serum proteins in both low- and high concentrations.[1,2] These protein-derived fragments or peptides represent a newly recognized amplified serum peptidome, which potentially could contain a valuable source of candidate biomarkers and biotherapeutic agents. Some of the best known examples of comparable

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peptidomes, the extracellular matrix (ECM) proteins, have been exploited as potent antiangiogenic agents. Some of these peptides are derived from terminal domain segments, whereas others are encrypted within molecular folds of large proteins and become exposed following a conformational change. Thus, the containment of a class of biologically active peptide segments intrinsic within circulating proteins of body fluids appear to be a recurring motif in the field of signal transduction, modulation, and growth regulation.[3,4]

The alpha-fetoprotein (AFP) derived growth inhibitory peptide (GIP) represents a prime example of a peptide segment encrypted within a circulating blood protein.[5–7] The encrypted growth inhibitory 34-mer GIP displays a biological activity that is distinct, or even opposite, to that of the protein of origin. The GIP epitope within the human AFP molecule has also been detected by immunoassay in human clinical studies involving liver cancer and patients with birth defects.[8] The full-length, native HAFP molecule is a well-established growth-enhancing protein, in contrast to its encrypted 34-mer segment[5,7] However, growth episodes during ontogenic development require both up- and downregulation in order to fine-tune cell proliferation and differentiation events in the developing embryo/fetal units, within their constantly changing environments. Similar requirements are also found in cancer growth during periods of cell proliferation and progression, metastasis, anoxia, and apoptotic escape. Because full-length AFP is a tumor-associated fetal protein, it possesses the properties for both up- and down regulation in the internal milieux of both fetal and tumor tissues.[9–14] The vast amount of bioactive peptide segments found throughout the HAFP molecule, including GIP-34, have recently been reviewed.[15] However, the biological activities of an 8-mer segment derived from GIP has yet to be reviewed in the scientific literature.

The 34-amino-acid (AA) segment of GIP lies buried within a molecular cleft of the folded, compact, circulating native AFP.[8,16] Randomly-obtained human cord blood of full-term pregnancies and sera from hepatoma patients have been found to contain a small percentage (5%) of the already exposed GIP as an activated or "conformationally" transformed version of HAFP.[8,17] Such exposed or activated forms of AFP occur at the fetal-placental interface as a result of exposure to high concentrations of polyunsaturated fatty acids localized there.[18–20] High serum levels of E2 during pregnancy and fetal/neonatal development can also induce the conformational changes required to expose the GIP segment on compactly folded full-length HAFP[8] (Table 1). In this regard, it has been demonstrated that a host of biochemical agents, such as excess fatty acids and steroids, can induce the conformational change necessary for exposure of the encrypted GIP segment on both human and mouse full-length AFP.[8,17,21] The protein conformational change that occurs is dependent upon the AFP/ligand molar ratios that exist in that particular compartment of the embryo, fetus, placenta, or neonate (Table 1).

Even though HAFP binds very little E2,[22] high concentrations of E2 and fatty acids are capable of diffusing into the molecular crevices of compactly folded HAFP and attaching to low-affinity hydrophobic binding sites, one of which is the "midpiece region" of the GIP segment.[23] In the compactly folded circulating HAFP, the hydrophobic portions of the molecule are tucked into the inside folds of molecular crevices to enhance solubility and create an outer hydrophilic surface for the circulating serum protein.[24] On- and offloading of excessive E2 or fatty acids at the hydrophobic midpiece site could initiate exposure of this segment of HAFP aided by potential serine/threonine phosphorylation sites serine 447 and theronine 472 and a Src homology-3 (SH3) motif at the terminal end of 34-mer GIP, namely, PVNP.[8,25] Thus, the GIP site on HAFP fits the description of a "hot spot" on the molecule that is sensitive to stress/shock conditions, such as excessive ligand concentrations, high insulin levels, pH and heat extremes, and hypertonic environments.[26] In protein-protein interactions, a molecular hot-spot has been defined as a small hydrophobic region (i.e., P149b) that dominates the free binding energy of a protein segment and is flanked by hydrophilic residues on either side, such as those found in the front and tail-piece of the 34-mer GIP.[27] It is germane to this discussion that the 34-mer GIP has been reported to exhibit an AA sequence identity/similarity to a number of stress and shock-related proteins.[23] Thus, the GIP-34 segment on HAFP appears to be a stress/shock sensitive site that emerges following HAFP exposure to adverse conditions in the embryo/fetal/placental units. The stress concept has been confirmed, in that the GIP segment on AFP has recently been employed as a pregnancy biomarker for intrauterine growth retardation.*

*Bartha J, et al. Fetal Diagnosis and therapy. (In press), 2007.

Table 1. Comparison of Biological Activities Between Growth Inhibitory Peptides (GIP-34, GIP-8) and Transformed and Nontransformed Human Full-Length Alpha-Fetoprotein (590 Amino Acids)

	Human alpha-fetoprotein (AFP)		*Growth inhibitory peptide*		
Biological activity	*Nontransformed*	*Transformed*	*GIP-34*	*GIP-8*	*Refs.*
1. E2-induced uterine growth (rodents)	No effect	Inhibits	Inhibits	Inhibits	5, 7, 8 30, 40, 41
2. E2-induced fetotoxicity	No effect	Reduces deaths	Reduces deaths	Reduces deaths	25, 29, 76
3. E2-induced MCF-7 foci formation (human)	No effect	Inhibits foci	Inhibits foci	Inhibits	7, 8, 28–30
4. T3-induced tail resorption (amphibian)	No effect	Inhibits resorption	Inhibits resorption	Not tested	17, 25, 30, 76
5. Ascites fluid accumulation (mouse mammary tumor)	Slight reduction	Slight reduction in fluid and cells	Reduces fluid and cell number	Reduces fluid and cell number	7, 8, 29, 30, 76
6. Breast cancer growth	No effect	Inhibits growth	Inhibits growth	Inhibits growth	7, 16, 29,
7. E2 receptor binding (hum)	No effect	Not tested	Binds (10^{-6}M)	Nonbinding	16, 76
8. Platelet aggregation (hum)	Inhibits aggregation	Not tested	Inhibits aggregation	No effect	28, 29, 30, 76
9. Insulin toxicity (chicks)	Not done	Slight suppression	Suppression	Not tested	28, 76
10. Fetal mortality (mice)	Slight reduction	Reduces deaths	Reduces deaths	Reduces deaths	8, 16, 23, 29, 49
11. Fetal malformation (chick)	No effect	Slight effect	Slight effect	Not tested	25, 29
12. Growth retardation (chick)	No effect	Mild reduction	Induces	Not tested	25, 29
13. Litter size (mice) (E2-induced reduction)	No effect	Prevents litter reduction	Prevents litter reduction	Prevents litter reduction	25, 29, 76
14. Brineshrimp hatchability	Not tested	Not tested	Inhibits	No effect	76
15. Angiogenesis	Enhances	Not tested	Inhibits	Inhibits	76
16. Immune response regulation	Inhibits T^- and B^- cell response	Not known	Enhances immune response	Invokes natural T-cell immune response	76

E2, estradiol; T3, Trüdothyonine.

The GIP-34 segment derived from human (H) AFP is well-documented in the biomedical literature as a growth suppressor/inhibitor of both fetal and cancer cells.[23,28] The GIP is a suppressor of cell proliferation and consists of 34 AA, which can be further parsed into three smaller biologically active peptides: the front piece (12 AA), the midpiece (14 AA), and the tail piece (8 AA). In prior publications, the term GIP was equivalent with the batch production number of P149; hence, the three peptides subfragment were named P149a, P149b, and P149c, respectively.[8] The GIP-34 itself has been physiochemically characterized as an amphipathic, beta-sheet peptide in its linear configuration; however, GIP can also exist in a cyclic form by the disulfide-bridging of its two constitutive cysteine residues (Fig. 1). Both forms display growth-suppressive properties, albeit at different concentrations.[29,30] Of the three GIP subfragments, the P149c tailpiece has displayed the greatest growth inhibitory potential against estrogen-sensitive cell and animal models encompassing both ontogenic and oncogenic cell growth.[28–31] To avoid further confusion in the present review, the GIP tailpiece EMTPVNPG (previous names P211, P149c, P472, AFPep, and so forth) will simply be referred to either as GIP-8 in its cyclic form or linear form. For purposes of the review, a 9-mer (nonapeptide) version formed by cyclization of an extra

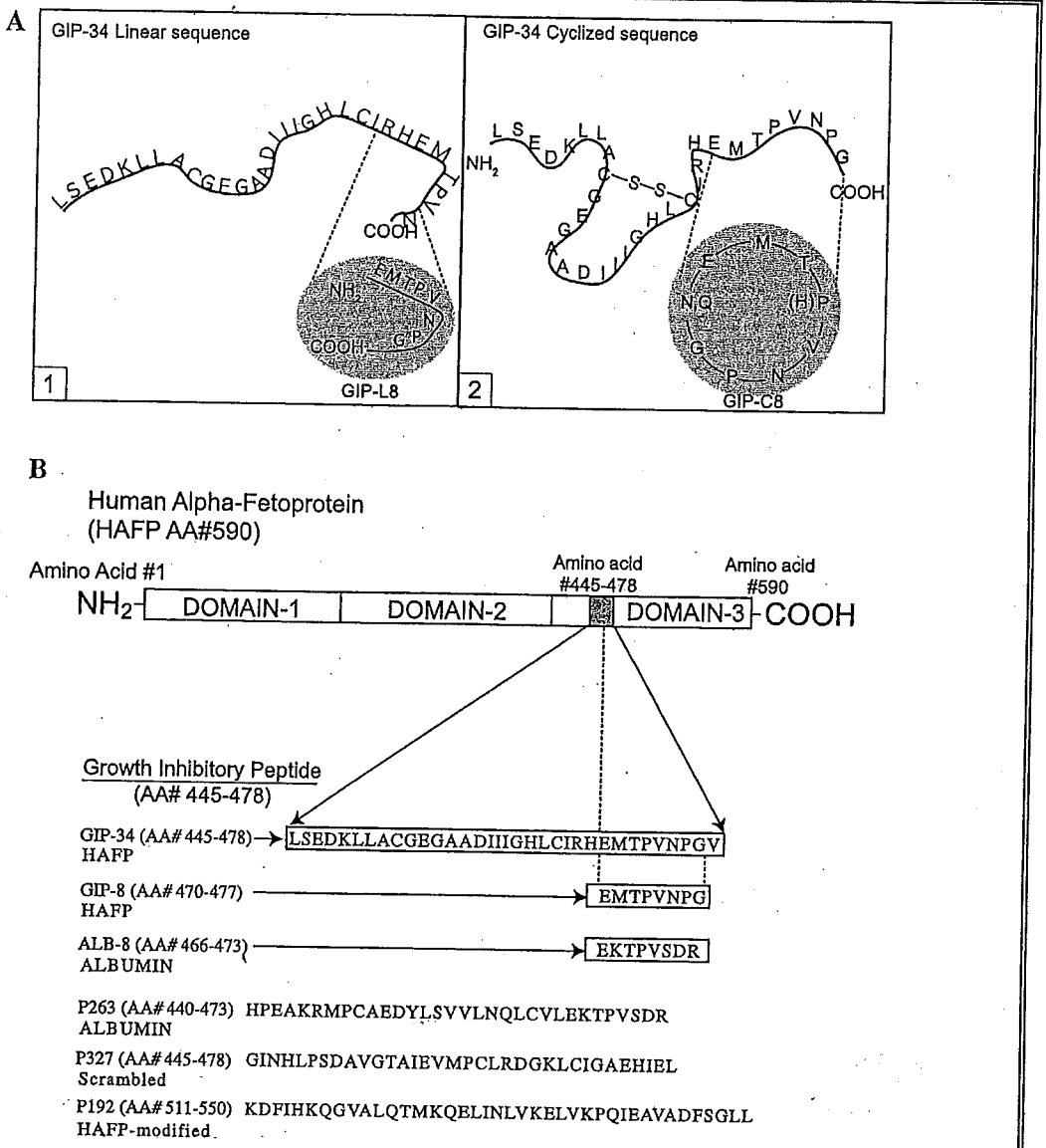

Figure 1. **(Panel A)** The linear and cyclic versions of the 34-mer and 8-mer growth inhibitory peptides (GIPs) are displayed in panels **A** and **A1** as nonsolvent computer-derived forms (see refs 31–34). **Panel A1** shows the amino acid sequence (single-letter code) of the linear versions; while **Panel A2** shows the amino acid sequence (single-letter code) of the cyclic versions. **(Panel B)** The three domains of human alpha-fetoprotein are shown in a bar configuration indicating the 590 amino-acid full-length protein. The telescoping segments of GIP-34 displayed from amino acid 445 to 478 (solid lines) are depicted in single-letter code sequences together with the tailpiece fragment comprising GIP-8 (dotted line). Control peptides P263, P327, and P192 constitute the bottom three peptides (ref. 7), while ALB-8 indicates the human albumin control peptide described in ref. 32. The minimal energy computer nonsolvent models of GIP were kindly provided by Dr. Curt Brennerman, department of chemistry, Rensselaer Polytechnic Institute, Troy, NY. GIP-L8, linear 8-mer; GIP-C8, cyclic 8-mer.

glutamine or asparagine residue will still be included under the original name of GIP-8, as the extra amino acid was intended for formulation and not for functional purposes.[31,32]

The report presented in this paper will demonstrate that both the linear and cyclic forms of GIP-8 can provide potential molecular platforms as anticancer agents, mainly against E2-dependent

growth, but also against selected nonestrogen sensitive growth systems. Although the linear and cyclic 34-mer GIP have been extensively surveyed in the biomedical literature, the GIP-8 forms have yet to be reviewed. This review will consider the origin, historical background, and development of GIP-8 as a potential estrogen-sensitive growth inhibitor/suppressor agent in comparison to the nonestrogen-dependent GIP 34-mer peptide growth regulator. Thus, the biological, biochemical, and physiochemical properties of the GIP-8 will be examined in comparison to its source of origin, the GIP-34. Finally, the possible utilization of GIP-8 in biomedical research will be explored in cancer therapeutics for a proposed use in molecular targeting. It is the propensity of GIP-8 for E2-sensitive growth inhibition that has provided the underpinning for utilization of both the linear and cyclic forms in a series of cancer and noncancer therapeutic studies described below.

Historical Background

Discovery and development of the GIP-34 segment, which includes GIP-8, was first reported in the author's laboratory in 1993 following an extensive search of Genbank amino acid matches on the HAFP molecule[7] (Table 2). Queries were performed in search of a binding site on HAFP for heat-shock proteins (HSPs-70, HSP-90), comparable to that found on the estrogen receptor (ER).[7,23,33] The HSP binding sites on the ER had already been localized at, or near, the ligand-binding domain positioned on the carboxyl-terminal side of the DNA binding domain and nuclear localization site, lying juxtaposed to the rodent E2 hydrophobic binding pocket.[34,35] An HSP-70 AA matched identity sequence on HAFP was found positioned adjacent to, and just downstream of, the E2-binding site modeled after rodent AFP.[7] More recently, HSP-70 was demonstrated to interact with, and to chaperone, full-length AFP molecule during folding of the fetal protein in the endoplasmic reticulum.[36,37] Those investigators found that HAFP coexisted in anti-HSP70 monoclonal antibody immunoprecipitates, whereas there was also HSP70 in those of the anti-HAFP monoclonal antibody precipitates. Moreover, soluble HSP-70 has been detected in amniotic fluid (AF), and together with HAFP, have been shown to positively correlate with AF-tumor necrosis factor-α levels.[38,39] The GIP-34 fragment of the presumptive HSP-70 binding site on HAFP has been chemically synthesized, purified, and its physiochemical properties characterized.[7] Following an assay of the biological activity of the synthetic GIP, growth inhibition of GIP-34 was demonstrated using a rodent immature uterine growth assay developed earlier as an assay of biological activity (Table 1) for both rodent and human AFP.[40,41]

Shortly after the discovery of GIP-34, it was reasoned that trypsin enzymatic digestion of the 34 mer GIP should result in three peptide subfragments owing to the substrate specificity of trypsin for arginine and lysine residues (Table 2). Surprisingly, the resultant 8-mer tailpiece fragment of GIP displayed an antigrowth potency in the E2-sensitive uterine assay that was comparable to, and sometimes exceeded, the 34-mer GIP.[7] From 1994 to 1995, the identity of the GIP tailpiece sequence was confirmed, subjected to bioassay, and the 8-mer peptide was synthesized as EMTPVNPG, known as P149C, now termed GIP-8[42,43] (Table 2). The linear forms of the peptides were studied from 1994 to 2000, encompassing both GIP-34 and GIP-8 and resulting in several reports.[7,44] Since that time, other investigators have added additional AAs to the segment and synthesized cyclic versions of GIP-8, named AFPep.[31,32,45–48] The anticancer potential of GIP-8 was first demonstrated in 1996, using a MCF-7 breast cancer E2-dependent cell-focus assay employing cell contact inhibition.[7] Later that year, GIP-8 was also found to inhibit a mouse mammary ascites *in vivo* tumor model; by 1998, it was found that GIP-8 also inhibited a mouse E2-dependent MCF-7 human breast cancer implanted as a kidney capsule xenograft (see 1998 row, Table 2). By the following year, all other fragments of GIP-34 had been synthesized, characterized, and bioassayed in both antigrowth and anticancer assays.[46] Outside laboratory confirmatory studies of GIP-8 as an anticancer agent appeared in 2000–2001, and multiple publications have continued to emerge.[29,30,48] A cyclized 9-mer version of the original GIP-8 was developed in 2001–2002 and demonstrated antibreast cancer activity in SCID mice implanted with MCF-7 cells.[45,47] Subsequent publications emerged in that same year demonstrating the increased scientific interest generated by GIP-8.[32,45,†] After more than a decade of development, the 8-mer fragment of the original 34-mer GIP has been confirmed as a novel E2-de-

† *Mesfin FB. Design, synthesis, and characterization of antiestrogenic, and anti–breast cancer alpha-fetoprotein-derived peptides. Ph.D. Dissertation; Albany Medical College Graduate Studies Program, Dept. of Biochemistry and Molecular Biology. In. Albany, NY, 2001.*

Table 2. Historical Background of the Development of Growth Inhibitory Peptide 8-mer Fragment (GIP-8)

Year	*Event/observation/report*	*Authors and references (#)*
1993	Concept of an AFP-growth inhibitory peptide conceived; GIP-34 synthesized, purified, characterized, and uterine bioassayed.	Mizejewski, (42, 43, 44)
1994	Trypsin digest of GIP-34; high-performance liquid chromatography purification, and uterine bioassayed antigrowth activity of HEMTPVNIPG demonstrated.	Mizejewski, (7, 42, 43)
1994	Linear EMTPVNPG (GIP-8) synthesized, purified, characterized, and uterine bioassayed.	Mizejewski, (7, 46)
1995–1996	GIP-8 suppresses growth of MCF-7 human breast cancer cells *in vitro*.	Mizejewski, et al (7)
1996–1997	GIP-8 suppresses growth of E2-independent mouse mammary ascites tumor.	Mizejewski, (17, 46, 44)
1997–1998	Two U.S. patents issued on GIP-34 and GIP-8[b,c]	Mizejewski[b]
1998	Linear GIP-8 demonstrates growth suppression of MCF-7 xenografts in nude mice in collaborative study.[a]	Mizejewski and Bennett[a]
1999	Anticancer activity of GIP-8 presented.	Mizejewski and Richardson (46)
2000	GIP-34 and GIP-8 anticancer activity reported in mouse mammary tumors *in vivo* and various human breast tumors *in vitro*.	Vakharia and Mizejewski (8)
2000	Linear GIP-8 antiuterotrophic and antibreast cancer activities confirmed.	Mesfin, et al. (31)
2001	Cyclic GIP-8 inhibits estrogen-dependent growth in MCF-7 xenografts in SCID mice.	Mesfin et al. (31) Mizejewski (33)
2001	GIP-34 and GIP-8 displayed as G-coupled receptor ligands drugs.	Mizejewski (6, 33)
2002	Cyclic GIP-8 prevents growth of E2-dependent breast cancers resistant to tamoxifen.	Bennett et al. (47, 111)
2002	Biological role of HAFP and derived peptides in anticancer therapy employing G-coupled receptors.	Mizejewski, (111)
2002	Peptides derived from alpha-fetoprotein demonstrate anticancer activities against both breast and prostate cancers.	Mizejewski, et al. (111)
2003	Alpha-fetoprotein-derived GIPs published as potential leads for cancer therapeutic agents.	Mizejewski and MacColl (28)
2003	GIP-34 and GIP-8 shown to reduce estrogen-induced fetotoxicity in mouse pups of term pregnancies.	Butterstein and Mizejewski (25)
2004	Cyclo- and linear GIP-8 shown to inhibit human breast cancer and mouse uterine growth by the PVNPG pharmacophore.	DeFreest, et al. (32)
2004	A proposed action of GIP-34 and GIP-8 was presented as inhibitory peptides of both estrogen and cytoskeleton factors.	Mizejewski et al. (23)
2005	AFP-derived GIP-34 and GIP-8 demonstrated to be biotherapeutic agents for cancer growth, progression, and metastasis.	Mizejewski, et al. (28, 29, 30, 32)
2005	GIP-8 shown to prevent induction of methylnitrosuria breast tumors in rats.	Parikh, et al. (48)
2006	AFP-derived GIP-34 and GIP-8 presented as cell-surface reactive agents in cancer proliferation, progression, and metastasis and oral activity.	Mizejewski and Butterstein (29, 71, 76)

AFP, alpha-fetoprotein

[a]Peterson JE, Bennett JA, Cavanagh KA, Mizejewski G. Studies of purified alpha-fetoprotein growth inhibitory peptides GIP-34, GIP-8 to determine anticancer activity. In: *Research Material Transfer*. Albany, NY: Collaboration and Confidentiality Agreement between Wadsworth Center and Albany Medical College, 1997.

[b]Mizejewski G, Growth inhibitory peptides. In United States Patent #5, 674,842, U.S. Patent Office Filing #US00567-4842, 1997.

[c]Mizejewski G, Methods of using growth inhibitory peptides: In United States Patent. U.S. Patent Office Filing #US0057-7-963A, 1998:707.

pendent antibreast cancer therapeutic agent (Table 2). Since the discovery of GIP-8 in 1995, many investigators have continued to pursue and characterize both the antigrowth and the anticancer activities of GIP-8.[28,29,31,45,48]

Amino Acid Sequence Matches

The GIP-8 AA sequence was subjected to a FASTA search in the Genbank (GCG Wisconsin Program) database, as described[54] (Table 3). The

Table 3. Matching of Amino Acid Region #471-480 of Human Alpha-Fetoprotein (AFP) with Conserved Sequences of Various Receptor/Binding Proteins

Protein	*Amino acid[a] sequence nos.*	*Amino acid sequences*	*Percent (%) ident/sim.*	*Percent (%) total*
Receptor/binding proteins				
Human AFP (GIP-8)	471–480	E M T P V N P G V G	100/0	(100)
Human DOPAR	253–262	L S N P V D P G V G	60/20	(80)
Human ANKY	1305–1413	H M T P L K P G X G	60/10	(70)
Rattine SOMATR	734–843	R T T P I A P G V R	50/20	(70)
Human SrctyK	1280–1289	E M A P I W P G A L	50/20	(70)
Murine Kinesin	595–604	G M T P L S P G T A	50/20	(70)
Rattine GlyR	338–347	E M T P V L G T E I	50/10	(60)
Murine FGFR	685–696	Q M T P A N P G Q L	50/10	(60)
Murine IGFIIR	30–39	R M T P L T P P L T	40/40	(80)
Rattine MeGLNR	518–529	E M K N M O P G D V	40/20	(60)
Human T-Cell CD28		R M T P L T P P L A	40/40	(80)
Transcription-associated factors				
Human AFP (GIP-8)	471–480	E M T P V N P G V G	100/0	(100)
Human Crumbs[b]	1652–1661	Q M T P V N P G V Q	70/10	(80)
Human PAX-3/FKH	512–421	I M T P V D P G V P	70/10	(80)
Human HOXG2	633–642	E M T P S T P G L Q	60/30	(90)
Murine WnT-7a[b]	315–324	Q H T P V R P G V A	60/20	(80)
Rattien Notch II	2378–2387	E M Q P L R P G A S	50/30	(80)
Human TF11D	163–174	P M T P A T P G S A	50/20	(70)
Human Src-TK	1280–1289	E M A P I W P G A L	50/20	(70)
Human Kid-TS	1145–1154	K S T G A N P G V P	50/10	(60)
Human Cad-TS	3718–3727	E M T P V L E A I I	50/0	(50)
Human FTZ-F1	628–637	K P T P I S P G Y Q	40/30	(70)
Human 1-Rel TF	528–537	E A S P S T P G R Q	40/30	(70)
Rattine Pou Dom[b]	90–91	Q K T P I F H C H T	20/40	(60)
Extracellular matrix–associated proteins				
Human AFP (GIP-8)	471–480	E M T P V N P G V G	100/0	(100)
Human α1 Coll IV	5–12	L S P L A P G N	38/50	(88)
C.Eleg Coll α3	10–17	X P P V A P G N	57/29	(86)
Human Coll XIII #5	5–12	L A P C L P G I	38/50	(88)
Human Coll-Sp	38–45	L M A P V X P Q L L	50/33	(83)
Human Elastin	400–406	P F P P G V G	71/14	(85)
Human Lamimin	88–97	Q G X D S N P G V S	40/20	(60)
Human Fibron	2070–2080	R P R P Y P P N V G	40/20	(60)
Human Coll-IV	90–98	G T P I G P G V	63/25	(88)
Human Coll-XIII	10–17	G T P I G P G V	63/25	(88)
Dros Laminin-A	660–668	L F I S S N P G V G	56/11	(67)
Human-VWF	1480–1486	T V S X V N L L L L	44/16	(60)
Chicken attach protein		L V G V G G G G V G	47/18	(65)
Rat VLA-1	755–760	S F L M L D L L L L	40/30	(70)
Human α-IP10	32–40	S N Q P V N P R S L	46/8	(54)
Human PG-IIIA	180–188	Q L G P V N P A L L	50/41	(91)

[a]Genebank-derived.

[b]homeodomain protein.

DOPAR, dopamine receptor; ANKY, ANKyrin repeat sequence; SOMATR, somatostatin receptor; SrctyK, Src tyrosine kinase; GlyR, glycine (linker) receptor; FGFR, fibroblast growth factor receptor; IGFIIR, insulin growth factor II receptor; MeGLNR, metabotropic glutamate receptor; Kinesin, motor protein (microtubule-associated); X, Unknown amino acid; Pax/Hox, homeobox protein; TF11D, transcription initiation factor (TATA-Box); Kid-TS, kidney tumor suppressor; Cad-TS, cadherin tumor suppressor; Pou Dom, pituitary-specific transcription factor; FTZ, F1-AFP transcription factor; 1-Rel TF, NF-Kappa-β transcription factor inhibitor; Src, proto-oncogene tyrosine kinase; Notch, membrane protein, ankyrin-associated, WNT-7a, frizzled receptor; AFP, alpha-fetoprotein; attach protein, attachment (adhesion) protein; Coll, collagen; C. eleg, *Caenorhabitis elegans*; Dros, *Drosophilia melanogaster*; Fibron, fibronectin; IP10, interferon-α-induced chemokine; VLA-1, integrin α1, (lamin and collagen receptor); VWF, von Willebrand's Factor.

GCG search found identity/similarity sequence matches to receptor-binding proteins, such as the fibroblast growth factor (FGF) receptor, insulin growth factor II receptor (IGFIIR), transforming growth factor-β, (TGF-β), and the dopamine (DOPA) receptor (Table 3). Other matches for transcription-associated proteins, including homeodomain proteins and FTZ-F1 (the AFP transcription factor), have been previously reported.[23] These AA matches provide evidence that the GIP fragments contain short recognition cassettes for possible G-coupled-receptor (GPR) involvement and interaction. Matches with cell-adhesion-related proteins were also found; these included collagen XIII, collagen IV, laminin, fibrinogen, and fibronectin. Finally, identities/similarities were identified with transcription-associated factors, such as Hox, c-myc, forkhead, and Pax (Table 3).

GIP-8 matches were found also with integrin-associated proteins, the ECM proteins, such as the avian attachment protein and other adhesion proteins (Table 3). Further identities were found with the integrin α/β chain proteins such as $\alpha_{11b}\beta_3$, $\alpha_1\beta_3$, and $\alpha_v\beta_1$ (Table 4).[30] The integrins serve as receptors for ECM proteins and are known to participate in cell-adhesion and migration (spreading) activities, as well as binding to disintegrins. Finally, matches were also made with ECM-associated proteins, such as the Von-Willebrand Factor, VLA-1, and PG-IIIa proteins, which are involved in cell adhesion, aggregation, and the action of metalloproteinases (i.e., the Adams family). Thus, GIP-8 shows an identity/similarity to integrins, basement membrane proteins, and ECM proteins, all of which are involved in cell-to-cell and cell-to-ECM interactions (see Tables 3 and 4).

GIP-8 physicochemical properties

Both GIP-34 and GIP-8 have been synthesized by classical F-MOC (9-fluorenylmethoxy-carbonyl)-protected solid-phase synthesis, as previously described in detail.[7,31,45] Following peptide syntheses, the lyophilized peptides were purified by reverse-phase high-performance liquid chromatography (HPLC), producing a peptide whose major peaks displayed molecular masses of 3573 (34-mer) and 844 Da (8-mer), as determined by electrospray ionization mass spectroscopy.[49–52] Cyclization of GIP-34-mer can be accomplished by reducing agents to form a disulfide bridge construct at the time of synthesis; cyclization of GIP-8 was performed using methods described by Kates et al. and others.[45,53]

Circular dichroism analyzed in the UV wavelength for GIP-34 displayed a negative maximum

Table 4. Growth Inhibitory Peptide (GIP) Amino Acid Sequences Matched in the Genbank to Various Integrin Alpha/Beta Chain Complexes and Compared to their Extracellular Matrix (ECM) Adhesion Inhibition by GIP

Integrin subunits chains	*GIP amino acid sequences**	*AA identity% (#AA)*		*ECM binding ligand*	*Tumor to ECM adhesion (% inhibition)*	*Cell/tissue and tumor distribution*
		α-chain	*β-chain*			
$\alpha_2\beta_1$	IIGHLCIRHE; MTPVNPGV	53 (17)	75 (8)	COLL, LAM	30–55	Epithelium, endothelium leucocytes
$\alpha_4\beta_7$	GEGAADIII; MTPVNPGVDI	78 (9)	56 (9)	FBN VCAM MADCAM	50	Endothelial, mucosal cells
$\alpha_6\beta_4$	IRHEMTPVPVNPGV	78 (8)	50 (12)	LAM-1 LAM-2	30–45	Keratinocyte malignancy
$\alpha_v\beta_5$	CGEGAADIIIG; HLCIRHEMTPVN; PGVGQ	67 (12)	80 (25)	VTN, FBN	95; 45–50	Epithelium, carcinoma cells
$\alpha_v\beta_8$	IRHEMTPVNPGG	67 (12)	50 (12)	Not known	Not tested	Reproductive tissues

Note. Many of the Integrins are expressed on a variety of tumor cells. Integrin data were obtained from References 5 and 30.
*, amino acid single letter code.
COLL, collagen; FBG, fibrinogen; FBN, fibronectin; LAM, laminin; VTN, vitronectin; (#AA), number of amino acid in sequence.

at approximately 201 nm. Computer analysis of the GIP-34 CD spectrum resulted in a secondary structure, comprising 45% β-sheets and turns, 45% random coil (disordered), and 10% α-helix. In comparison, computer analysis predicted the carboxyterminus (GIP-8) linear fragment to exhibit a random coil (disordered) structure; energy minimization computations of the octapeptide suggested that the linear peptide, with a terminal β-hairpin loop, had the potential to form a horseshoe-shaped pseudocyclic structure[31] (Fig. 1). Thus, the data indicated that GIP-8 had an innate propensity to form a cyclic configuration[45] (Fig. 1). The addition of an aspargine or glutamine to the carboxy terminus of GIP-8 allowed the formation of a cyclized 9-mer, resulting in a planar macrocyclic nonopeptide.[32,45] In comparison of the two 34-mer peptides, the linear GIP-34 had the potential to form a twisted fish-hook structure, whereas the disulfide-bridged 34-mer construct formed a two-tailed cyclic configuration[23,29,30] (Fig. 1A).

In further studies, the AA constituents of GIP-34 and GIP-8 were subjected to extensive substitutions in order to determine the active site of the molecule.[28,49–51,54] Studies by Bennett et al. parsed the entire GIP-34 sequence for estrogen-sensitive growth inhibitory activity, and then further assayed the linear GIP-8 and the cyclic nonopeptide.[31,45] The minimal portion of the cyclized GIP pharmacophore appeared to encompass the PVNP sequence and favored the hydrophobic branched side chains of Ile and Leu as valine substitutions.[32] The presence of valine and proline was proposed to produce a hydrophobic pocket. Although the biologic activity of the GIP nonopeptide could withstand proline-$_4$ and glycine-$_8$ substitutions, proline-$_7$ and aspargine-$_6$ could not be mutated without loss of growth inhibitory activity. Thus, the presence of the imino acid at proline-$_7$ is required to maintain growth inhibitory potency. Substitution of hydroxyproline for proline was also tolerated and resulted in increased hydrophicity and shelf-life of the GIP-cyclized nonapeptide.[31,32,45] The positions of Glu_1-Met_2-Thr_3 and Gly_8 were not found to constitute the pharmacophore nucleus of the cyclized GIP-8.

The pharmacophore nucleus of GIP-8 appears to involve most, if not all, of the PVNP sequence, which represents a PXXP motif found as a Src Homology-3 (SH3) domain in many proteins and peptides.[55,56] The PXXP domain allows for participation in phosphorylation events employing serine/theronine SH3 kinases. The SH3 motifs recognizes sequences bearing the AA sequence PXXP (X = any AA), which participates in diverse signaling pathways involving protein-protein interactions, such as ligand binding, microtubule association, protein scaffolding, cell adhesion, and signal sorting.[57,58] The action of the mechanoenzyme dynamin during endocytosis is mediated, in part, through the association of its proline-rich regions with SH3 domains containing single or repeat sequences of PXXP.[59,60] The same SH3 domain phenomena are also observed in microtubule-associated proteins. The SH3 proteins/peptides employ PXXP as a docking motif, which, in turn, provides an interface for ligand binding, recognition, and selectivity.[61] Members of the p53 protein family, including p73, utilize one to two PXXP motifs for transactivation and to suppress growth,[62,63] whereas such sequences are further required for p53 to induce apoptosis during tumor chemotherapy.[64–66] BRCA1 and HSP70 are also known to contain an essential C-terminal PXXP motif involved in the mechanism of response to DNA double-strand breaks, genome surveillance, and repair.[67,68] In the control of mitosis and tumor pathogenesis, an SH3-interacting domain containing a double PXXP motif was found on a human pituitary tumor transforming protein, known as securin.[56] Finally, a molecular mechanism of Src kinase enzyme activation involving interaction with the estrogen receptor (ERα) and associated scaffolding proteins has been reported.[69] The Src enzyme can be further activated either by tyrosine kinases or serine/theronine kinases, the latter of which display the PXXP motif. Mutation of the PXXP sequences abolishes the activation of Src kinase activity and stimulation of ER transcriptional activity of the A/B domain (Ser 118) and tyr 537 phosphorylation of the ERα.[70] Thus, mutation of these motifs can prevent ER-scaffolding and protein-protein complex formation, and can eliminate activation of the Src/MAPK (ERK1 and ERK2) pathways. Recent studies have also shown colocalization of steroid-linked scaffolding proteins with ERα receptor by the PXXP domain.[69] The PXXP segment on GIP-8 might mimic a portion of the pharmacophore that is involved with the nonligand transactivation (A/B) domain of the ER, as GIP-8 does not bind to the ER, yet is inhibitory for E2-stimulated growth and MAPK signaling cascades. It is of interest that GIP-8 has been found to reduce SER 118 phosphorylation of the human ER.[71] Previous

studies have shown that binding between the Src homology (PXXP) to the SH3 domain results in a suppression of kinase activity.[72] Thus, the PXXP motif on GIP could interfere, or compete, with the rapid formation of stable protein signal (kinases) complexes and the mediation of ligand binding specificities.[73,74]

Biological Activities

Linear and cyclic GIP-8 antiuterotrophic growth properties: In vivo *immature mouse uterine assay*

Linear forms of GIP-8 displayed considerable activity as an inhibitor of normal E2-sensitive growth of the immature rodent uterus (Tables 1 and 2). The original observation of suppression of E2-dependent growth in the 23-hour immature mouse uterus assay observed in 1993 produced a GIP-34 antiuterotrophic activity of 38%–42%; this was the first demonstration to show that synthetic GIP-34 did not require E2 incubation for growth inhibition as did the full-length AFP.[7] In 1994, studies revealed that the 8-mer fragment of GIP-34 had potent uterine growth inhibitory properties. The linear GIP-8 was even shown to display an inhibitory potency (40%–45%) equal to, or greater than, that of GIP-34. Between 1995 and 2001, both linear and cyclic GIP-8 were firmly established as being capable of suppressing E2-supported growth of the immature mouse uterus, and of inhibiting MCF-7 breast cancer growth similarly to GIP-34.[7,8,45] A similar uterine growth assay for both GIP-34 and GIP-8 were also demonstrated in an adult uterine growth model.[75]

Linear GIP-8: activities independent of estrogen sensitive growth: Estrogen receptor-α binding

The non-E2-sensitive activities of GIP-8 have been described in several prior publications and have been discussed within the context of GIP-34. Further, GIP-8 has been subjected to estrogen receptor (ER-α) binding affinity studies (Fig. 2A), and two independent studies have confirmed that GIP-8 does not interfere with, or compete for, E2 binding to the human ER,[8] the rabbit ER, and the progesterone receptor.[31,46] Human recombinant αER bound GIP-34 with an $IC_{50} = 8 \times 10^{-6}$ M, whereas GIP-8 showed no binding affinity whatsoever.[8,30,76] In later studies using rabbit uterine cytosols, LY156758, 4-hydroxytamoxifen, and raloxifene displayed their well-documented competition with E2 for binding to the ER. In contrast, GIP-8 did not bind to the human ER over a peptide concentration range of 10^{-5} M to 10^{-10} M. The authors stated that the mechanism of growth inhibition by which GIP-8 interferes with the response to E2 signaling is clearly different from tamoxifen and agents that compete directly with E2 for binding to the ER.

HAFP receptor binding

It was previously proposed[7] that GIP-8 and GIP-34 might bind to a human AFP receptor isolated by several different investigators.[78–80] A cell-surface receptor for HAFP was isolated and purified from plasma membranes of human breast cancer cells (MCF-7) and has been described on several other types of cancer cells.[81,82] Both high (10^{-9} M) and moderate to low (10^{-6} M) affinity sites have been reported, with binding site numbers (N) ranging from 2000/cell to 100,000/cell. By the use of microtiter plates coated with MCF-7 AFP receptor-enriched cell membrane preparations, biotinylated HAFP (B-AFP) was found to bind to the AFP-receptor (Fig. 3B) using a streptavidin-horseradish peroxidase substrate. Neither cyclic GIP-34 and linear GIP-34, nor linear GIP-8 could displace, or compete for the binding of, B-AFP to the putative HAFP receptor, whereas full-length HAFP did bind (Fig. 2B). Although GIP-34 and its fragments have been shown to be highly reactive at various cell surfaces, they do not appear to bind the AFP receptor-rich membrane isolates prepared from MCF-7 cells.

Antiangiogenesis: Chick allantoic membrane assay

The GeneBank sequence matching data indicated that both GIP-34 and GIP-8 showed an AA sequence identity/similarity matching to ECM and blood vasculature components (Table 3).[29,76] These observations led to the testing of GIP-34 and GIP-8 in assays employing chick embryo blood vessel angiogenesis (Table 5). The chick allantoic membrane (CAM) assay is a measure of blood vessel formation in the chicken inner eggshell membrane.[76] Thus, the CAM assay is a means to measure angiogenesis in the chorioallantoic membrane system, which develops from incubation days 3 to 5. Vessels subsequently grow throughout the inner surface of the egg shell membrane, which encompasses both the yolk sac and the embryo unit. The CAM assays are per-

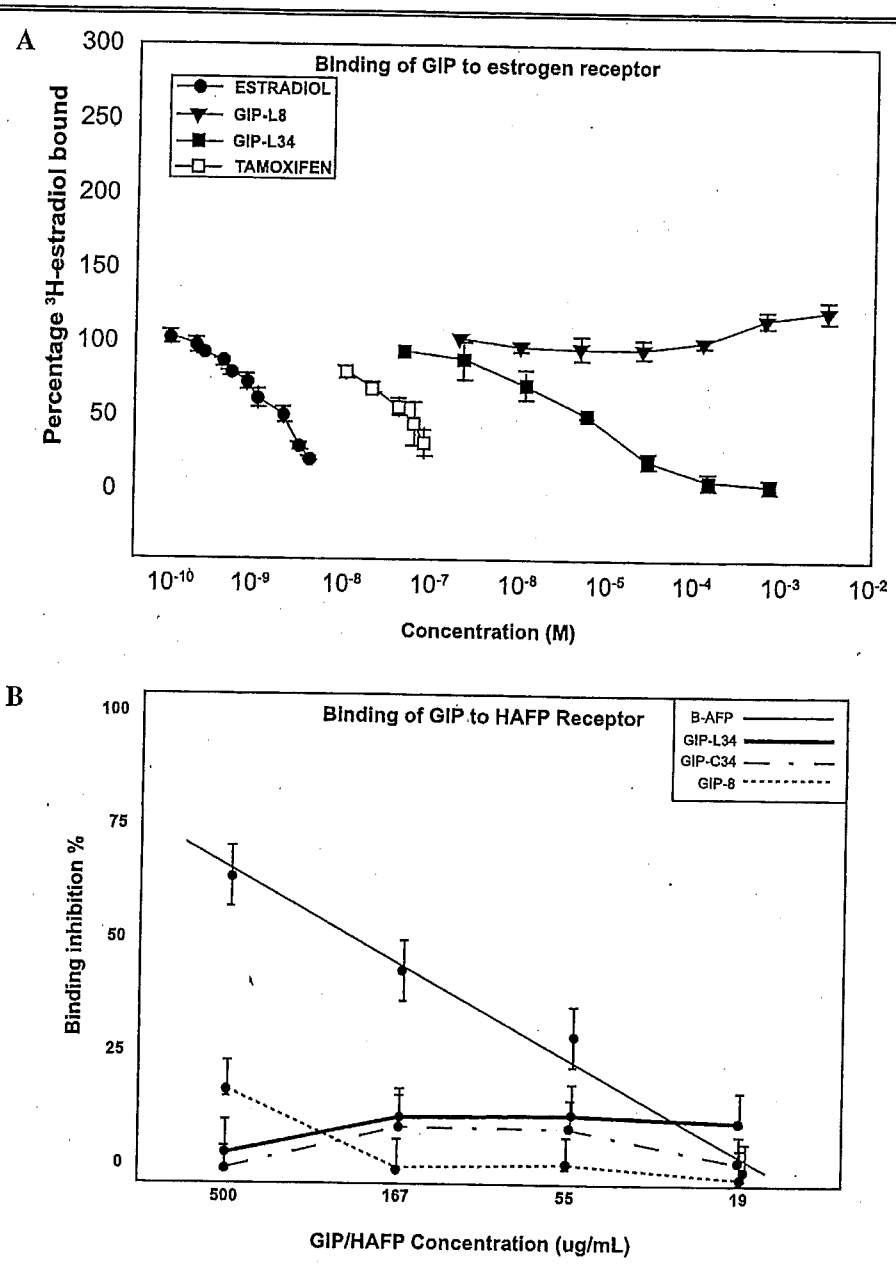

Figure 2. **(Panel A)** Binding of GIP [growth inhibitory peptide]-34 and GIP-8 to human estrogen receptor (HER) was analyzed as depicted. The binding of HAFP-derived peptides to human estrogen receptor (HER)-alpha is a competitive displacement hydroxyapatite assay using recombinant HER and tritiated estradiol (^{3}H-E2). Values represent mean ± standard deviation of triplicate observations. AFP-peptides were tested as equimolar-mixtures to displace 2nM ^{3}H-E2 bound to 1.2 nM HER-alpha. Nonlabeled estradiol and tamoxifen used as positive binding controls for the assay. **(Panel B)** Microtiter plates coated with HAFP receptor-rich preparations from MCF-7 cell membranes (50×10^{-5} cells/mL) bind biotimylated HAFP (B-AFP) in a dose-dependent fashion, as shown in the diagonal line (solid, nonbold). Color changes derived from strepaviden-horseradish peroxidase-ABTS substrate were monitored at 492 NM wavelength and recorded as optical density. Using a B-AFP solution of 500 μg/mL, nonlabeled HAFP was titrated in 3-fold dilutions from 50 μg/mL to 1.9 μg/mL, which displaced labeled AFP with the nonlabeled form. Linear GIP-34 (L-34) and GIP-8 (L-8) and cyclic GIP-34 (C-34) were used as competitive binding displacement agents.

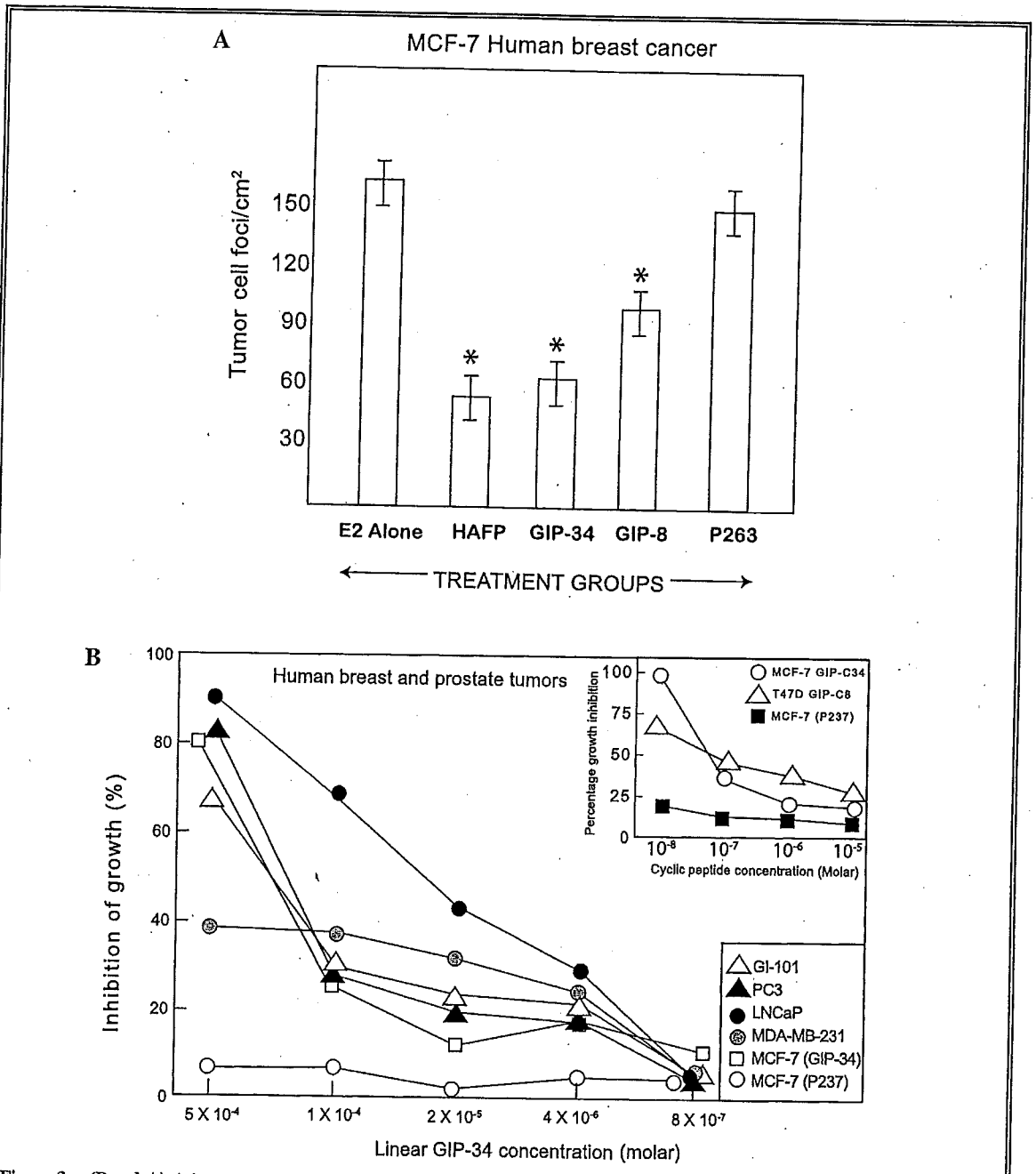

Figure 3. **(Panel A)** A bar graph is displayed showing human AFP, GIP[growth inhibitory peptide]-34, and GIP-8 assayed in the MCF-7 breast cancer cell foci assay using E2 (10^{-8}M) supplemented media. Unrestrained cells pile up, one upon another, forming microscopic mounds called foci. Tumor-cell foci are enumerated microscopically by means of a cell-colony counter and expressed as foci/cm^2. HAFP, GIP-34, and GIP-8 reduce and/or prevent the loss of tumor-cell contact inhibition. The P263 control peptide is derived from human serum albumin. Data are expressed as mean ± 1.0 standard deviation. * = <0.05 statistical significance **(Panel B)** Both human breast and prostate tumors were studied *in vitro* using a sulforhodamine-B cell proliferation assay to assess cyclic and linear GIP-34 and cyclic GIP-8 growth inhibitory potency. Linear GIP-34 is capable of growth inhibition in a variety of prostate- and breast-cancer cells in a dose-dependent fashion ranging from 10^{-4} to 10^{-7} Molar concentrations. P237 is a scrambled peptide version of GIP-34. **(Panel B Inset)** The effect of cyclic GIP-34 and GIP-8 on MCF-7 and T47D human breast-cancer cells is depicted. Note that the cyclic AFP peptides **(inset)** are effective at lower concentrations (10^{-8}M) than the linear GIP (10^{-4}–10^{-5}M) in the whole picture of Panel B. Data for cell proliferation are expressed as percent of control cell growth in sulforhodamine-stained preparations.

formed during the 6–9 day incubation period concomitant with the embryo-to-fetus conversion observing changes in pattern, density, and size distribution of the CAM blood vessels, compared to FGF and TGF stimulation, used as the positive controls.[83,84]

As listed in Table 5, both cyclic GIP-34 and linear GIP-8 preparations substantially inhibited the angiogenic CAM response in chick eggs that occurred at molar concentrations of 1.0–10 μM. Whereas cyclic GIP-34 inhibited 95%, linear GIP-34 was only capable of a 40% angiogenesis inhibition. GIP-8 was similar to cyclic GIP-34, showing >90% inhibition of angiogenesis. In comparison, the albumin control peptide, P263, and a carboxy-terminal HAFP fragment (P192) were both found to be unreactive at these same concentrations.[84] The antiangiogenic activity displayed by GIP is comparable to that of endostatin, a much larger fragment derived from collagen.

Tumor cell adhesion to extracellular matrix

Tumor cell adhesion to the ECM is an essential step during tumor cell migration and metastases, providing a means for migrating cancer cells to transiently or permanently attach to the connective tissue substratum.[28,83] A tumor cell adhesion ECM assay was utilized to assess whether the AFP peptides could influence or modify tumor cell attachment to a protein substratum or matrix. Various ECM proteins were adsorbed to the walls of microtiter plates and screened for their ability to serve as a substratum for enhanced tumor cell adhesion, as compared to non-ECM protein-coated microtiter plates. With 6WI-1 mouse mammary tumor cells, substantial cell attachment was observed with vitronectin, laminin, fibrinogen, fibronectin, and collagens I and IV after 2.0 hours of incubation at 37°C (Table 6). GIP-34 and GIP-8 were then tested for their ability to compete with tumor cell adhesion to the ECM substratum.[29,85]

Whereas GIP-34 was capable of inhibiting most ECM proteins from 40% to 68%, GIP-8 was selective for fibronectin, fibrinogen, and collagens I and IV, and largely lacked the ability to inhibit polylysine, chondrotin sulfate, and gelatin.[28,85] GIP-8, at 1.0–10 μM, inhibited mouse tumor cell adhesion to fibronectin by 73%, fibrinogen by 78%, collagen-I by 40%, and collagen IV by 60% (Tables 6 and 7). When T47D human breast cancer cells were similarly tested against 1.0 μM of cyclic GIP-8, growth inhibition of the E2-dependent cells showed 31% and 34% inhibition using cells cultured on fibronectin and collagen-I plates, respectively, and 49% inhibition of cells plated on collagen IV.[85] No sig-

Table 5. Summarized Activities of Growth Inhibitory Peptide (P149) in Normal Blood Vessel and Tumor Angiogenesis using the Chick Chorioallantoic Membrane (CAM) Assay[a]

	CAM-Angiogenesis Inhibition (%)			
Peptide tested: no. designation	*Normal blood vessel (CAM) angiogenesis*	*Tumor blood vessel angiogenesis*[b]	*Tumor cell adhesion inhibition (%)*	*Platelet aggregation inhibition (%)*
1. GIP-34 linear	40	45	60–70	90–95
2. GIP-34 cyclic	95	95–100	40–50	90
3. GIP-8 linear	90	90–95	45–55	<10
5. P263 (ALB)	0	0	<15	0
6. P192 (CT-AFP)	0	0	0	0
7. P237 (Scram)	0	0	<10	0
8. α_2AP	ND	ND	<10	85–90
9. Ristocetin	ND	ND	ND	100
10. Ovalbumin	ND	ND	<20	ND

Note. Percent inhibition of blood vasculature formation on chick embryo extraembryonic membrane is displayed for P149, its fragments, and control peptides. For peptide number designations, see the footnotes to Table 3.

AFP = alpha-fetoprotein; ALB, albumin (human); CT-AFP, carboxy terminal end of AFP (human); Scram, scrambled peptide of P149 amino acid sequence; α_2AP, α2-antiplasmin; ND, not done.

[a]$N = 5$ each per experiment.

[b]Mouse melanomas (see text).

Table 6. Growth Inhibitory Peptide (GIP-34, GIP-8) Interaction with and/or Binding to Various Ligands and Organic/Inorganic Agents

Interacting or binding agent	*Growth inhibitory peptides*		
	GIP-34	*GIP-8*	*References*
1. Human estrogen receptor (HER) α (recombinant)	Binding	Nonbinding	8, 46, 75, 76
2. 17 β-estradiol[b] fetotoxic doses	Reduces fetotoxic effect (73%)[a]	Reduces fetotoxic effect (37%)	25, 76
3. (Estradiol-induced) MCF-7 foci formation	Inhibits foci (65%)	Inhibits foci (40%)	7, 28–31
4. Congo red, ANS	Midpiece binding	Nonbinding	23
5. HAFP receptor	Nonbinding	Nonbinding	77
6. Heavy metals: cobalt, zinc, copper, and iron	Binding with all cations listed	Nonbinding	29–31
7. HSP-70 chaperone interaction	Binding	Binding	112, 33
8. Survivin protein interaction	Not tested	Binding	112, 33
9. Extracellular matrix[c] binding	Inhibits	Inhibits	29, 76, 85, 111
a. Collagen I, IV	30–45	40–60%	
b. Vitronectin	55	Not done	
c. Laminin	<10	Nonbinders	
d. Fibronectin	50–60	73%	
e. Fibrinogen	50–55	78%	

ANS, anilinonapthalene-sulfonic acid
[a]Insulin-induced teratogenic doses in the fetal chick.
[b]Estrogen-induced fetotoxic doses in the perinatal mouse.
[c]MCF-7 cells and TD-47 cells.

nificant growth inhibition was noted when T47D cells were cultured on laminin, polylysine, or uncoated plastic. With cyclic GIP-8 doses of 1.0–100 μM, fibronectin coatings were inhibited by 37%, collagen I by 38%, and collagen IV at 47%. These data suggested cross-"talk" signaling between the ECM and the tumor cell membranes. Overall, cyclic GIP-8 was capable of inhibiting both the attachment of tumor cells to the substratum and the subsequent growth of remaining tumor cells on that particular ECM. Based on the ECM adhesion data, tables of integrin-association with GIP fragment are presented, which reveals integrin α- and β-chain interactions (Tables 4 and 6).

GIP immunoregulatory activity

Recent studies have reported that not all AFP-specific T-cell clones are deleted during ontogeny, and that AFP antigenic sites persist and are recognized by both murine and human T-cells. During the last decade, several research groups have succeeded in assaying the T-cell immunodominant epitope sites on HAFP.[86–90] These research groups have reported that four major HLA-A epitotopic sites, and several more minor epitopic determinants, can be localized throughout the three domains of HAFP. Computer-generated HAFP AA sequences 9–10 AA in length, comprising 74 potential peptides, were screened, and they produced 14 positive and 10 negative peptides representing major histocompatability complex (MHC) sites of the HLA-A epitopic type.[88,91] Five peptidic segments of the positive 14 corresponded to known major T-cell epitopes that had been described in previous reports.[92,93] Of the remaining nine antigenic determinants, five qualified as minor epitopic sites. Interestingly, two of the major epitopes corresponded to overlapping AA sequence constituents of GIP-8, namely, CIRHEMTPV and PVNPGVGQC. Each peptide proved capable of inducing specific T-cell lymphocyte responses *in vitro* from normal human HLA-A *0201 donor lymphocytes and recognized HLA-A *0201/AFP primed tumor cells in both cytotoxicity assays and in interferon (IFN-α) induction studies.[94]

Table 7. Human and Rodent Breast Tumors Suppressed by Growth Inhibitory Peptide (GIP) Using *In Vivo* and *In Vitro* Models

Tumor designation	*Cancer type*	*Receptors present*	*Treatment duration (days)*	*Optimal conc. (M)*	*Percent growth suppression*		*Reference*
					GIP-34	*GIP-8*	
I. In vitro: *Cell cultures*							
1. MCF-7, human	Glandular adeno carcinoma	Estrogen	6	10^{-7}	80	>90	7, 8
2. T-47D, human	Ductal carcinoma	Prolactin, progesterone, estrogen, androgen	6	10^{-5}	25–30	65	8, 29, 31, 32
3. BT-549, human	Ductal papillary	Estrogen	6	10^{-6}	40–67	ND	8
4. MDA-MB-231, human	Glandular adeno carcinoma	Non-E2	6–9	80	(59, 60)	0	30, 31, 47
5. MDA-MB-435, human	Glandular adeno	EGF, TGFα	6	10^{-7}	70	ND	8
6. GI-101	Ductal carcinoma	EGF, estrogen, pS2	6	10^{-8}	75	ND	29, 30, 76
7. MCF-7 foci	Grandular	Estrogen	14	10^{-12}	65	40	7, 8
8. CNS tumor	Glial blastoma	NR	5–6	10^{-6}–10^{7}	45	>50	30
9. Human kidney	Renal carcinoma	NR	6	5×10^{-4}	70	ND	UD
10. Hut, B-cell	Lymphomas	NR	2–3	10^{-7}	85	ND	UD
11. HEPG2	Hepatomas	AFP, HGF	2–3	10^{-8}	45	ND	UD
II. In vivo: *Isografts, xenografts*							
1. EMT-6, mouse	Mammary-derived sarcoma	EGF, TGFβ	30	0.5 μg/day	10–20	ND	30, 31
2. 6WI-1, mouse a. Mouse adult	Adenocanthoma	NR	12	1.0 μg/day	45–70	50	29–31
3. Mouse pup	Adenocanthoma	NR	6	0.5 μg/day	56	29	31, 76
4. MCF-7 mouse xenograft[c]	Glandular adeno carcinoma	Estrogen	15–30	0.5–10.0 μg/day	>80	>90	30, 31, 32
5. GI-101 tamox-resistant	Grandular ductal adenocarcinoma	Estrogen	60	0.5 μg/day[b]	>70	ND	ND
6. T47D	Ductal carcinoma[a]	Estrogen, progesterone,	30	2.0 μg/day	ND	>95	31, 32
7. MCF-7/Tam Tamox-resistant	Grandular adenocarcinoma	Estrogen	30	2.0 μg/day	ND	>95	47
8. MDA-MB-31	Grandular adenocarcinoma	Non-ER	30	0.5 μg/day	~10	0	29, 30, 47, 50
9. MCF-7 mouse xenograft[b]	Grandular adenocarcinoma	Estrogen	20	10 μg/day	ND	>90	31, 32
10. MNU-induced rat tumor	Breast adenocarcinoma	ND	90	0.03–0.27 mg/rat/day	ND	60	48
11. MCF-7 mouse xenograft s.c.	Grandular adenocarcinoma	Estrogen	30	10 μg/day	ND	>90	71
12. MCF-7 mouse xenograft (oral)	Grandular adenocarcinoma	Estrogen	30	10 μg/day	ND	>90	71

EGF, epidermal growth factor; TGF; transforming growth factor; NR, not reported; CC, cell culture; Wnt, wingless *Drosophila* homolog; acanthoma, adenoid squamous-cell carcinoma; UD, unpublished data (G.J. Mizejewski and M. Dauphinee, Rambough-Goodwin Cancer Institute); S.C., subcutaneous; HGF, heptocyte growth factor; ND, not done; ER, estrogen receptor, E2, estradiol.

[a]Estrogen pellets applied.

[b]EMTOVNOGQ.

[c]EKTOVNOGN.

AFP peptide-specific T-cells were also identified in the spleens of mice immunized with dendritic cells transduced with AFP-expressing adenovirus. This study and others concluded that the human T-cell repertoire is capable of recognizing AFP in the context of MHC class I immune responses, even in an environment of high circulating HAFP levels, in both hepatoma and cirrhotic patients.[90,95] Unlike GIP-34, the GIP-8 is a sufficiently shorter segment that can generate an MHC class I immune response and can sensitize host T-cells against these epitopic sites. Although the AFP epitopes provide a rationale for T-cell-based immunotherapy against hepatomas (AFP-producing cells), two overlapping GIP-8 antigenic epitopes are immunogenic and could potentially induce an antigenic immune response, resulting in host antibody production during the course of therapy. Thus, the eventual production of circulating antibodies and/or an immune cell response to GIP-8, including the T-cell responses described above, might interfere with, or provide a long-term obstacle to GIP-8 anticancer peptide therapeutic drug delivery. Aside from the T-cell responses described above, actual *in vivo* antibody production against GIP-8 has yet to be demonstrated.

Anti-Cancer Activities: Linear GIP-8: E2-dependent growth/cell contact inhibition

The first published report on the E2 regulatory GIP-34 included studies of growth inhibition of MCF-7 cells *in vitro*[7] (Tables 1 and 8; Fig. 3A). GIP-34-inhibited tumor cell foci formed as a result of the accumulation of breast cancer cells *in vitro*, which clump and pile up as a result of cell overgrowth.[96] MCF-7 cells in culture lose their contact inhibition properties when exposed to physiological concentrations (10^{-8} M) of E2.[97] Whereas anchorage dependence of cell growth is an integrin-associated process, it has been reported that contact inhibition in MCF-7 cells is mediated through cell-cell adhesion molecules, such as cadherin and catenin.[98] In MCF-7 cells, E2 exposure releases the constraints maintained by cadherin/catenin-containing cells to allow migration. The migrating cells then stack up to form mounds termed foci, and these can be quantitated using a colony counter. In the absence or elimination of E2, cell proliferation is halted after the cultured cells establish cell-cell contact, so cell foci formation does not occur. Therefore, the MCF-7 focus assay has been used for the evaluation of GIP-34 and GIP-8 antiestrogenicity potency.[7] Results showed that both HAFP and GIP-34, at 10^{-11} to 10^{-12} M, were capable of suppressing MCF-7 cell focus accumulation by nearly 75% and 70%, respectively[76] (Fig. 3A). Although not as potent, the linear GIP-8 produced a 30%–40% inhibition, albeit at higher (10^{-6} M) concentrations (Table 8; Fig. 4).

It is evident from Fig. 3B (inset) that both cyclic GIP-34 and GIP-8 were inhibitory against two breast cancers, MCF-7 and T47D, as determined by sulforhodamine cell proliferation assays. Although linear GIP-34 has been shown to be active against human prostate cancer (Fig. 4B), GIP-8 has yet to be studied in prostate cancer cell culture assays. It is evident that the GIPs have a growth inhibitory range from 40% to 90% against a multiplicity of breast and prostate tumors.

GIP-8 was also utilized for studies of E2-dependent breast cancer cell growth both *in vivo* and *in vitro*.[31,32,45] For a complete listing of tumor models assayed using GIP-34 and GIP-8, see Table 7; & Figures 3 and 4. GIP-8 was shown to suppress E2-dependent breast cancer growth, as previously reported for GIP-34 (Table 2). Furthermore, both linear and cyclic GIP-8 inhibited E2-dependent growth of MCF-7 cells implanted in severe combined immunodeficient (SCID) mice[31] (Table 8). A hydroxyproline-substituted GIP-8 analog also completely prevented the growth of xenografted tamoxifen-resistant sublines of MCF-7 (Fig. 4A), but it was not able to suppress the growth of the E2-independent MDA-MB-231 human breast cancer cell line.[47] Interestingly, the octamer peptide was further reported to inhibit the uterotrophic effect of tamoxifen in the host mouse following tumor transplantation. Thus, GIP-8 simultaneously inhibited tamoxifen-resistant breast cancer while suppressing the uterotrophic side-effect of tamoxifen. It is interesting that both linear and cyclic GIP-8 forms are active only against E2-dependent breast cancer growth, whereas the GIP-34 peptide is active against both E2-dependent and E2-independent growth (Table 8; Fig. 3B); this suggests differing mechanisms of action for the two peptides. The authors of the above tamoxifen studies forwarded three potential uses for GIP-8. First, it might be utilized as a replacement for tamoxifen, to eliminate that drug's side-effects of uterine cancer and blood clotting. Second, it might be employed to as an adjunct drug to be used together with tamoxifen treatment. Third, it could

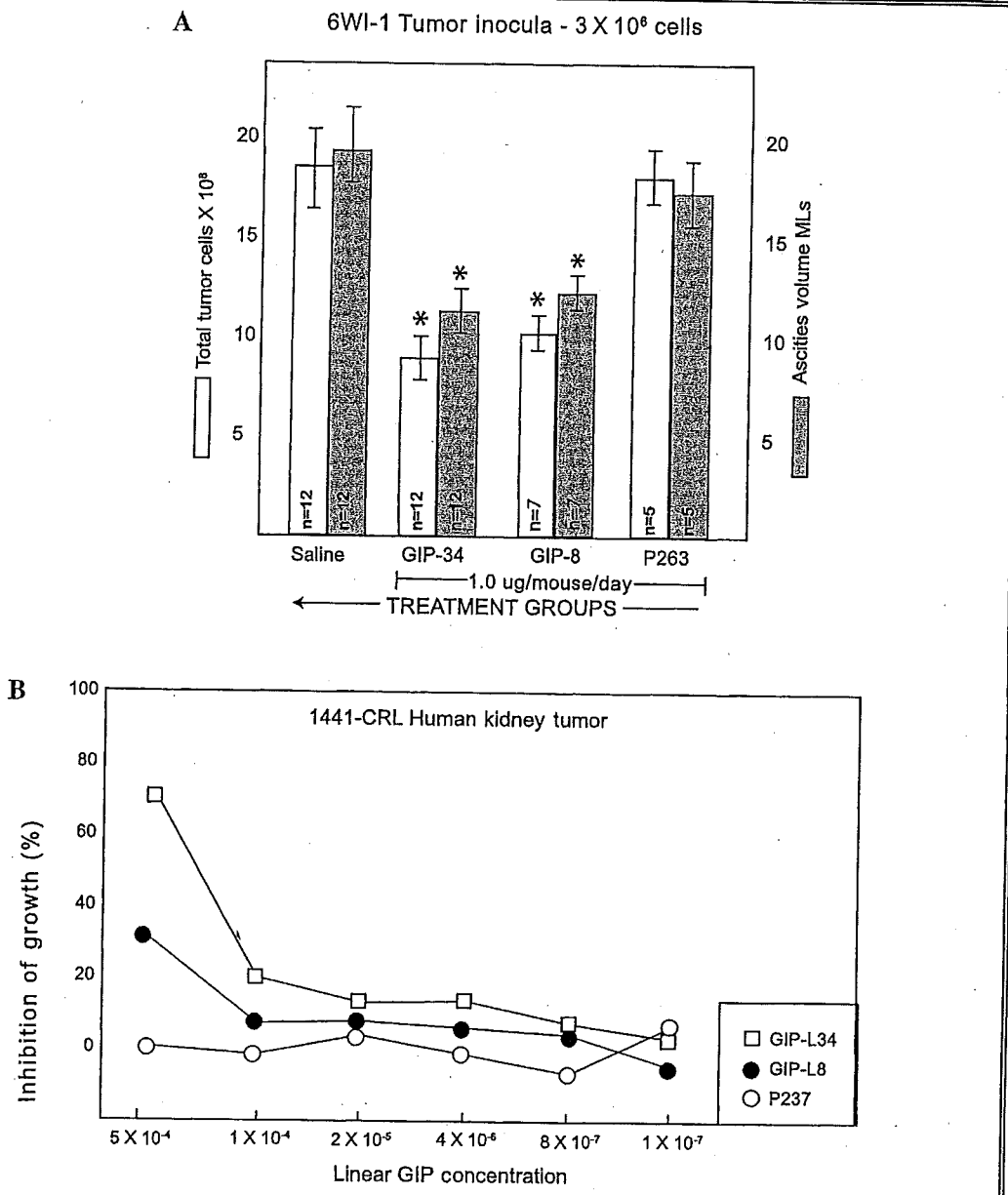

Figure 4. **(Panel A)** The *in vivo* growth suppressive activity of linear GIP[growth inhibitory peptide]-34, linear GIP-8, and control peptide P263 (all peptides, 1.0 (g/day) in the 6WI-1 mouse mammary tumor isograft is demonstrated. Tumor-growth suppression by the AFP-derived peptides was assessed by the peptide's ability to reduce both tumor-cell proliferation (numbers) and ascites fluid (volume) accumulation in the body cavity of Nylar mice. Asterisk, statistical significance at $p < 0.05$. P263, albumin control peptide. Saline used was phosphate buffered saline, pH = 74. **(Panel B)** *In vitro* cell growth of human kidney tumor 1441-CRL, following exposure to linear (L) GIP-34 and linear (L) GIP-8, was assessed in the sulforhodamine-B cell proliferation assay (see text). The kidney tumor is a non-estrogen–dependent cell line that requires high concentrations of either of the linear peptides to produce cell growth suppression (5×10^{-4}M). Data are expressed as percent of nontreated tumor cell proliferation.

be used to treat tamoxifen-resistant breast cancer patients. However, it is impractical to propose that tamoxifen would be replaced by any such drug in the near future.

In a subsequent study using the cyclic GIP-8 in (2.0 μg/day) MCF-7 SCID mouse xenografts, the cyclic GIP-8 suppressed total growth of the tumor for a 20-day period.[45] The mice also received E2 supplementation by silastic tubing E2 implants (Fig. 4A). An 8-mer human albumin control peptide (EKTPVSDR), derived from the homologous AA region on HAFP, was employed as a control peptide; it had previously tested negative in the immature mouse uterus assay. Whereas the presence of E2 produced a 3-fold increase in tumor volume in all control mice after 20 days, cyclic GIP-8 completely prevented tumor growth over this time period. Finally, a recent report by GIP-8 investigators demonstrated that the cyclic 8-mer could be administered to MCF-7 xenograft-bearing mice by oral delivery in addition to the parenteral routes.[71]

In a further study the cyclic GIP-8 was tested in a model of N-methyl-N-nitrosourea-induced breast cancer to determine whether it could prevent the induction of mammary tumors in rats.[48] GIP-8 prevented the appearance of the tumors in a dose-dependent manner (Fig. 4B). It was determined that cyclic GIP-8 treated rats displayed significantly longer mean tumor-free days, lower tumor incidence, and lower tumor multiplicity (number of tumors/rats). Host toxicity was not evident when body weight, fur texture, and organ weight were used as criteria in the cyclic peptide-treated animals. In addition, acute GIP-8 toxicity studies in nontumor mice showed no effects on total body weight or individual organ weight following a single intravenous (i.v.) injection of 10 mg peptide/mouse or following 5 successive day treatments of 2 mg peptide/day and determined by necropsy. The results indicated that the protection provided by cyclic GIP 8-mer was similar to the exposure levels measured in human epidemiological studies of breast cancer risk reduction by full-length HAFP.[99,100] Representative samples of the induced tumors examined by immunohistochemistry demonstrated that GIP-8 had prevented growth of tumors comprising 80% adenocarcinomas and 20% fibroadenomas.[48]

GIP-8 E2-independent cancer growth: Mouse Ascites mammary tumor

A mouse-induced mammary tumor 6WI-1 of adenoacanthoma (squamous cell) origin was studied, as described.[8] The growth suppression of the mammary ascites tumor by GIP-34, GIP-8, and control peptides was determined in non-E2-supplemented assays that measured both the tumor cell number and the ascites volume of 6WI-1 mammary tumors transplanted into NYLAR/nya mice.[28–30,76] Tumor cell inocula, ranging from cell concentrations of 0.3 to 3.0 $\times$ 10^6 cells; produced host mortality at 12–14 days and were lethal in 100% of the mice (Fig. 5A).

Inoculation of mice at any of the cell doses led to increases in body weight from 25 g (day 0) to approximately 45 g by day 12, owing to the accumulation of tumor cells and ascites fluid in the intraperitoneal cavity. It was observed that a dose of 1 μg/day GIP-34 for 11 days suppressed the tumor-associated body weight gain at the two higher cell doses and totally suppressed weight increase at the low cell dose (Fig. 5A; Table 8). Mice in the totally suppressed group lived an additional 30 days or more until sacrificed, a substantial increase for the 2-year lifespan of a mouse. The GIP-34 or GIP-8 significantly suppressed the accumulation of tumor cells and ascites fluid volume (40%–45%) at a tumor cell inocula of 1 $\times$ 10^6 cells (Fig. 5A; Table 2). A scrambled version of GIP-34 and an albumin control peptide (P263) totally lacked anticancer activity in the mouse mammary isograft model (data not shown). As an alternative model of the 6WI-1 mammary tumor in adult mice, an inoculum of 1.0 $\times$ 10^6 tumor cells was injected into 15-day-old neonatal mice for a 6-day, rather than a 12-day, period; it replicated the adult tumor growth suppression produced by GIP (Table 8).

In addition to the mouse ascites tumor, GIP-8 was tested against a non-E2-dependent human kidney tumor cell line; it demonstrated only a 30% inhibition of growth, compared to 70% by GIP-34 (Fig. 5B). In addition, both linear and cyclic GIP-8 failed to prevent and/or suppress growth of the MDA-MB-231 human breast cancer (a nonestrogen receptor) cell line; however, GIP-34 was able to suppress growth by 40%.[28–30] Thus, GIP-8, unlike GIP-34, appears to be inhibitory mainly for E2-dependent reproductive cancers, such as breast tumors (Tables 6 and 7).

Mechanism of action

The site of action of GIP-34 has been proposed to initiate at the plasma membrane where GPRs are located; hence, GIP could serve as a possible decoy peptide ligand and perhaps occupy a receptor binding site.[28] Decoy ligands, in this case,

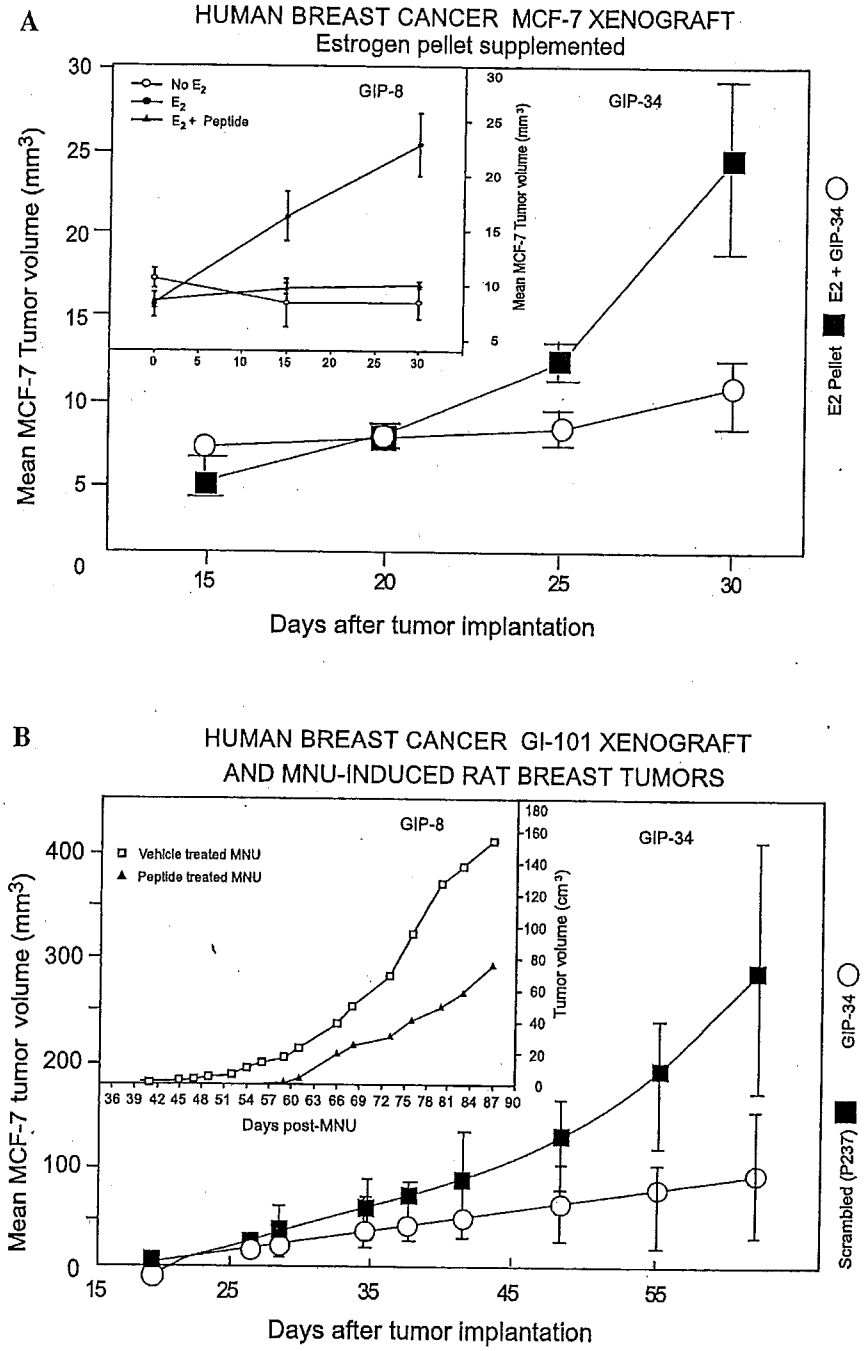

Figure 5. **(Panel A)** Suppression of the MCF-7 E2-supported mouse xenograft by GIP[growth inhibitory peptide]-34 is displayed in the **total picture** of the figure. Correspondingly, suppression of a similar MCF-7 xenograft model by GIP-8 is shown in the **boxed inset.** GIP-34 suppression used a time release pellet of 0.25 μg/mouse/day for 30 days, while the GIP-8 suppression utilized 2.0 μg/mouse/day injections with a similar suppression. Both models required E2 pellet supplementation. **(Panel B)** Two separate *in vivo* models of GIP suppression of breast cancer are displayed. The **total picture** model depicts the suppression of a tamoxifen-resistant human ductal breast-cancer xenograft in mice by GIP-34 (0.5 μg/mouse/day) for 60 days. The boxed inset model shows reduction of tumor burden of methyl-nitrosourea (MNU) induction of a rat mammary tumor by GIP-8 over a 90 day period. GIP-8 was administered in a dose of 0.03–0.27 mg/rat for 20 days. This figure represents a composite redrawn from refs. 30, 31, 48, and 76.

are defined as molecules that bind to a cell-surface receptor and block or neutralize, rather than initiate, receptor signal cascade activation (Fig. 6). Alternatively, a molecule can bind to a cell membrane protein (nonreceptor) and present as a circulating soluble receptor to block signal transduction. Such cases have been described in the literature for Duffy chemokines and their receptors.[101–103] The G-protein-coupled receptors (GPRs) are located at the cell surface and are responsible for the translation of an endogenous signal into an intracellular response through heterotrimeric G-proteins that target other proteins, often enzymes, that influence the level of intracellular second messengers. A recent computer modeling study using GIP-8 demonstrated that the peptide was potentially capable of docking with a 30-Kd GPR30 cell surface G-coupled receptor, a rhodopsin-family molecule that serves as an estrogen-binding cell membrane receptor.[104] The computer program modeled GIP-8 as fitting into a binding or docking pocket between the third and fourth extracellular loops of the 7-transmembrane G-coupled receptor (Fig. 6). GPR30 has indeed been shown to transduce E2-activated signaling from the cell membrane to the nucleus in a variety of breast cancer cells.[105–107] Thus, GPR30 and/or other G-coupled receptors could provide possible leads for the identification of specific therapeutic molecular targets of GIP-8.

The lack of cytotoxic activity and the demonstration of cytostatic effects on E2-dependent tumors suggest a nontoxic growth inhibitory mechanism, often observed as a result of homologous/heterologous desensitization of GPRs (Fig. 6). Both GIPs have never shown toxicity in any model studied. In the process of GPR desensitization, and/or receptor occupancy, receptors are normally withdrawn from the cell surface owing to repeated overstimulation and are then either recycled or subjected to ubiquitin-protoeosomal degradation pathways.[108] Regarding the process of desensitization, it has been recently reported that a family of guanosine triphosphate (GTP) activating proteins for heterotrimeric G-protein alpha-subunits, termed regulators of G-protein signaling (RGS), are responsible for the desensitization of GPRs.[108,109] The RGS proteins serve as unidimensional desensitizing agents for GPR signal transduction and also provide for scaffold assembly and signaling coordination.[110] It is tempting to speculate that GIP-8 and GIP-34 bind to G-coupled receptors, and then following continued stimulation by GIPs to the cell-surface receptors, activate an RGS protein coupled to the internal side of the GPR receptor at the plasma membrane; this might initiate uncoupling of the E2-activating signal transduction MAPK pathway (Fig. 6). Future therapeutic targets could include the various portions of the RGS proteins, such as the RGS domain, the kinase domain, and the pleckstrin homology domain, in addition to enzymes such as phospholipase alpha, beta, and epsilon types. It is notable that various interactions of GPRs with full-length HAFP have already been described in earlier reports.[6,111]

The blocking of E2 signal-transduction pathways interferes with the initiation of cell proliferation and growth in estrogen-sensitive tissues, including the mouse uterus and human breast cancer cells, both *in vitro* and *in vivo*. It has been reported that GIP-8 reduces the levels of MAPK kinase activity, thereby initiating the pathway leading to cell mitosis.[28,47] This action of GIP-8 was reported to restrict the phosphorylation of the ER, which is MAPK kinase-dependent, whereas phosphorylation involving ERK1 and ERK2 is needed to make the ER fully operational.[48] Examination of electrophoresed MCF-7 tumor tissue extracts obtained 2 hours after 8 days of treatment by GIP-8 (using Western blots), showed a decrease in phosphorylation of the ER and a concomitant increase in the phosphorylation of p53 (Ser 15) in the GIP-8-treated mice.[48] Thus, GIP-8 was shown to block or reduce ER phosphorylation of serine-118 but increased p53 phosphorylation at serine-15. Blockage of serine-118 at the aminoterminus of the human ER by the PXXP motif of GIP-8 could involve the A/B domain for the nonligand activation of the E2 receptor. Therefore, the blockade of GPR-receptors and/or the activation of RGS protein by GIP-8 could blunt the subsequent estrogen-induced signal transduction MAPK pathways, thereby reducing mitosis and cell proliferation, ultimately leading to growth arrest.

CONCLUSIONS

Both GIP-34 and GIP-8 have been demonstrated to be nontoxic and to suppress ontogenic and oncogenic growth in cell culture and various animal models. However, GIP-8 can be distinguished from GIP-34 by its apparent selectivity for estrogen-sensitive ontogenic and oncognic growth suppression. The inhibition of estrogen-dependent growth would appear to reduce the

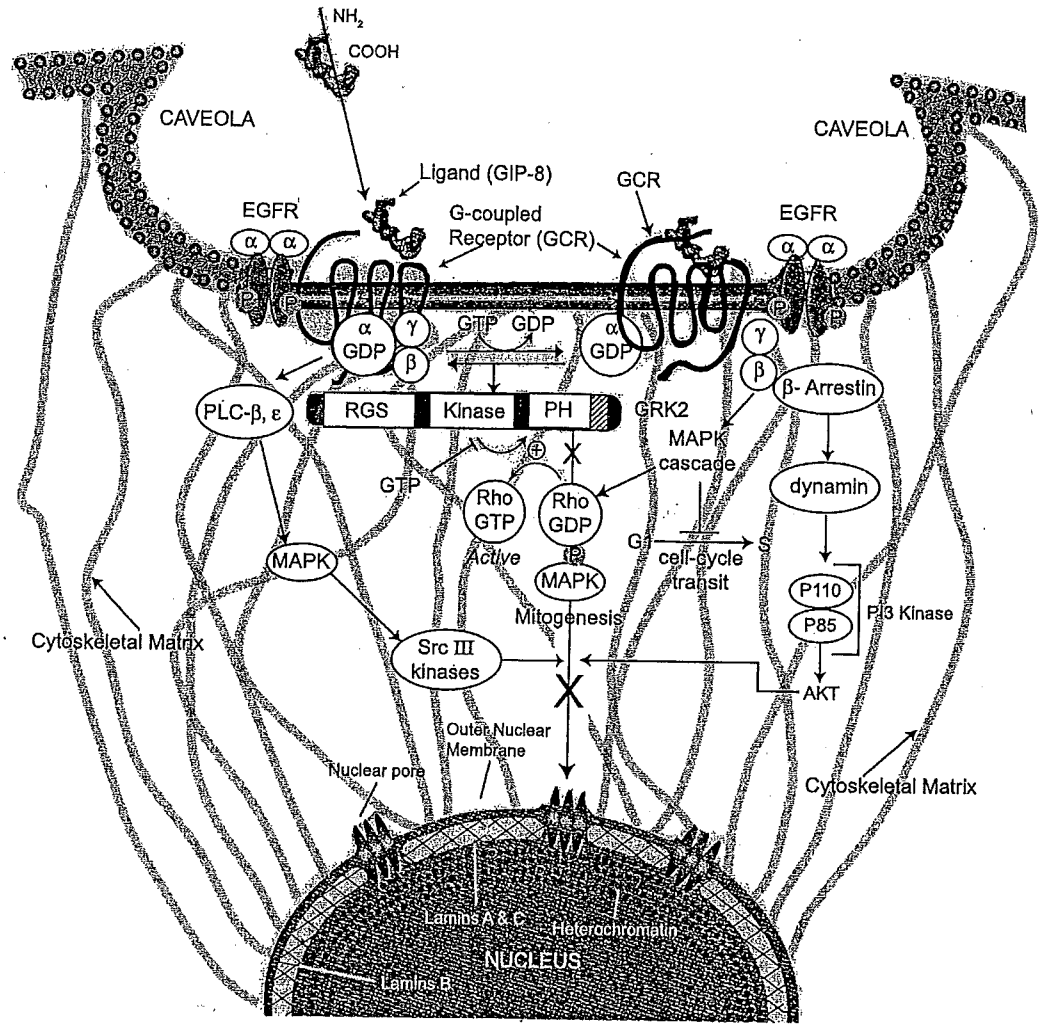

Figure 6. An artist's rendition of plausible docking of the growth inhibitory peptide 8-mer fragment (GIP-8) onto a G-coupled receptor, such as a heptahelical transmembrane protein member of the rhodopsin family. Some G-protein coupled receptors (GCRs) are linked to epidermal growth factor receptor (EGFR) signaling cascades. Ligand binding to a G protein-coupled receptor (GPCR) changes the conformation of the receptor's intracellular loops to promote the exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the $G\alpha$ subunit and the subsequent dissociation of $G\alpha$ from $G\alpha\beta$. The hydrolysis of $G\alpha$ is the rate-limiting step in the reaction. The flow diagram (top to bottom) depicts the general scheme of signal transduction by the heptahelical GPCRs and the subsequent signal termination (desensitization) by the regulators of G-signal transduction (RGS) proteins that serve as GTP-activating proteins for the heterotrimeric G-protein α subunits. Homologous desensitization of ligand signaling by the regulator of G-signaling (RGS) protein (containing an RGS domain, a kinase domain, and a pleckstrin homology (PH) domain) uncouples the signaling to the mitogen-activated protein kinase (MAPK) cascade through PLC-(β, ε) and/or secondary Rho/GTP/GDP pathways. While clathrin, β-Arrestin and dynamin are involved in receptor endocytosis and trafficking, arrestins can further serve as scaffold proteins that link to the MAPK cascades. At least 12 or more different RGS proteins are now known but their complete mode of action has yet to be determined. Epidermal growth factor receptor (EGFR), Src III, Src homology III serine/threonine kinases; PI3, phosphoinositol-3-kinase; GRK, G-coupled receptor threonine/serine kinases; PLC, phospholipase-C; AKT, oncoprotein signaling agent; RHO, GTP binding protein-associated kinase. The spider-like lines represent cytoskeletal protein fibers. The diagram was derived from multiple figures and redrawn as a composite, from refs. 23, 30, and 108.

therapeutic scope of action of GIP-8 to treatment of only E2-dependent, ER-positive tumors. Such tumors represent approximately 50%–55% of human breast cancers in the clinic. The growth-suppressive activities of the GIPs, as discussed above, could occur at the plasma membrane level, in the cytoplasmic MAPK signaling cascade, or in the nuclear/cytoplasmic compartment of the ERα or combinations thereof. Dual therapies using both GIP-34 and GIP-8 would, in theory, address both E2-dependent and E2-independent tumors. The mechanisms of action of GIP-8 and GIP-34 appear to be different, even though both must confront a plasma membrane to gain entry or activate the tumor cell's proliferation pathways. The GIP-34, by the use of fluorescent anti-GIP antibodies in histochemistry studies, have been clearly demonstrated to bind to the cell surface and to become internalized into the cytoplasm, eventually lodging in a perinuclear position.[28] Whereas GIP-8 has not yet been localized by histochemical procedures, computer modeling has suggested the plausible docking of GIP-8 with a GPR-cell surface receptor.[104] Evidence linking GIP-8 to MAPK kinase downregulation, and to the *in vitro* prevention of estrogen-receptor phosphorylated activation, further suggests that GIP-8 might be active in nuclear/cytoplasmic compartments following receptor-mediated endocytosis. Because GIP is known to require several days[8] and several cell cycles for activation (6-day assays), it would seem that dissolution of the nuclear membrane during mitosis might allow free access of GIP to nuclear transcription machinery at some point during the cell-cycle phases.

Future Studies

Because the GIPs act on various proliferating tissues and a multitude of cancer types, both GIP-34 and GIP-8 could potentially provide site-directed targeted drug delivery to a variety of different types of cancer. Because the GIPs have been shown to target to specific cells,[23,28] the peptide could be employed as a carrier molecule using GIP conjugated to chemotherapy drugs, heavy metals, toxins, antimetabolites, and radioisotopes. Because GIP-8 has shown promise in studies to bypass drug resistance,[47] GIPs may be capable of skirting the ABC transporter system to avoid multidrug resistance. Small-molecule organic peptide mimics could also be modeled from GIP-8 and GIP-34 to produce second-generation drugs, preferably with oral modes of delivery, as recently described for GIP-8.[71] The GIP-derived peptides could further find utility in the process of identifying molecular targets for drugs intended as biomodulation cancer therapies and to provide "proof of concept" in identification of targets for future peptides and/or mimics.[51] Finally, AFP-derived peptides could find utility as templates or lead compounds for the development of anticancer drugs.

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