Survey of Functional Activities of Alpha-fetoprotein Derived Growth Inhibitory Peptides: Review and Prospects

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Abstract: Alpha-fetoprotein (AFP), known largely as a growth-promoting agent, possesses a growth-inhibitory motif recently identified as an occult epitopic segment in the third domain. The present study reviews the multiple biological activities of this AFP-derived peptide segment termed the Growth Inhibitory Peptide (GIP), which is a 34-amino acid fragment taken directly from the full-length 590 amino acid molecule. The GIP segment has been chemically synthesized, purified, characterized, and subjected to a variety of bioassays. The GIP has a proven record of growth suppression in both fetal and tumor cells, but not in normal adult cells. Even though the mechanism of action has not been completely elucidated, GIP participates in various biological activities such as endocytosis, angiogenesis, and cytoskeleton-induced/cell shape changes. In this review, a survey of the functional roles of the GIP is presented which encompasses multiple organizational levels of GIP involvement, including the 1) organism, 2) organ, 3) tissue, 4) cell, 5) plasma membrane, 6) cytoplasm, and 7) the nucleus. At the cell membrane interface, the actions of GIP are discussed concerning cell aggregation, agglutination, adhesion, and migration in light of GIP serving as a possible decoy ligand and/or soluble receptor. Regarding cytosolic activities, GIP has been reported to inhibit various cytoplasmic enzyme activities, modulate apoptotic events, and regulate cytoplasmic signal transduction (MAP kinase) cascades. Concerning the nuclear compartment, GIP is capable of complexing with the estrogen receptor and binding estradiol, but does not affect estradiol-induced estrogen receptor transcription. In overview, efforts were made to review the multiple biological activities reported for GIP in order to prioritize likely physiological activities and present an updated consensus of functional roles for this AFP-derived peptide.

Keywords: Alpha-fetoprotein, receptors, peptides, plasma membrane, antigrowth, cell surface.

A) INTRODUCTION

Alpha-fetoprotein (AFP) is a tumor-associated fetal mammalian glycoprotein present during ontogenic and oncogenic growth [1, 2]. Human (H) AFP is secreted by the yolk sac and fetal liver during pregnancy, but can be re-expressed in adult teratomas, hepatomas, and yolk sac tumors of the ovary [3, 4]. In the clinical laboratory, HAFP has been employed as a tumor and gestational age-dependent fetal defect marker with dual utility as a screening agent for neural tube defects and aneuploidies [5, 6] , and as a serum tumor marker for liver, yolk sac, and germ cell cancers. In recent years, AFP has been determined to be a growth factor for both fetal and tumor cells [7, 8] and enhances growth in both cellular environments. HAFP is a 70-kDa single-polypeptide chain containing 3-5% carbohydrate; it exhibits a triplicate domain structure configured by intramolecular loops dictated by disulfide bridging, resulting in a helical V- or U-shaped structure first demonstrated by Luft and his associates [9].

During folding and unfolding of newly synthesized polypeptides in the endoplasmic reticulum, chaperoned proteins can pass through multiple transient or intermediate forms (resembling a molten globular phase) prior to attaining their final tertiary structure [10]. During folding of proteins destined for secretion, hydrophobic amino acid residues are tucked into the protein's interior crevices, while hydrophilic residues predominate on the protein's exterior surfaces which provides solubility in serum compartments [11, 12]. This inner hydrophobic/outer hydrophilic structure has been described for HAFP in which compactly-folded globular proteins are packaged and processed through the trans-Golgi network and exocytosed (secreted) to the extracellular fluid environment. The vast majority of serum proteins, such as HAFP, circulate as compactly folded forms in the vascular and interstitial fluids until stress/shock environments are encountered [13]. Then, some proportion of these compactly folded proteins have the propensity to partially unfold and assume a molten globular intermediate form, especially during transmembrane passage, exposure to high ligand concentrations, and stress/shock environments.

Human AFP has been reported as a serum protein capable of assuming a molten globular configuration following exposure to stress/shock environment and excessive on-and off-loading of ligands [10, 14]. This often occurs following activation of a "hot spot" on a protein; the hot spots are characterized as buried within the molecule and being tightly packed [15]. It is the third domain of HAFP that contains the growth inhibitory peptide (GIP) amino acid (AA) segment that lies encrypted in the protein's native, compactly folded form [16]; this site is thought to become accessible via a conformational change involving a rotational hinge [17]. The

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encrypted growth inhibitory peptide (GIP) epitope on HAFP is not dectable using present day commercial polyclonal or monoclonal antibodies. This hidden site has been shown to be decrypted by partial unfolding of the native full-length protein following exposure of the fetus to stress/shock environments [18, 19]. The 34-amino-acid sequence of the occult segment, produced as a synthetic peptide has been purified, characterized, and assayed for biological activity [16, 20-22]. The major property of the 34-mer synthetic peptide was found to be growth suppressive in both neonatal and tumor cells; hence the name, Growth-Inhibitory Peptide growth inhibitory peptide (GIP). The synthetically produced version of the 34-mer peptide segment in its initial batch production was designated as number P149, and this number ID has been equated to GIP in the present manuscript. The P149 fragment was further subdivided into segments and synthesized as three separate fragments: 1) an amino-terminal 12-mer peptide termed P149a; 2) a 14-mer midpiece peptide labeled P149b; and a carboxy-terminal 8-mer designated P149c (see Fig. **1**). All control peptides used in this study [16, 18] [3] have either already been described and/or are listed in the abbreviation codes on the title page of this report.

Since the discovery of GIP in 1993 in the author's (GJM) laboratory and its subsequent published findings in 1996 [23]

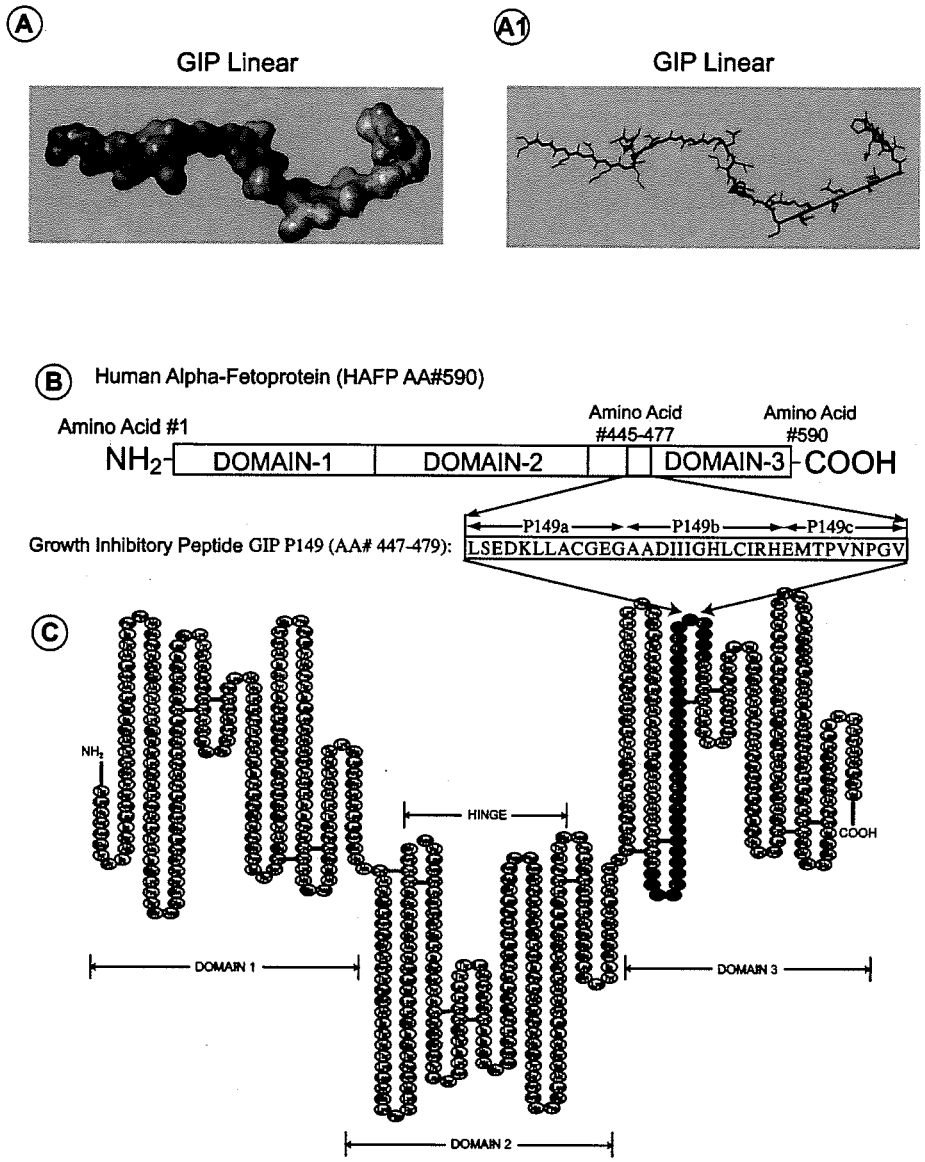

Fig. (1). Panel-A: The linear version (A and A1) of the 34-mer Growth Inhibitory Peptide (GIP) is displayed in Panels A and A1 as "electron "cloud and stick" computer models, respectively (See Ref. #48). Panel-B. The three domains of human alpha-fetoprotein are shown in a bar-configuration representing the 590 amino acid full-length protein. Panel-C: The telescoping segment displayed, from amino acids #445-477, is depicted in its single letter code amino acid sequence on the 3-domain structure of HAFP with the three fragment constituents of P149 comprising P149a, P149b, and P149c on HAFP domain-3. The minimal energy non-solvent stick computer models of the linear peptide were kindly provided by Dr. Curt Brennerman, Dept. of Chemistry, Rensselaer Polytechnic Institute, Troy, NY.

[16], a multitude of biological activities has since been described for this AFP derived synthetic peptide. Even though growth inhibition of fetal and tumor cells has been consistently demonstrated, other non-growth functional activities have been described [3, 20]. Such biological roles of GIP have been observed and documented at various organizational levels from the organism to the cell including even subcellular events. It is the purpose of the present review to categorize, list, prioritize and survey these varied biological activities in order to assess and elucidate the fundamental role(s) in which AFP-derived peptides function. Thus, the varied activities that were surveyed will be evaluated in order to determine the fundamental activities of this peptide. The multiple activities will be grouped in order to determine trends and patterns that point to the more prominent functions. The survey will encompass the following organizational levels: 1) the organism; 2) organ; 3) tissue; 4) cell; 5) cell surface; 6) cytoplasm and 7) the nucleus. Ultimately, the goal of this present review was to examine the various activities of GIP to determine mechanisms of action so that molecular targets could be identified for therapeutic use in human diseases. Since GIP was first recognized as an occult epitope on the unfolded HAFP molecule, some of the biological activities described in the present review will refer to both the 34-mer synthetic GIP itself as well as the same GIP segment inclusive on the full-length (590 AA) HAFP molecule itself (i.e., transformed AFP). A direct comparison between the 34-mer synthetic version of GIP and the GIP site exposed on the full-length AFP molecule (transformed AFP) will be discerned when appropriate. See Fig. **1** for GIP to HAFP spatial relationships and for amino acid sequence stretch comparisons of P149a, P149b, and P149c, and Table **2** for GIP to HAFP activity comparisons.

B) AMINO ACID SEQUENCE MATCHES

The GIP amino acid sequence was subjected to a FASTA search in the Genbank (GCG Wisconsin Program) database (Table **1**). FASTA employs a Z-based statistics algorithm in order to demonstrate similarities (relationship) between protein and/or peptide amino acid sequences. The statistical significance of analyzing short peptide sequences (<30 amino acid) from GCG criteria has been presented elsewhere [24, 25]. In brief, a word score of 1 to 2 is employed with a default set at 2.0 and above. With the word score set, an E-value is generated by the program; a value between 1 and 10 is considered significant. Thus, only short peptide sequences that scored within the appropriate E-values were selected for presentation in Table **1**.

The GCG search found identity/similarity sequence matches to angiogenesis-associated compounds such as FGF receptor, IL-1b, TGF-β, and non-receptor chemokine Ip-10. Other matches for cytoskeleton-associated protein, including gephyrin, neurofilaments, ankyrins, cadherin, and the motor filament kinesin, have been previously reported [3]. These amino-acid matches provide evidence that the P149 peptides contain short recognition cassettes for cytoskeletal involvement and/or docking. The Ache receptor matches further lend credence to the proposal that AFP-peptides might be involved in the activation of cell shape changes [4]. Matches with cell adhesion-related proteins were also found which included collagen XIII, collagen IV, laminin, chondrotin sulfate proteoglycans, fibrinogens, and fibronectin [3]. The GenBank matches in the programmed cell death portion of this review appear to reflect matched sequences of apoptosis regulatory proteins on the P149-peptide. Such proteins encompass the bcl-family of proteins, cysteine aspartases (caspases), annexins, and tumor necrosis factor-α. The P149a and P149b fragments provided the best sequence match to the bcl-2 protein. Searches for enzyme-associated proteins aligned with GIP also revealed AA identities with ATPases, kinases, synthases, and P450 enzymes. Finally, identities/similarities were identified with transcription-associated factors such as Hox, c-myc, forkhead and PAX.

C) BIOLOGICAL LEVELS OF ORGANIZATION

Both HAFP and its derived Growth inhibitory peptides are active at multiple levels of biological organization ranging from the organism to the cell. Several examples of biological action will be cited at each level with accompanying tables and figures to more fully substantiate the activities of HAFP and GIP. Table **2** exemplifies the GIP to transformed HAFP relationship. During the last decade, a multitude of studies have established full-length AFP as a growth regulator during tumor growth and progression [26-29]. Research findings now support the concept that full length AFP is largely a growth-enhancing agent by means of a cyclic AMP-protein kinase A activation pathway [30-32] [9, 11-14]. Indeed, if HAFP were a negative regulator of growth, few pregnancies would ever come to full term. However, growth is a complex process that requires fine - tuning, utilizing both up-and down-regulation to function correctly during a defined period or event, including both pregnancy and cancer. Although sustained growth of the fetus is required for full-term pregnancy to be achieved, the fetus encounters situations that require periods of temporary or prolonged growth cessation, such as differentiation and prevention of organ/tissue overgrowth [32]. Thus, fetal growth may require a temporary cessation until homeostasis is gradually achieved or until compensated signal transduction pathways are reestablished.

In its native form, HAFP displays mainly growth-enhancing properties, regardless of whether the tissue is of fetal or neoplastic origin; not surprisingly, AFP at physiological/pharmacological dose levels has been reported to enhance tumor growth [7, 31, 33]. Moreover, native HAFP has been shown to possess pro-angiogenic properties that enhance neovascularization and growth in both fetal and tumor tissue [34]; [35]. Reports also indicate that HAFP can stimulate the expression of certain oncogenes (c-Fos, c-Jun, and n-Ras) to enhance growth of human hepatoma cells [36]. However, HAFP has been shown to inhibit platelet aggregation and erythrocyte hemagglutination, properties also exhibited by GIP [37, 38]. Such reported properties suggest that the molten globule form of the full-length HAFP molecule exists in a blood circulating form; this form has been detected and identified and was named "transformed AFP (tAFP)" [25]. The tAFP is an unfolded molecular form in which the GIP epitope lies exposed on a conformationally - altered form of HAFP. This transformed version of HAFP might have future utility as a tumor and fetal defect marker.

Table 1. Genebank (FASTA)Matching of Amino Acid (AA) Sequences from AA #445-480 of Human Alpha-fetoprotein (AFP) with Conserved Sequences to Angiogenesis, Enzymes, and Transcription-Related Proteins

I. Angiogenesis Related		Percent (%) Identity/ Similarity	Percent (%) Total
Hum AFP #445	L S E D K L L A C G E G A A D I I I G H L C I R H E M T P V N P G V G	100/100	100
Fragment P149	P149a P149b P149c		
Mus IL-1B	E D R L V L C L X G A D V X P V G E L C R L K L H F L D F Y L V	36/18	54
Hum CYR61	L V V X T L L H C P E G E A X P K A P G V L	45/18	63
Hum TGF-β	L S E D Q L L	86/0	86
Col. Liv. Annexin-I	L S D E K L L L	63/25	88
Rab TNF-α	A S E N G V L X K A P V S P G S L	44/25	69
Hum IL-4	L L A S G L L I P E G H L C L N D	47/12	59
Hum IL-2	I X S I I V G H L	56/11	67
Hum Bld Factor XIII	Q S X I I V G H L	55/33	89
Hum Bld Factor VIII	I Q S I I V G H L	63/13	76
Hum Bld Factor IX	I Q S I I V G H L	63/25	88
Hum α, BIFN Recept.	I Q S I I V G H L	63/25	88
Hum IFN-α	C I S I S N Q P V N P R S	46/8	54
Chemokine IP-10	C I S I S N Q P V N P R S	46/8	54
Mus FGF Recept.	K Q M T P A N P G Q	60/20	80
II. Enzyme-Related			
Xenopus ATPase	L S C T R L I A C	56/22	78
Hum Cas Kin-α	I L L P C G E G A	78/0	78
BOV Cas Kin-II	V L L P C G E G A	78/0	78
Rat Creat. Kin-β	S L L P C S E G	63/13	76
Hum 6-PO4 Fruc Kin	I L L L C G E G	67/0	67
Hum β-acetyl G.A.T	L T G E G Q A D L T L V S P G L	57/25	82
Bov. 6-PO4 Fruc Kin 3	L C G E G T X D	71/14	85
Artemia ATPase-6	A A N I I A G H L	78/11	89
ATP synthase-A	A A N L T A G H L L I Y	50/25	75
Coumarin P450	I I M A Y I C I R	56/33	89
Nitric Acid Synthase	C I Q H G N T P G N G R	58/8	66
Mus Aspart AT	E M S P G G P G S	66/22	88
III. Transcription Related			
Por. Hox 2.4	C S I C E L L Y C G E G	58/8	66
bcl-2 complex	E S E T K L C I I V F H L C I	66/13	79
Hum Rel I-KB	G C G G G L G E	38/38	76
Hum C-myc	I I V G H L C W	75/13	88
Mus bcl-2 (α)	G H R C V R T P L I P C P P G	47/20	67
Nuclear FTZ-F1	K P T P I S P G Y	40/30	70
Mus WnT-7A	H T P V R P G V	60/20	80
Hum Forkhead	E M T P V D P G V	60/10	70
Hum PAX-3	I M T P V D P G V	70/10	80
Hum TFIID	P M T P A T P G S	50/20	70
Hum Src-tyr	E M A P I W P G A	50/20	70

Abbrevatives: Table-1 Aspart. AT = aspartate aminotransferase; Bcl-2 = B-cell lymphoma; BOV = bovine; Cas Kin = casein kinase; Col. Liv = parasite organism; Creat = creatine; CYR61 = connective tissue growth factor; FGF = fibroblast growth factor; Forkhead = transcription factor; FTZ-F1 = AFP transcription factor; GAT = galactosylaminyltransferase; Hox = homeodomain protein; Hum = human; IFN = interferon; IL = interleukin; IP-10 = interferon induced chemokine; Mus = mouse; PAX = homeodomain protein; PO_4 Fruc = phosphor fructor; Rab = rabbit; Rel IKB = NFKB inhibitor protein; SRC = kinase related complex; TFIID = transcription factor-II; TGF = transforming growth factor; TNF = tumor necrosis factor; Tyr = tyrosine; Wnt = wingless oncogene product.

D) THE ORGANISM LEVEL

Metamorphosis and embryonic differentiation are two biological processes that include histogenesis and organogenesis as integral components of each process. In the present review, the inhibition by GIP on the hatching of brine-shrimp cysts, frog metamorphosis, and chick embryo development will be surveyed.

D1) The Brine Shrimp Bioassay

Some metazoans, like the arthropods and nematodes, share the characteristic of periodic molting or ecdysis and hence are referred to as the ecdysozoans. In the molting/ecdysis process, embryos are released as a gastrula, enclosed in a hard shell-like capsule (cyst) that enters diapause,

Table 2. Comparison of Biological Activities between Growth Inhibitory Peptide (34-amino acids) and Transformed and Non-transformed Human Full-Length Alpha-Fetoprotein (590 Amino Acids)

Biological Activity	Growth (GIP) Inhibitory Peptide	Human Alpha-fetoprotein (AFP)		Refs.
		Transformed	Non-Transformed	
1. E2-induced uterine growth (rodents)	Inhibits growth	Inhibits growth	No effect	16, 52, 53, 107, 110
2. E2-induced fetotoxicity	Reduces deaths	Reduces deaths	No effect	18
3. E2-induced MCF-7 foci formation (Human)	Inhibits foci	Inhibits foci	No effect	16, 18, 36
4. T3-induced tail resorption (Amphibian)	Inhibits Resorption	Inhibits Resorption	No effect	3, 19
5. Ascites fluid accumulation (volume & cell number)	Reduces fluid and cell number	Slight reduction in fluid & cells	No reduction in fluid & cells	3, 16, 25
6. Breast cancer growth (rat)	Inhibits growth	Inhibit growth	No effect	25, 52
7. E2 Receptor binding (Hum)	Binds (10^{-6}M)	Not significant	No effect	3, 25
8. Platelet aggregation (Hum)	Inhibits aggregation	Not known	Inhibits aggregation	3, 38
9. Insulin toxicity (chicks)	Reduced	Slight reduction	Not done	3, 18
10. Fetal mortality (Mice)	Reduces deaths	Reduces deaths	Slight reduction	3, 18
11. Fetal malformation (Chick)	Defects Reduced	Defects retarded	No effect	3, 18
12. Growth retardation (Chick)	Growth Retarded	Slightly retarded	No effect	3, 18
13. Litter size (Mice) (E2-induced reduction)	Prevents Litter reduction	Prevents Litter Reduction	No Effect	3, 18, 27
14. Estrogen toxicity (Mice)	Reduces	Reduces	No effect	18, 27

a reversible state of dormancy [39]. Metabolic activity in the diapause cysts is extremely low and those embryos are remarkably resistant to physiological stress for extended periods of time. The ecdysis-associated steroids, such as 20-hydroxyecdysone, are steroid ligands of the DNA-binding transcriptional factors (nuclear receptors) that control development, molting, segmentation, and metamorphosis in insects and arthropods [40]. One such transcription factor is the HOX homeoprotein; another is the beta FTZ-F1 transcription factor (Table **1**), which also serves as a nuclear factor for the induction of the HAFP gene in mammals [41, 42]. The mutation or disruption of these nuclear factors results in incomplete molting and dysfunctional metamorphosis. Mutation of other nuclear receptors leads to uncoupling of the molting/ecdysis process from subsequent metamorphosis in the ecdysozoans. The Brineshrimp test (crustacean bioassay) is a simple and inexpensive phototaxic assay that has been used to determine cytotoxicity versus cytostasis of animal and plant chemicals on mammalian tumors such as mouse leukemias [39, 43, 44]. The brine shrimp, Artemia salina, exhibits an oviparous (diapausal cyst production) mode of reproduction. The diapausal cysts are hatched by placing them in sea water at ambient temperatures under photoperiods of 10 hr and 14 hr of light/dark cycles for 72 hr [45-47]. Following ecdysis, the hatched larvae (nauplii) swim about vigorously in 60-mm diameter petri dishes containing 10 ml of sea water. The incubation dishes contain preweighed amounts of P149 to obtain peptide concentrations ranging from 1 mg/ml to 100 μg/ml per dish. Dishes were examined twice daily for time of a) hatching b) larval emergence, and c) the active larval swimming stage (Fig. **2A**). After 72 hr, the developmental stages of Artemia in the dishes are recorded as total unhatched cysts remaining, emerged larvae, and number of the swimming larvae nauplii. The P149 peptide was found to inhibit the hatching stage of Artemia salina in a dose-dependent manner; nauplial mortality rates at 72 hr were not affected. Although hatching takes 12-24 hrs, larval emergence and nauplial swimming requires 72 hr, Artemia hatching was the stage most susceptible to treatment with both cyclic and linear P149 peptides (Fig **3A** and **B**; Table **3-1**). The hatching of brine shrimp was about 70% in untreated groups. Cyclic P149 cyst hatching suppression was comparable to the linear P149 (~ 40% inhibition), while the scrambled peptide was similar to the non-treated control (Fig **3**; Table **3**). It was evident by 72 hr, that the Artemia bioassay represented only P149 hatching inhibition.

D2) AFP Peptide Inhibits Frog Metamorphosis

Stage 25 developing *Rana catesbiana* bullfrog tadpoles were employed to study thyroid-induced tail loss metamorphosis [19]. Results showed that untreated tadpoles had a mean tail loss of about 8% over 5 days, while tadpoles treated with 300 ng/ml of triiodothyronine (T_3) lost 32% of

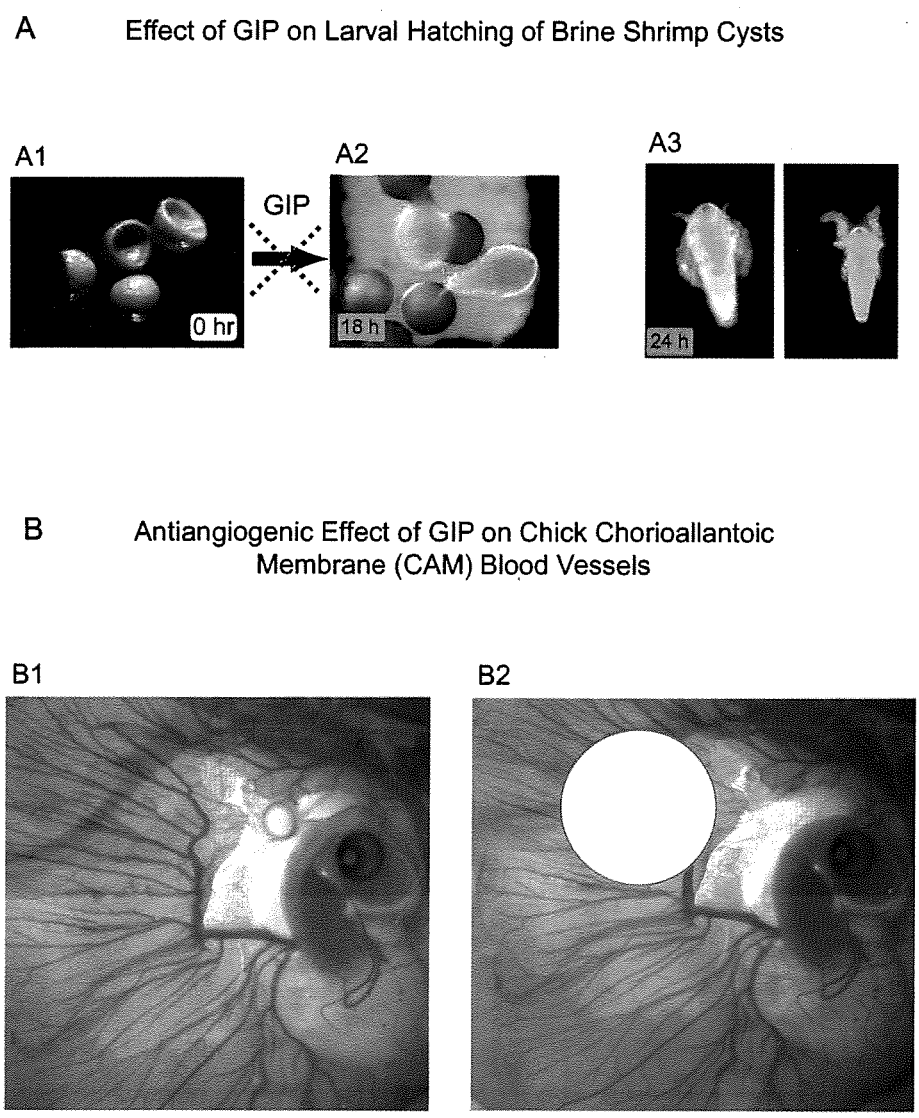

Fig. (2). Whole organism bioassay of brine shrimp cyst hatchability and analysis of chick fetal angiogenesis are depicted. Panel – A: The hatching, larval emergence, and naupliu swimming stages are shown in panels A1, A2, and A3, respectively. Note that the GIP inhibited the hatching of the cysts from 12 to 18 hrs of incubation in seawater. Pictures were adapted, modified, and reformatted into a composite picture derived from website eeob.iastate.edu. Panel-B: Assay of blood vascular formation (angiogenesis) in the chorioallantoic membranes (CAM) of the chick embryo/fetus is shown. Panel-B1: Normal angiogenic development of the blood vessel network of the 6-day chick fetal CAM is displayed. Panel B2: Placement of a Teflon disk, coated with GIP, is depicted during blood vessel tubulogenesis at incubation day-6. The GIP-coated Teflon disk is 14mm in diameter. A blanched area (under the disk) on the CAM is observed at day 9 after disk removal. See text narrative for method details and references.

their tail mass (Fig. **4A**). This difference was substantial after 3 days of T_3 treatment. Human AFP, at a concentration of 70 ng/ml, completely inhibited the T_3 induced tail loss when AFP was preincubated with T_3 for 1.0 hr. Other mammalian proteins (at 70 ng/ml concentrations), including mouse and human albumin, bovine fetuin, transferrin and alpha-1-acid glycoprotein, failed to inhibit tail reduction by T_3 (Table **3** #2). When AFP and T_3 were added separately to the tadpoles without co-reagent incubation, inhibition was not observed suggesting that HAFP must be T3-transformed to have an effect or that HAFP somehow nullified the T_3 effect.

Similarly, tadpoles exposed to the purified P149 completely abrogated the tail resorption response similar to that observed with transformed full-length HAFP (Fig. **4A**). Moreover, the P149 peptide displayed a dose response while the scrambled version of the same peptide had no effect [19]. The P149 peptide-inhibited response mimicked that of the full length transformed HAFP at a concentration of 33 ng/ml. In summary, the P149 peptide appeared to account for most of the inhibitory response demonstrated by full-length AFP.

D3) GIP Inhibits Chick Fetal Insulin Toxicity Producing Growth Restricted Fetuses

The chick embryo fetal toxicity assay for insulin was performed as described by Landauer and Bliss [48, 49] with the initial injection of insulin on day one. On day 8, treated

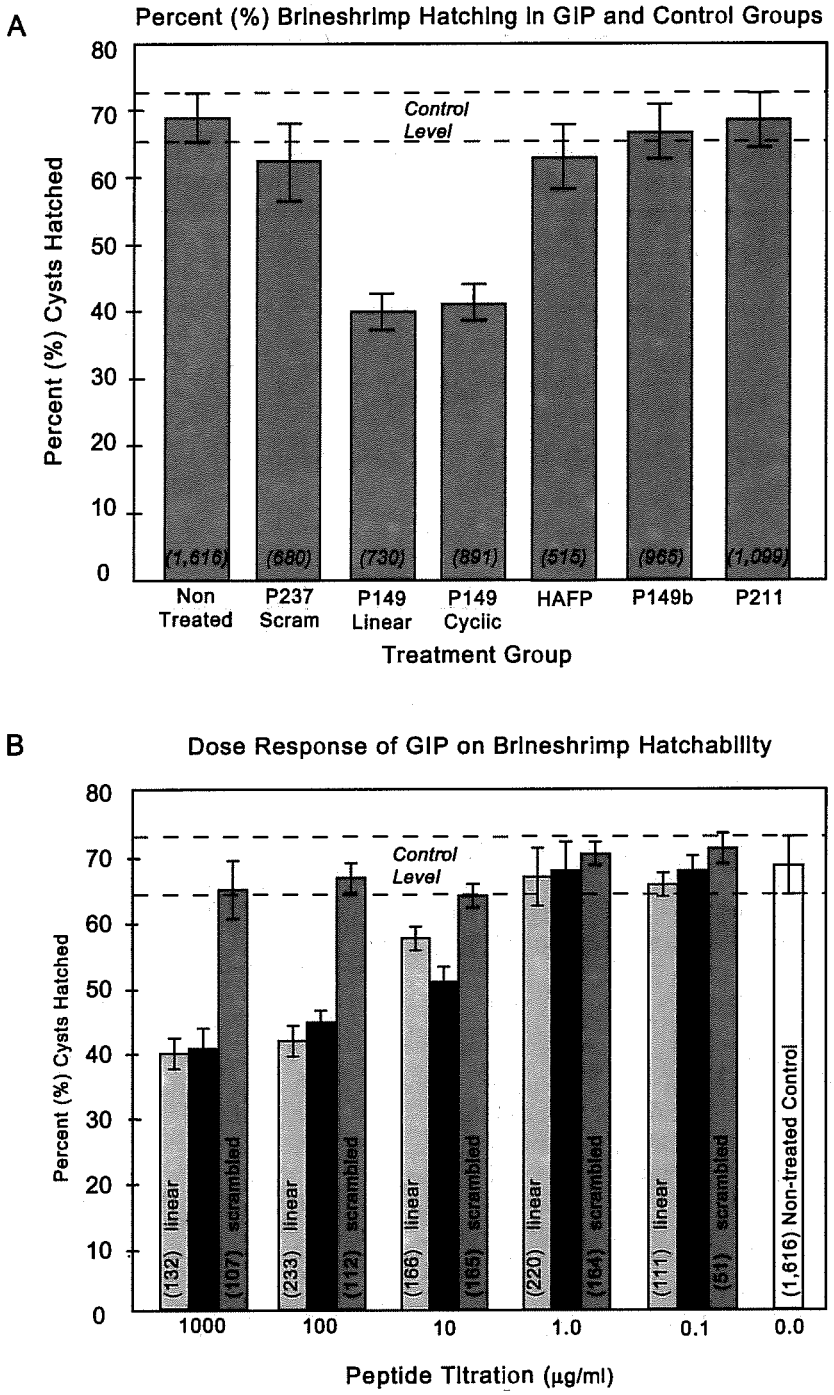

Fig. (3). The effect of GIP on the Brine shrimp bioassay of cytotoxicity/cytostasis is depicted. As noted, GIP is not cytotoxic and affects only hatchability stages of the brineshrimp cysts. Panel-A: The percent (%) brine shrimp hatching in seawater is shown using non-treated and treated cysts. Peptide-treated groups encompassed linear, cyclic, scrambled (P237) and P149 fragment peptides. It is evident that both linear and cyclic, but not GIP fragments, inhibit about 40% of brine shrimp hatchability. Panel-B: A dose response histogram of the GIP treatment on brine shrimp hatching is displayed. It is evident that GIP elicits an abrupt but stepwise dose response from 1.0 mg/ml down to 10 ng/ml. An optimal concentration of GIP required for hatching inhibition would be 50 µg/ml.

and non-treated eggs were opened and examined; crown-rump measurements and body weights were recorded. Chick fetuses were developmentally staged in accordance with the criteria of Hamilton [50]. The assay for chick fetal toxicity and malformations using insulin alone resulted in the death of nearly one-third of the fetuses after 7 days of incubation [18]. Fetal malformations were observed in these chicks; with defects including dwarfism, cyclopia, orbital agenesis, and micromyelia. Accompanying these fetal defects, growth retardation was also observed in the insulin-injected treat-

Table 3. The Organism Level. Summary Activities of HAFP and Growth Inhibitory Peptides (GIP) in Various Growth and Development Assays

Bioassay Type/Name	Peptide Biological Response/Activity			
	Inhibition, Suppression	Enhancement, Activation	Minimal or No Effect	Not Tested
1. Brineshrimp Hatchability	P149 ln P149 cy	P237	HAFP P149b, P211, P237	P149a
2. Frog tail Resorption	HAFP P149 ln	None	P237	P149cy, P149b, P149b, P149c
3. Chick Insulin Toxicity	HAFP P149 ln	None	P237	P149cy, P149b, P149a, P149c
4. Mouse Estrogen Toxicity	HAFP, P149a, P149 l, P149b, P149c	None	P237	P149 cy
5. Bactericidal test, E. Coli	None	None	P149 ln, P237	P149 cy , P149b, P149a, P149c
6. Fruitfly larval development	None	None	P149 ln, P237	P149 cy, P149b, P149a, P149c

HAFP = human alpha-fetoprotein.
Growth Inhibitory Peptide (GIP) = 34-mer peptide designated P149.
P149 cy = 34 mer cyclized version of GIP.
P149 ln = 34 mer linear version of GIP.
P149 a = 12 mer-aminoterminus of GIP.
P149b = 14 mer-midpiece report of GIP.
P149c = 8 mer-carboxyterminus of GIP.
P237 = 34 mer-scrambled amino acid version of GIP.

ment group (Fig. **4B**; Table **3** #**3**). In comparison in chicks injected with HAFP or P149 plus insulin, the fetal death rates were reduced by 60%, respectively. The presence of fetal malformations was also substantially reduced in the HAFP plus/insulin group and in the P149 peptide plus/insulin group. Fetal defects were observed only in treatment groups that received either insulin alone or insulin in combination with proteins or peptides. In comparison, growth restriction occurred in the HAFP plus insulin group and in the P149-peptide plus insulin group, which exhibited a retardation rate nearly 1.5 times that of insulin alone. The total body weights and crown-rump lengths served to confirm the observations of congenital malformations and growth retardation among the treatment groups. Thus, it was shown that transformed HAFP and/or P149 could decrease fetotoxicity and congenital malformations, at the expense of growth retardation, in developing chick fetuses.

D4) GIP and the Murine Estrogen Fetotoxicity Assay

High doses of E_2 injected into 16-day pregnant mice produce fetal death in approximately 30% of the affected newborns as compared with a 2% death rate in the saline-treated controls [18]. However, if estrogen injections were immediately preceded by injections of transformed HAFP or P149, the death rate of fetuses was reduced to less than 10% (Fig 4C). Injection of the scrambled version of the P149 peptide following estrogen injection did not reduce the newborn death rate, nor did injection of unincubated (untransformed) native HAFP. However, if HAFP was first transformed by incubation with E_2 (tAFP) for 1.0 hr prior to injection, the presence of the transformed HAFP reduced rates to 12-15%. When peptidic subfragments (P149a, P149b, and P149c) of the GIP were separately assayed following E_2 injection, P149a and P149b produced effects comparable to P149, while P149c was slightly less effective (Table **3** #**4**). Administration of estrogen had no effect on body weight, as previously reported [18], but it did result in reduced litter size. P149 and its fragments protected against litter-size reduction. Although the protection by P149 fragments against litter reduction approached that of P149, no individual AFP fragment protected as well as the full-length 34-mer GIP [18].

D5) P149 Peptide Effect on Bactericidal Tests Using *E. coli*

The P149 peptide was tested for bactericidal activity by a modification of the Minimum Bactericidal Activity Test for antibiotics (GJM's unpublished data). Mueller-Hinton broth and agar were used for testing. The organism employed was *Escherichia coli* ATCC 25922. A stock solution of 1 mg substrate/ml of broth was utilized. In summary, no inhibition of growth, stimulation of growth, or killing of bacteria was detected using P149 peptide (Tables **3** #**5**). Thus, it was concluded that P149 does not possess inherent antimicrobial activity; that is, GIP does not demonstrate antibacterial peptide activity against *E. coli* in culture [51].

D6) Effect of GIP on Drosophilia Development

As in the *E. coli* experiments (see above), the testing of GIP influence on Drosophilia development also displayed no effects. The P149 peptide in solution (1 mg/gram dry weight) was added daily to the food during a period of 13 days.

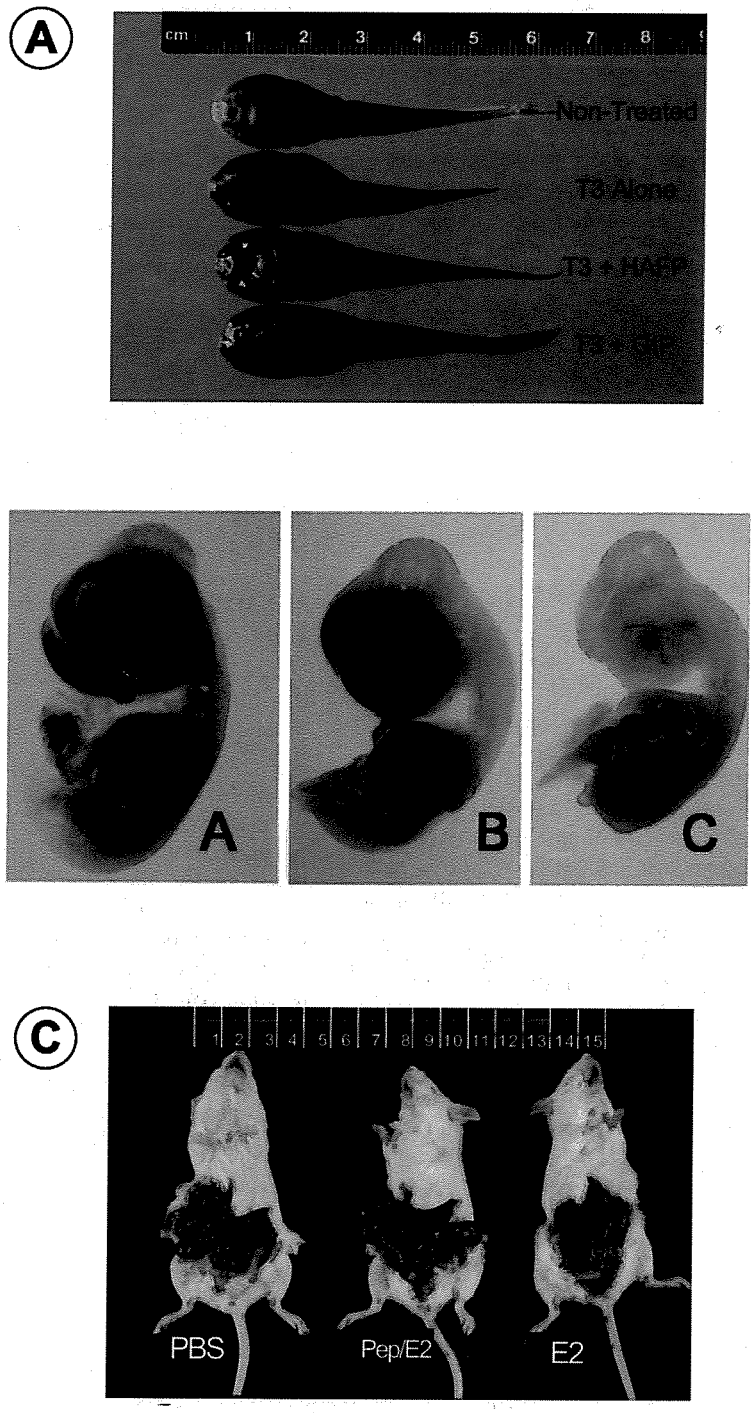

Fig. (4). Three whole organism bioassays using GIP are displayed in Panels A, B, and C. In each instance, GIP (P149 peptides) was administered to the organism via injection or environmental exposure. Panel-A Photograph of four representative tadpoles. Treatments were as indicated in the photograph labels. Note that full-length AFP and GIP blocked the triiodothyronine (T3) effect and were similar to the non-treated tadpole controls. Panel B: Three representative chick fetuses are displayed from the various insulin treatment groups. Observe that the insulin plus P149-treated fetus is a smaller but normal fetus. A) Non-treated control; B) Insulin plus P149; C) and insulin alone (eye orbital agenesis). Panel-C: Biopsied body cavity exposure of mice treated with saline, GIP plus E2 and E2 alone. Note that the P149 (PEP) plus E2 treatment prevented E2-induced fetotoxicity. E-2 alone-treated groups resulted in 30% fetal estrogen toxicity and evacuation of uterine chamber (see text for details).

During this time, complete developmental cycles (egg to adult) occurred in both experimental and control bottles. However, no observable changes in Drosophlia development could be discerned between the treatments versus the control group at the time interval tested (Table **3-6**).

D7) An Organism Level Overview of GIP Exposure

At the structural level of the organism, GIP was demonstrably active in modifying apoptosis, morphorgenesis, differentiation, larval molting, fetal toxicity, and teratogenicity. The larval hatchability of brineshrimp cysts was blocked by more than 40% in the GIP-treated groups. GIP inhibited amphibian tail resorption, a process which encompasses both apoptosis and differentiation. The teratogenic impacts of high insulin and estrogen concentrations in the chick and mouse fetus/embryo were also blunted by GIP administration. However, GIP was not bactericidal against E. coli and had no effect on fruit fly development. Thus, it is evident that the environment in which the organism exists can be highly influenced by the presence of GIP.

E) THE ORGAN LEVEL

AFP-Peptides Inhibit Cell Growth and Proliferation in Normal Uterine Tissues:

E1) *In Vivo* Mouse Uterine Growth Bioassay

An immature mouse uterine bioassay to measure the growth regulatory properties of AFP and its derived peptides was previously developed in the author's (GJM) [52-55] laboratory. AFP-Peptides (P149) and its fragments P149a and P149c were found to suppress estradiol-induced uterine growth (Table 4 #1). In contrast, P149B did not substantially affect uterine growth. Control peptides consisting of the scrambled P237, and an albumin homologue of P149 (P263) also did not inhibit uterine growth [16]. It is of interest that the P149 peptide suppressed uterine growth by transformed human, mouse, and rat full-length AFP. In a previous report using the uterine growth assay, P149 peptide failed to inhibit E2 stimulated growth in the presence of high concentrations of divalent cations, (0.17 gm/L) such as calcium, manganese, and magnesium; in contrast, inhibition was synergistically improved by the addition by anti-integrin antibodies [3]. These results (both the antibody and cations) indicate that integrin-mediated cell surface events somehow play a role in an interaction of P149 with the surface of uterine cells. Anti-AFP- peptide antibodies nullified the inhibitory effect of P149, as did divalent cations, suggesting a cell-surface steric hindrance and/or an ion-associated blockage event [3].

E2) *In Vivo* Adult Mouse Uterine Growth Bioassay

The uterine assay applied to the immature mouse pups was modified for use in adult mice [56]. Timed pregnancies (vaginal plug) were employed to obtain groups of pregnant mice which delivered at the same time intervals. At postnatal day 18, nursing mice were culled from their pup litters and isolated for 14 days to allow for serum prolactin reduction. After this period, normalized uterine-to-body weight ratios in PBS-treated mice were established. Using 2.5 μg E2, a UT/BW ratio increased 2.5 times the baseline value at 24 hrs following E2 injection (Table 4 #2). When GIP was titrated over a range of 100 μg to 0.1 μg peptide injected per mouse, uterine growth inhibition attained 61% over this dose range. Thus, the results were comparable and as effective as the inhibitory growth suppression observed with P149 in the immature uterine assay (See Section E1 above).

E3) GIP Effect on Partial Hepatectomy of the Mouse Liver

The P149 peptide was administered to adult mice immediately following partial hepatectomy, which surgically removes two-thirds of the liver mass [57-59]. During the first 3 days, the mice received doses of GIP from 50 ng to 200 μg per mouse. On the 4th day following surgery, the animals were injected with H^3-thymidine and sacrificed 3 hrs later; portions of the remaining liver were removed, fixed, and prepared for histochemical autoradiography [59] and [60]. After an appropriate time exposure, the slides were developed for autoradiography and the tissues stained with H & E; for mitotic indices, parallel slides were examined under light microscopy for both mitotic figures (histopathology) and radiographic silver grains representative of cell division (Table-#4, Fig. 5). Elevated serum AFP concentrations normally follow initiation of hepatocellular proliferation. Serum AFP levels rise on day-1, peak 3 days follow partial hepatectomy [60, 61], and return to normal by 8 days. Results revealed that the liver parenchymal cells of the GIP-treated livers were substantially growth-suppressed compared to regener-

Table 4. The Organ Level: Summarized Activities of Human Alpha-fetoprotein (HAFP) and Growth Inhibitory Peptide (P149) in Various Growth and Development Assays

Bioassay Type/name	Peptide Biological Response/Activity			
	Inhibition, Suppression	Enhancement, Activation	Minimal or no effect	Not tested
1) immature rodent uterus	HAFP, P149a, MAFP, RAFP, P149c	P192	P149 cy, P237, P263, P149b	None
2) adult mouse uterus	HAFP, P149 In P149a, P149c	P192, P149b	P237, P263, P149b	P149cy
3) mouse liver regeneration	P149 In	None	P237	P149cy, P149a, P149b, P149c

AFP = alpha-fetoprotein; P192=HAFP carboxyterminal third domain.
H = human; M = mouse; R = rat; P263 = human albumin homolog.
* For other peptide number designations see footnotes to Table-3.

ating liver cells not treated with GIP (Table **4-3**). It is evident in (Fig. **4**) that GIP growth-suppressive effect was specific for the liver parenchyma cell population rather than the stromal connective tissue cells (see figure legend).

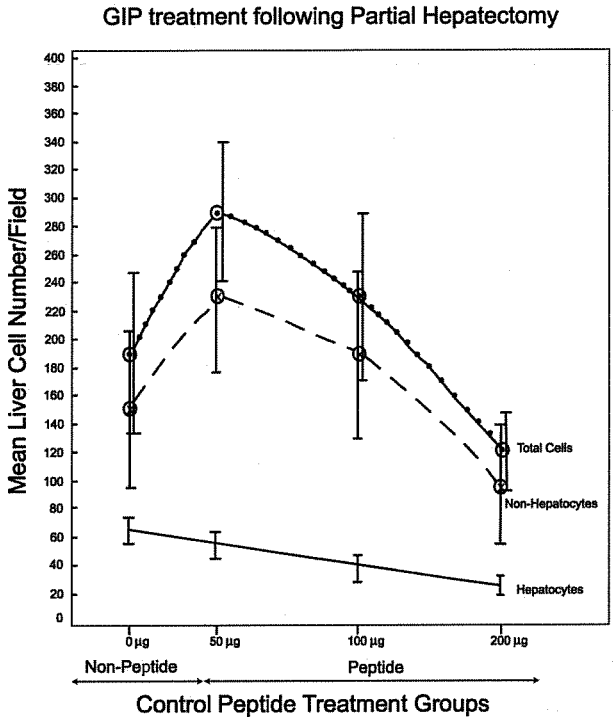

Fig. (5). Following partial hepatectomy in adult mice, GIP was administered in a dose response fashion. Autoradiographic analysis of mitotic figures in parallel with H&E stained tissues were counted employing light microscopic analysis of mouse hepatectomized liver sections. As shown in the figure, increasing amounts (50 to 200 µg) of GIP decreased the number of dividing parenchymal cells while non-parenchymal cell numbers could not be adequately discriminated from those of hepatectomized control (non-GIP treated) cells. Non-parenchymal cells included Kupffer cells, connective tissue cells fibroblasts, blood vessel and sinus endothelia, and bile duct lining cells. Parenchymal cells included liver hepatocytes, Oat cells, and peribiliary duct cells.

E4) An Organ Level Overview of GIP Treatment

At the structural level of the organ, GIP was shown to be effective in modulating steroid-induced neonatal and adult organ growth and differentiation; in addition, adult organ regeneration was greatly affected. Inhibition of estrogen-induced growth in the uterus of the immature mouse pup was clearly inhibited (40-50%) by GIP and its peptide fragments. Growth suppression (60%) of the adult uterus was also suppressed in post-lactation cycling female mice. Finally, administration of GIP in adult mice during the post-surgical period following liver ablation displayed growth suppression in the regenerating hepatic tissues. Thus, it is apparent that administration of GIP in conditions affecting specific target organs can be highly effective in influencing those organ responses.

F) THE TISSUE LEVEL

F1) Alpha-fetoprotein Derived Peptides as Anti-Angiogenic Agents

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is required for growth of solid tumors and during pregnancy, wound healing, tissue repair, placentation, and embryonic development [62]. The process is composed of complex, multi-step stages, encompassing four major events: 1) cell migration, 2) proliferation of cells (endothelial), 3) cell survival, and 4) vessel tube assembly/formation [63] The examples cited in this review will refer to blood vessel formation in cancer and vasculogenesis in normal developing cells. Vasculogenesis is regulated by multiple factors, which may include the secretion of chemical agents (proteins, peptides, prostaglandins, etc); these promote the stepwise progression of events leading toward and inducing neovascularization. Such agents are termed pro-angiogenic factors (PAF) in contrast to their opposing agents, the anti-angiogenic factors (AAFs). The PAFs can include various families of growth factors (Table **1**) such as transforming growth factor-β1 (TGF), basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived endothelial growth factor (PDGF), insulin growth factor (IGF)-II, and a recently described ECM growth factor termed CYR61 [64]. This CYR61 protein is a secreted ECM growth factor that both induces and promotes angiogenesis [65]. Additional agents that function as PAFs can include nitric oxide, insulin, hypoxia, and molecules that act directly on vascular endothelial cells. Thus, angiogenic factors fall into two subgroups: (i) those that act directly on endothelial cells to stimulate migration and mitogenesis, and (ii) those that act indirectly on host tumor and/or normal cells to induce endothelial growth factors for these activities.

The activities that underlie the progression toward angiogenesis are subcomponents of the four stages (described above) leading to successful neovascularization in tissues, tumors, and wound granulomas. These subcomponent events of pro-angiogenesis occur in cells which involve the following structures and activities: basement membranes, adherent cells of the ECM constituents, cytoskeleton-induced cell shape changes, cell aggregation, receptor clustering, ECM interaction with integrins, and involvement with cell proliferation, differentiation, and survival [66-68]. Antagonism of the above subcomponent constituents qualifies a compound as an anti-angiogenic factor (AAF). Inhibition of most of these angiogenic events would prevent the assembly of endothelial cells into capillary tubes, i.e., tubulogenesis, the endpoint of the angiogenic process and the fourth step of the process. That is, without the first three stages of angiogenesis (cell migration, proliferation, and cell survival), capillary vessel tube assembly does not ensue. These data demonstrate that the P149 peptides display all properties of the subcomponents and direct activities of anti-angiogenic factors in both normal and cancer tissues. The first three events concerning GIP have been previously addressed [3, 14], and the fourth event (blood vessel formation or inhibition) is presented below.

A. The Chick Allantoic Membrane Assay (In Vivo) of Egg Shell Membrane Vascularization

The chick allantoic membrane (CAM) assay is a measure of blood vessel capillary structure formation of the inner membrane of the chicken eggshell membrane [69]. Thus, the CAM assay is a rapid screening method of measuring angiogenesis in a living system. The chorioallantoic membrane system emerges on incubation day 3-5, and its vessels subsequently grow throughout the inner surface of the egg shell, which encompasses the yolk sac and the embryo unit (Fig. **2B**). The CAM assays are best conducted at the 6-9 day incubation period just prior to the embryo-to-fetus conversion. Changes in patterns, density, and size distribution of the CAM vessels are recorded by planometric measurement. Values were calculated as percentage of control using FGF stimulation as the positive control in accordance with methods developed by Folkman and others [66].

As listed in (Table **5-1**), the cyclic GIP preparation inhibited the angiogenic response in chick eggs substantially; this occurred at molar concentrations of 1.0 to 10 μM. While the cyclic P149 inhibited 95%, the linear P149 version was only capable of 40% inhibition of angiogenesis. The peptide fragment of P149c, (last 8 amino acids) and P149b (middle 14 amino acids) also displayed varied results. The P149c fragment was similar to the cyclic 34-mer showing >90% inhibition, whereas, the mid-piece (P149b) was largely inactive (20%). Finally, the albumin control peptide P263 and the carboxyterminal AFP fragment (P192) were both found to be unreactive.

B. GIP and Tumor Angiogenesis

The 34-mer GIP and several of its fragments have also been assayed for their anti-angiogenic properties in tumor angiogenesis models using the CAM assay in chick eggs [70]. In the CAM assay, mouse C5B1 melanomas were implanted on the egg shell membranes on incubation day 6, and observed 72 hr later for angiogenic patterns surrounding and attaching to the tumor surface (Table **5-2**). Angiogenesis is induced by means of basic FGF application according to the above published protocols. The results indicated that the cyclic GIPs were capable of 95-100% inhibition of tumor vascular ingrowth compared to FGF-positive controls established with the transplanted tumor. The carboxy-terminal fragment of GIP P149c also produced 90-95% inhibition, while the mid-fragment peptide piece P149b was essentially non-active (<20% inhibition). Finally, upon inspection of the chick fetuses subjected to GIP exposure, no developmental side-effects (harmful or otherwise) were discerned in treated as compared to sham-control fetuses.

In summary, the data derived from both the CAM blood vessel assay and the CAM-tumor assay clearly demonstrate that the GIP disulfide-bridged cyclic version, and to a lesser extent, the linear version, are antiangiogenic factors. Since the GIP has amino acid sequence identity to the chemokine factors (chemical attractant cytokines), it is conceivable that GIP and its fragments serve as angiogenic inhibitors (chemokine mimics or decoys) by blocking chemoattractant stages of the angiogenesis process. Decoy ligands are defined as binding agents that attach to receptors but do not elicit a physiological response.

F2) A Tissue Level Overview of GIP Treatment

At the structural level of the tissues, angiogenesis provided an excellent example of GIP's modification of biological responses. Since the total process of angiogenesis requires cell migration, proliferation, survival, and tubulogenesis, present results indicated that GIP greatly influenced one or more of these stages. Indeed, it was evident that GIP blocked tubulogenesis in that vascular branching and anastomoses were absent from the tissue treatment areas. It will also be demonstrated in subsequent sections of this report (Section G), that GIP suppresses not only cell migration,

Table 5. The Tissue Level: Summarized Activities of Growth Inhibitory Peptide (P149) in Normal Blood Vessel and Tumor Angiogenesis using the Chick Chorioallantoic membrane (CAM) assay*. Percent Inhibition of Blood Vasculature Formation on Chick Embryo Extra embryonic Membrane is displayed for P149, its Fragments, and Control Peptides

Peptide Tested: # Designation	Angiogenesis Inhibition (%)	
	Normal Blood Vessel (CAM) Angiogenesis	Tumor Blood Vessel Angiogenesis**
1. P149 linear	40	45
2. P149 cyclic	95	95-100
3. P149b (P232)	20	20
4. P149c (P211)	90	90-95
5. P263 (ALB)	0	0
6. P192 (CT-AFP)	0	0
7. P237 (Scram)	0	0

*N=5 each per experiment; AFP = alpha-fetoprotein; ALB = albumin (human); CT-AFP – carboxy terminal end of AFP (human); Scram = scrambled peptide of P149 amino acid sequence.

** = mouse melanomas (see text).

For peptide number designations, see footnotes to Table-3.

proliferation, and survival but also cellular ECM adherence of tumor cells. Thus, GIP is demonstrably active in inhibiting tissue developmental events as evident by the blockage of blood vessel neogenesis.

G) THE CELL LEVEL

G1) Peptide P149 Affect Cell Shape

Cytoskeletal Alternations

The cytoskeleton of a cell determines the shape of a cell, much as the metal tubes of a camping tent dictate its shape and form [71]. Integrins ($\alpha_5\beta1$), in turn, are responsible for the anchorage-dependent attachment to the ECM substratum, which reinforces the cell infrastructure [72]. Because the two systems are linked, if the attachment is weak, the cells may round up (change shape) in preparation for detachment and subsequent migration. Epithelial and endothelial cells are not capable of proliferating when denied attachment; eventually they undergo a programmed cell death called anoikis [23]. The cytoskeletal elements such as actin micro-filaments, motor filaments such as dynein, kinesin, and the intermediate filaments such as ankyrin and other neurofilaments, act in concert to induce changes in cell shape [73]. The focal adhesions and other endocytotic signaling (kinases) in the cell membrane dictate which changes in cell shape can occur. Data in previous reports and Table **6** demonstrate that the AFP-peptides can induce changes in cell shape by alteration of the microenvironment that the cell inhabits [16].

Astroglial cells may modulate neural signals by physically changing the extracellular space in the central nervous system. A GIP study was reported in which astroglial cells changed their morphology following exposure to the AFP-peptides [4]. In that report, astroglial cell cultures were prepared from four brain regions: cerebellum, hippocampus, cortex, and brain stem. Astroglial shape change was elicited by the P149 peptide in three of the four brain regions. No response was observed from astroglial cells derived from the brain stem. The largest number of cells reacting to the GIP was observed in the hippocampal cultures. An intermediate number of cells responded in the cerebellum, while the fewest cells reacted in the cortical cell cultures. Overall, dose-dependent responses of GIP were observed in cerebellar, hippocampal, and cortical cultures, with EC_{50} values calculated for these responses to GIP of 30, 3, and 3 nM respectively (Table **6-1**).

G2) Inhibition of Cell Migration or Spreading *in vitro* by AFP-Peptides

Cell adhesion receptors and their ligands, i.e., ECM proteins, provide the traction and stimulus for the migration and spreading of cells [74]. Cell migration depends on a delicate balance between cell adhesion and cell detachment factors. In general, most cells, including tumor cells, use adhesion molecules to execute cell migration; that is termed cell spreading in cell culture [75]. The integrins mediate migration of adherent cells such as fibroblasts, epithelial cells, and tumor cells on the ECM. Migration of cells occurs on weak or moderately attached cells, whereas strong cellular attachment tends to immobilize the cells. Cell migration requires multivalent binding of integrins to a matrix bound ligand such as collagen, laminin or fibronectin [76]. Integrins are internalized during cell migration; the integrins are taken in at the trailing edge of the cell and re-inserted at the leading edge in a calcium-dependent fashion. This process results in

Table 6. The Cell Level: **Summarized Activities of Human Alpha-fetoprotein (HAFP) and Growth Inhibitory Peptides (P149) in Various Growth and Development Assays**

Bioassay type/name	Peptide Biological Response/Activity			
	Inhibition, Suppression	Enhancement, Activation	Minimal or No effect	Not tested
1). Cell shape and form	P149 In	None	P237	HAFP, P149c P149cy, P149b, P149c
2) Cell migration and spreading	P149 In	None	Ovalbumin	HAFP, P149a, P149cy, P149b, P149c
3) Cell foci formation	P149 In, HAFP, P149c	None	P237, P149a, P149b	P192, P263
4) Cancer cell proliferation a) in vitro	P149In, P149a, P149cy, P149b, P149c	None	P237	P192, P263
b) in vivo	P149In, P149c	None	P237	P149a, P149b, P149c
c) ascites tumor	P149 In, P149c	None	P149cy, P149a, P149b	P192, P263
5) Cell death and rescue (apoptosis)	HAFP, P149In	None	P237	P149a, P149b, P149c
6) Immuno-regulatory Response a) T-cell	None	P149In	P237	P149cy, P149a, P149b, P149c
b) B-cell	None	P149 In	P237	P149c

* For other peptide number designations, see footnotes to Table-3.

the cell being moved forward, much as the track-belt propels a military tank. Of course, cell-to-cell adhesion is also involved in the process utilizing integrins. Whether a given cell remains stationary or migrates depends on the cooperation and cross-talk between the various types of adhesion receptors [77]. AFP-peptides appear capable of disrupting the cross-talk between integrins and other adhesion receptors [78].

Analysis of a coverslip cell migration assays revealed that the P149 peptide, at a coating concentration of 1μg/ml to 5.0 μg/ml, inhibited more than 60% of the MCF-7 cancer cells' spreading and migration over the surface of the coverslips (Table **6 #2**). This effect exceeded the ovalbumin control peptide by 3-fold. Microscopic observation revealed that the MCF-7 cells did not uniformly spread over the surface of the coverslip, but they only were observed to form islets of cells having jagged cytoplasmic processes. The cells that were unable to migrate displayed distorted morphology such as star-shaped configurations, cytoplasmic spiking, surface spiny spheres, membrane ruffling, and extensions of cytoplasmic processes concomitant with low cell viability. In comparison, ovalbumin inhibited only 10-20% of the cell spreading.

G-3) AFP-Peptide Inhibition of Estrogen-Induced Focus Formation of MCF-7 Breast Cancer Cells

Human breast cancer cells (MCF-7) in cell culture tend to lose their contact inhibition properties when exposed to physiological concentrations of estrogen (10^{-8}M). While anchorage dependence of cell growth appears to be an integrin-related process, it appears that contact inhibition is mediated through cell-cell adhesion molecules such as cadherin and catenin [79]. In the MCF-7 cells, estrogen exposure releases the constraints by catenin stimulated cells, to allow unrestrained migration; the cells stack-up to form mounds termed foci; these can be quantitated via a colony counter. In the absence of estrogen, cell proliferation stops after the cultures establish cell-cell contact, and thus no foci are found. Thus, the MCF-7 focus assay has been used for the evaluation of the peptide for antiestrogenicity properties [16]. MCF-7 cultures were fed four times (with peptide) over the 14 days of culture with varying concentrations of peptide (10^{-8}-10^{-13} M) in estrogenic DF_5 medium, which contains high levels of endogenous E_2 (5% fetal bovine serum supplemental). Results indicated that both HAFP and P149, at $10^{-11}$to 10^{-12}M, were capable of suppressing cell focus accumulation by nearly 70% while HAFP produced nearly 75% inhibition (Table **6-3**). Slight estrogenic enhancements of the focal reaction were also noted with HAFP and to a lesser extent, GIP at 10^{-8}M [16].

G4) Fluorescent Localization of GIP on Tumor Cell Surfaces and in Cytoplasm

Previous reports using MCF-7 cells indicated that both P149 and HAFP, when supplied to the cell culture, first bound to the cell surface and then migrated into the cytoplasm; they thereafter accumulated in the perinuclear regions of the endoplasmic reticulum [20, 80]. Two different types of fluorescence were observed when the cells were viewed (Fig. **6A**). First a diffuse, flowing fluorescence was seen on the cell surface and throughout the cytoplasm. This was later also seen in the perinuclear area, immediately surrounding the nucleus (Fig. **6A**). The second type of fluorescence was seen as a particulate, punctate granular fluorescence. This particulation was also found surrounding the nucleus, in the cytoplasm, and on the cell surface. The punctate fluorescence of AFP-peptide on the cell surface is indicative of cell surface peptide and/or receptor clustering. Due to the fact that both diffuse and punctuate types of fluorescence were observed on the cell surface and in the cytoplasm, it was concluded that the added P149 AFP peptide readily binds to the MCF-7 cell surface and is apparently endocytosed. Pulse-chase studies confirmed that GIP uptake does indeed occur in the MCF-7 cells [20]. Since the peptide localized in the cytoplasm in about 1.0 hr and the fluorescence ultimately appeared to be perinuclear, it was conceivable that vesicle-bound (endosome) peptide was transported from the cell surface in an endosome, which could fuse with the endoplasmic reticulum (ER) for deposition in the perinuclear region of the ER; the perinuclear region of the cell is known to be continuous with the lumen of the ER. Many internalized cargo peptides localize to the perinuclear spaces for compartmentalization, sequestration, and storage [20, 81]. It was interesting that the binding and the uptake of HAFP in isolated hepatoma cells exhibited similar fluorescence patterns (Fig. **6B**).

G5) AFP-Peptides Can Suppress Cancer Cell Proliferation Both *In Vivo* and *In Vitro*

The AFP-peptides are capable of suppressing cancer growth both *in vitro* and *in vivo*. Cancer cell growth is promulgated and maintained by continued growth factor receptor stimulation from the extracellular milieu. Growth factor receptors are cell surface transmembrane proteins, many of which are G-protein coupled signal transduction pathways linked to kinase enzymes. Some are coupled to tyrosine, serine/threonine, and MAP-kinase cascades that regulate the molecular switches for mitosis, and hence, cell proliferation. The blunting or uncoupling of the receptor from its signal transduction pathway can set up a blockade of the cell to prevent further growth stimulation. In the physiological process called "desensitization", a cell can become refractory to persistent agonist stimulation, and can shut down its binding/internalization mechanisms to peptides, hormones, cytokines, kinins, and other agonists. Cancer cells are likewise susceptible to agonist desensitization; thus, receptor ligand peptides can be employed as decoy drugs to induce a persistent state of desensitization by continuous drug administration. Such a compound (i.e., GIP) would constitute a physiological, non-toxic growth suppressor for cancer cells that commonly employ cell surface receptors linked to G-coupled signal transduction pathways (see Concluding Statements). In theory, this should include many cancer cells regardless of tissue origin.

If the growth of cancer cells can be inhibited regardless of tissue type or origin, then a common mechanism of suppression is probably involved. The synthetic P149 Peptide exhibits antagonist activity, in contrast to the variable agonist activity of the full-length AFP molecule. An *in vitro* growth suppression assay was performed which utilized sulforhodamine-B as a measure of cultured cell viability [20].

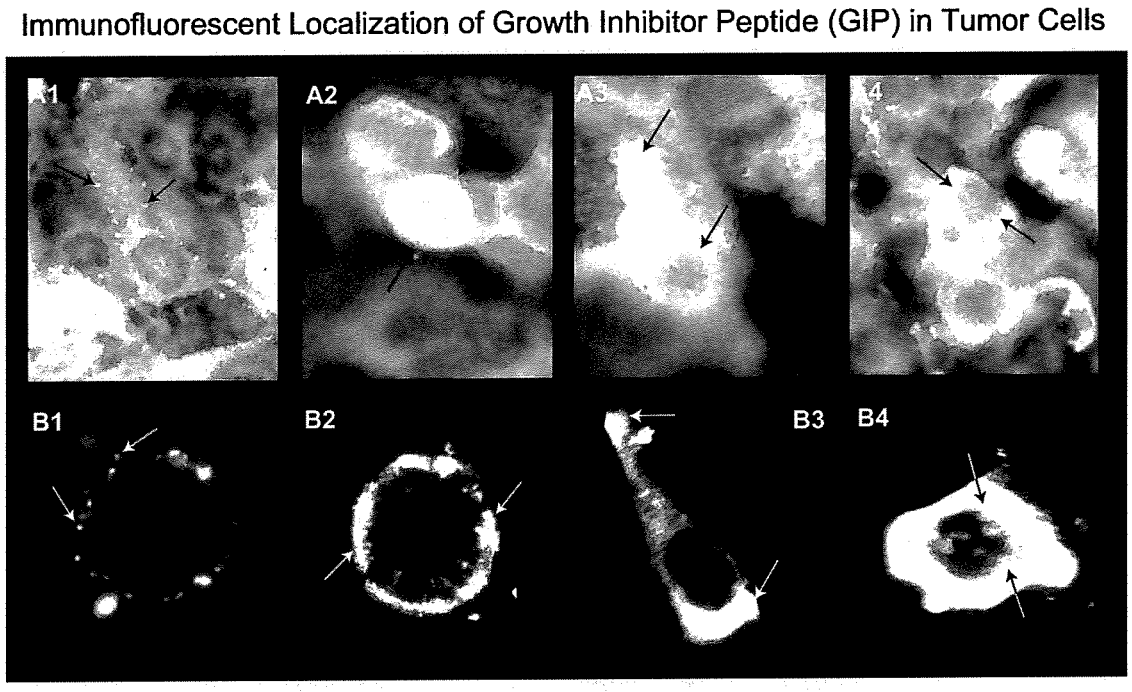

Fig. (6). Immunofluorescent Localization of P149-Peptide and HAFP in random MCF-7 tumor cells are displayed. In order to localize the AFP-peptide on and within MCF-7 cells, the cells were stained by immunofluorescent methodology [20]. Panels A1, to A4, displays P149 peptide on the MCF-7 cell surfaces and periphery, in cyplasmic polar distributions, and heavily distributed throughout the entire cytoplasm including the perinuclear region. HAFP localized in mouse BW7756 hepatoma cells are shown in a single cell suspension which display similar localization to the P149 peptide-exposed to MCF-7 monolayers (Panels B1 to B4).

GIP was subsequently tested *in vivo* in nude mice transplanted subcutaneously with Gl-101 and MCF-7 cells whose growth was monitored by tumor volume [81]. Results of the sulforhodamine-B assay demonstrated that cyclic GIP-induced cytostasis occurred in estrogen (E)-insensitive human MCF-7 cancer cells at doses ranging from 10^{-7} to 10^{-8}M (Fig. 7A). The cell culture screen concurrently revealed that linear GIP suppressed breast cancer growth in the GI-101 cell line, with growth suppression ranging up to 80-40% at doses of 10^{-5} to 10^{-7}M GIP. The dose response followed an inverted U-shaped curve, which is often seen in animal and human bioassays. Finally, *in vivo* studies showed that GIP repressed growth of the Gl-101 xenograft by 70% over a 60-day period, while the MCF-7 tumor showed growth inhibition of 60-70% over a 30-day period [78]. These data signified that GIP induces cytostasis in human breast cancer cells both *in vivo* and *in vitro*, possibly desensitizing the growth response of both E-sensitive and insensitive human breast cancer cells (Table **6-4**). It was further proposed that the GIP serves as a decoy ligand for G-protein receptors that desensitize or uncouple extracellular receptor kinases (ERK), thereby disrupting the growth response to various map kinase protein-to-protein cascades of cellular proliferation [20].

Tumor Suppression and Ascites Reduction in the Mouse Mammary Tumor 6WI-1

In the 6WI-1 mouse mammary ascites tumor assay, injection of mice with 3 x 10^6 tumor cells in 0.2 ml volumes leads to a 2-fold increase in body weight by day 12, due to the accumulation of cells and ascites fluid in the intraperitoneal cavity [25]. The average cell count in the accumulated ascites fluid on day 12 was 20 x 10^8 cells/ml. Mouse mammary tumor cell number has been reported to be proportional to ascites fluid volume over the 12-day period [82]. It was observed that the suppression of tumor-induced body weight gain (via ascites fluid and tumor cell increases) was significant ($p < 0.05$) at GIP doses of 1.0 μg peptide per day (Fig. **7B**, Table **6-4C**). At this concentration, linear P149, but not cyclic P149, suppressed the accumulation of both tumor cells and ascites fluid. Tumor growth and ascites fluid accumulation were suppressed by approximately 40% when the linear GIP peptide was administered at high inoculum doses. From these experiments, it can be deduced that P149 peptide can inhibit vascular permeability, as indicated by measurement of total ascites fluid, since plasma fluids pool and accumulate from leaky capillaries in the body cavity.

G6) GIP Effect on Apoptosis

The epithelial/endothelial cell can respond to its environment by attachment or detachment by cellular adhesion mechanisms. Cells that detach from their substratum often round up and do not participate in cell proliferation but become subject to cell death (anoikis). Programmed cell death, or apoptosis, is a physiological process of cellular demise rather than necrosis or autophagy, (the latter is cell death due to lysis, infection, or inflammatory disease). During embryonic/fetal development, cellular apoptosis occurs in the process of forming cavities, vessels, and ducts during histogenesis-and organogenesis. Organs such as the thymus gland normally involute via apoptosis at time of maturation in hu-

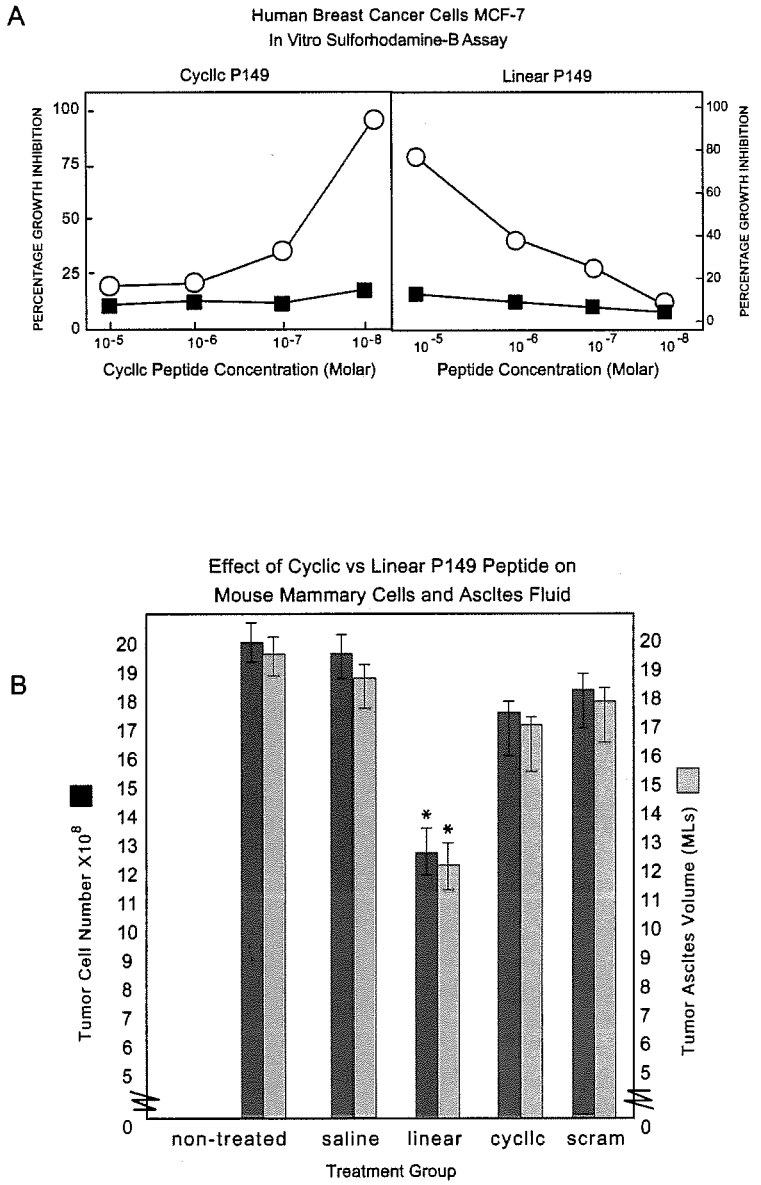

Fig. (7). The growth suppressive activities of GIP (P149) and control peptide are depicted in MCF-7 human breast carcinoma and mouse mammary tumors. The growth suppressive activities of the isoforms of P149, cyclic versus linear, are depicted in the *in vitro* model of the MCF-7 human breast cancer cells and the *in vivo* model of the 6WI-1 mouse ascites tumor. Panel-A. Results indicate that the linear peptide was effective at high concentrations (10^{-4}, 10^{-5}M) against the MCF-7 human breast cancer cell lines, while the cyclic linear P149 showed the best growth inhibition against MCF-7 cells at lower (10^{-8}M) concentrations o = P149 peptide; ■ = scrambled peptide. Panel-B: NYLAR/Nya mice were inoculated with $3x10^6$ 6WI-1 mouse mammary adenoacanthoma tumor cells. An inoculated control group received daily injections of saline while the treatment groups received different P149 and control peptides (μg day) for 12 days. The growth suppressive activity of the linear but not the cyclic P149 in the mouse mammary tumor isograft is demonstrated by the peptide's suppression of tumor cell proliferation and ascites fluid accumulation. Use of the linear, but not the cyclic peptide, was statistically significant (asterisk) at $P<0.01$; P263 = control peptide; GIP = P149.

mans. Full-length HAFP has been shown capable of inducing apoptosis in various cancer cell lines [83]. For survival, cells undergo rescue from apoptosis by activation of a gene family of proteins called bcl-2 [14] [23]. Thus, there is delicate balance between cell death and survival, regulated by different families of proteins. The P149 peptides described in this review are capable of affecting the cell death/rescue balance in various cells.

P149-Peptides Can Affect Apoptosis Induced by full-length HAFP

The synthetic P149 peptide, shares an amino acid identity with an anti-apoptotic protein Bcl-2, which rescues cells from apoptosis. Human B-lymphoma cell line Raji, which is highly sensitive to HAFP-mediated apopotosis, was used to assess biological activity of the P149 peptide [84]. The P149 AFP-peptide was tested as to whether it could modulate

apoptosis induced by full-length HAFP. Raji cells were pre-treated for 1.0 hr with 10^{-4}M P149 peptide, and thereafter various doses of pure HAFP were introduced into the same cultures. It was found that P149 peptide clearly inhibited AFP-mediated cell apoptosis, showing 2.5-fold decrease of the total cytotoxic effect in comparison with pure HAFP. Control scrambled peptide p237 had no effect on the AFP-mediated programmed cell death. These data indicate that AFP-peptide could specifically modulate AFP-mediated apoptosis by interfering with intracellular mechanisms of the signal transduction pathway. It is conceivable that GIP can bind or complex with HAFP to modulate the uptake and function of the full-length fetal protein (see binding studies in Sections H9 and H10).

P149 Peptides Alone Can Influence Apoptotic Events

In a second set of experiments, GIP and apoptosis were assayed by *in vitro* flow cytometry in isolated human lymphocyte, monocyte, and thymocyte cell populations (Table **6-5**, Fig. **8A** and **B**). Apoptosis can be induced either by dexamethasone administration or by gamma irradiation. The effect of AFP-peptide involvement was assayed in non-tumor cell suspensions incubated overnight at 37°C in dexamethasone or after exposure to gamma radiation in the presence of 10^{-8}M to 10^{-10}M peptide. Following flow cytometric analysis, it was found that P149 enhanced apoptosis in irradiated thymocytes, but not in lymphocyte or monocyte cell populations [3]. This suggests that P149 can be a cell radiosensitizing agent. In summary, it was revealed that

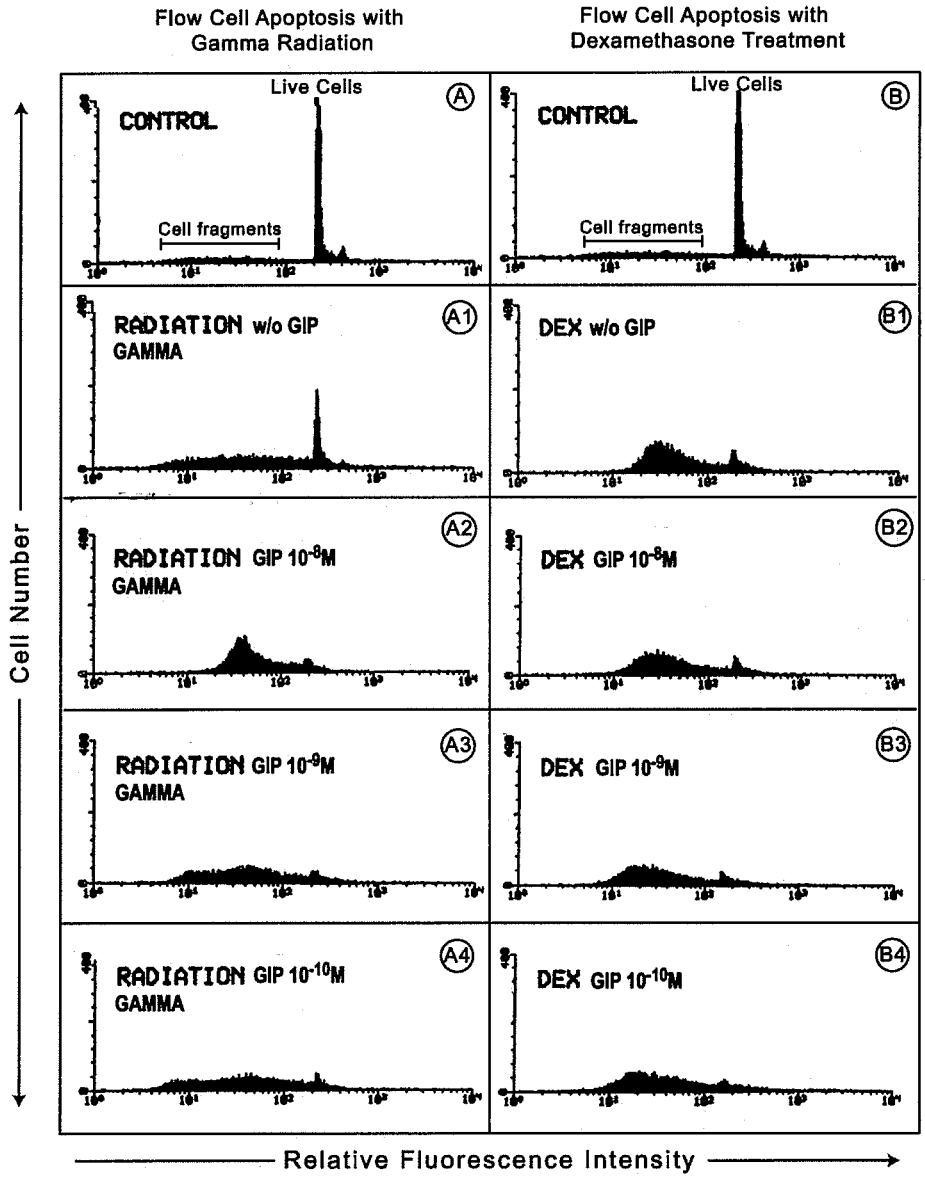

Fig. (8). Effect of GIP (P149) on murine thymic Cell Apoptosis is displayed. Left Side Gamma-irradiated thymocytes plus GIP at 10^{-8} to 10^{-10}M; Right Side: dexamethasone plus GIP at the same doses. Note that prior cell irradration in conjunction with GIP treatment induced apoptosis while GIP plus dexamethasome treatment yielded results similar to dexamethasone alone. The cell fragmented region represents cells having undergone apoptosis.

AFP-peptide had no effect on dexamethasone-treated cell populations, showing that P149-induced apoptosis can be induced in thymocytes after exposure to gamma-radiation; however, it also interferes with HAFP initiated apoptosis [84].

G7) Analysis of the Immuno-Regulation of Human Alpha-Fetoprotein and GIP

The immunoregulatory functions of GIP were assessed in two assays designed to study its effect on immune function. First, the HAFP and GIP activities were studied by ConA-induced proliferation of murine spleen lymphocytes *in vitro* [85]. The proliferative response was measured by [H3]-thymidine T-cell incorporation. ConA-induced lymphocyte proliferation was suppressed by 60% when purified full-length HAFP (50 μg/ml) was used alone. The influence of P-149 on ConA-induced proliferative T cell response was analyzed and results indicated that titrated P-149 had no suppressive activity and lacked a dose response; however, it produced a slight enhancement over controls at all dose levels (data not shown). Second, experimentation with P149 was performed to include both T-cell (Con-A) and B-cell (PWM) responses using lymphocyte blast transformation assays [86]. It was apparent from these latter assays that both P149 and P187 showed a slight enhancement but no dose response to both T-cell and B-cell lectin-induced proliferation (data not shown). In contrast to HAFP, immunosuppression was not observed in any of the P149 preparations (Table **6-6**).

G8) A Cell Level Overview of GIP Exposure

At the structural level of the cell, GIP was shown to be an effective altering agent of the cell cytoskeleton system which influences cell spreading and matrix attachment. In this regard, GIP was found to significantly modulate responses such as cell shape, migration, contact inhibition, and proliferation. In addition, GIP was found to affect apoptosis, immunoregulation, and radiosensitization of cells of the immune system. Overall, GIP was capable of influencing many situations whereby cancer cells attempt to adapt or adjust to different environments.

H) THE CELL SURFACE LEVEL

H1) AFP-Peptide Inhibition of Human Platelet Aggregation

Method:

Platelet Aggregation inhibition (PAI) was analyzed using AFP-peptides and its subfragments in freshly prepared human platelet rich plasma (PRP) preparations as previously described [78]. The three platelet aggregation agonists used were adenosine diphosphate (ADP), arachidonic acid, (AA) and collagen-II (Col-II) at 5 μM in platelet rich-plasma (PRP). The doses were "threshold doses", which titrated as the minimal agonist concentrations that elicited full platelet activation *in vitro* (i.e., platelet shape change, aggregation, and granule secretion). Peptide at 0.30 mg/ml PRP inhibited ADP induced platelet aggregation by 93%; at 0.1 mg/ml no inhibition was detected however, at 0.3 mg/ml, P149 peptide inhibited collagen-induced platelet aggregation by 96%, at a collagen concentration of 2 μg/ml PRP (Fig. **9A, B**). No significant inhibition was observed with peptide P149c, but peptides P149a and P149b at 1.0 mg/ml inhibited both ADP and collagen to an extent similar to that as seen with peptide P149. The P149b subfragment effects on ADP-induced aggregation were also studied. While inhibitions of aggregation were not as dramatic as those of other agonists, inhibition did occur in the 125 μg of P149b per reaction vessel.

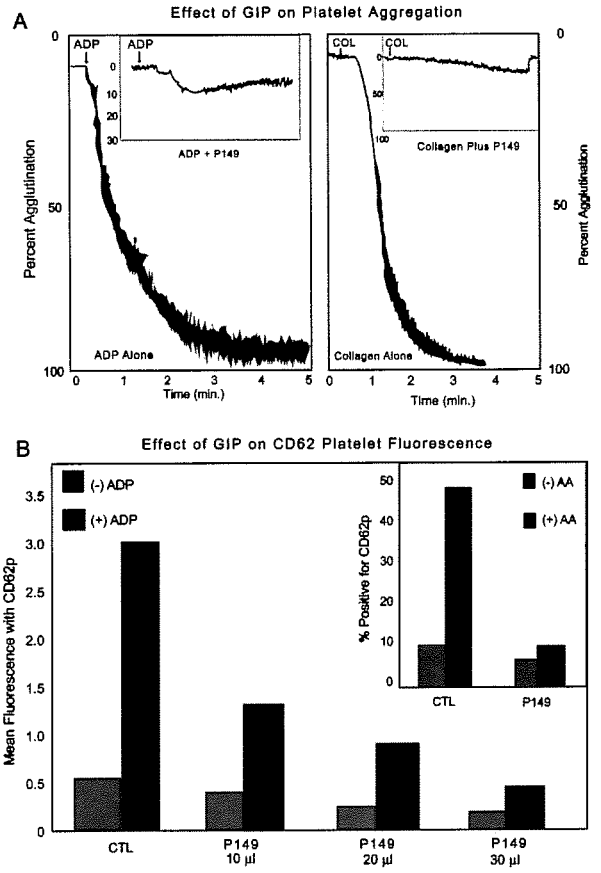

Fig. (9). Panel A: A platelet aggregation study is depicted in treatments involving 0.5 ml of normal human platelet-rich plasma with 300 μg of P149 suspended in $2.5x10^6$ platelets per reaction vessel. After 2 minutes of stirring to obtain equilibrium at 37°C, adenosine diphosphate (ADP) was added to the reaction vessel (Panel-A left side): ADP incubated with P149 inhibited all phases of platelet aggregation; Panel A Right side: P149 inhibited platelet aggregation by collagen in a similar fashion to ADP. Studies employed the use of an optical-based aggregometer. Panel-B: CD62P flow cytometry fluorescence quenching by P149; ADP alone and ADP plus P149; inset: arachionic acid (AA) and AA plus P149a showing aggregation diminution.

A second study involved treatment of 0.5 ml of the normal PRP with arachionic acid (Aa) and 300 μg of the AFP-peptide preparation (Fig. **9B**, inset). After 2 min stirring for temperature equilibration at 37° Aa (10uL) was added. At this time, no aggregation occurred (100% inhibition), and even when a second 10 μl of Aa was then added, no aggregation took place. This inhibition was 100% complete; however, when 10 μl epinephrine (non GIP-reactive) was added,

an immediate aggregation reaction occurred. Ristocetin controls, used as a positive aggregation control, also showed 100% aggregation of the platelets (Table 7-1). In summary, these results show that the 34-mer AFP peptide and two of its subfragments (P149a, P149b) are capable of PAI (Table 7 #2).

H2) AFP-Peptides Influence Erythrocyte Aggregation/ Agglutination

The P149-peptides have been shown to influence, modulate, and regulate various molecular and cell aggregation events (Table 7 #2). Although similar to receptor clustering, these events occur either in intracellular or extracellular settings. Antibodies to red blood cell (RBC) antigens such as the ABO cell surface antigens offer an example of the agglutination of circulating RBCs in the bloodstream. Proteins or peptides that interact with the RBC surface would naturally interfere with or obstruct antigen-antibody reactions at the cell surface. In a previous study, the P149 peptide was admixed with A-type RBC and anti-A antibody serum, diluted in a titration series, and incubated for 30 min [3]. Results indicated that the P149-peptide, at 150-600 μg/ml, inhibited RBC agglutination over a range of five serial dilutions greater than control tubes. That is, the GIP inhibited RBC antibody-induced agglutination at 2-fold dilutions: 1:32, 1:64, 1:128, 1:256 and 1:512 at which point the control was negative (Table 7 #2). These results indicate that the AFP-peptide can inhibit or interfere with cell-to cell (cell surface) agglutination exhibited by erythrocytes.

H3) AFP-Peptides Can Inhibit Cellular Adhesion of Breast Tumor Cells to Various ECM Proteins

A) Tumor Cell Adhesion to ECM Proteins

A recent study screened various human ECM proteins for inhibition or enhancement of tumor cell adhesion in the presence of P149 peptide and its fragment [3]. The adhesion was utilized as a means to assess whether the peptide could influence or modulate tumor cell attachment to a protein substratum or matrix. The GIP was subjected to cell adhesion studies employing ECM proteins (Table 7). Various ECM proteins were adsorbed to microtiter plates and screened for their ability to serve as a substratum for enhanced tumor cell adhesion, as compared to non-coated microtiter plates. The ECM proteins differed in their ability to serve as attachment surfaces for two breast cancer cell lines human MCF-7 and murine 6WI-1. The mouse mammary 6WI-1 tumor cell attachment occurred up to 60% for all ECM protein matrices, with laminin demonstrating the greatest adhesion ability. Fibrinogen ranked second as an adhesion protein, with fibronectin and the collagens close behind. The non-ECM protein attachment factors such as gelatin, chondroitin sulfate, and polylysine exhibited somewhat decreased cellular adhesion in comparison (20%-40%) to the ECM proteins (not shown). By use of MCF-7 cells, most ECM proteins were found capable of supporting tumor cell adhesion to some degree. Cell adhesion in the MCF-7 cells also occurred up to 58%, with fibronectin achieving the highest percent cell attachment. While lamii in supported little MCF-7 cell attachment (<5%), collagens I and IV ranged from 30 to 50%, and fibrinogen displayed 20%. Interestingly, the AFP peptide (10 μg/ml) itself was capable of supporting about 25% cellular adhesion with both tumor cell types. The attachment of GIP approached that of the adhesion matrices such as gelatin, chondroitin sulfate, and polylysine which ranged from 30 to 35%.

B) GIP Inhibition of Tumor Cell Adhesion to ECM Proteins

Cell adhesion inhibition via P149 peptide was found to be 4-fold higher in human tumors than in the murine breast

Table 7. <u>The Cell Surface Level:</u> **Summarized Activities of Human Alpha-fetoprotein (HAFP) and Growth Inhibitory Peptide ((P149) in Various Cell Surface/Membrane Interactions**

Process/Activity Studied	Peptide Biological Response/Activity			
	Inhibition, Suppression	Enhancement, Activation	No effect	Not tested
1) platelet aggregation	HAFP, P149In, P149a, P149b	None	P149c, P237	P149cy
2) Erythrocyte agglutination	P149 In	None	P237	P149cy, P149b, P149a, P149c
3) Cell adhesion to ECM (Inhibition)	P149 In, P149b	P149 In, P149b	P149c, P237	P149cy, P149a
4) Tumor cell Immunofluorescence	None	P149 In	P237	P149cy, P149b, P149a, P149c
5) Uptake by MCF-7 whole cells	P192	HAFP, P149 In	P149 In, P149a, P149cy, P149b, P149c	P149cy, P149b, P149a, P149c
6) Binding to HAFP receptor extracts	None	HAFP	P149 In, P149a, P149cy, P149b, P149c	P237

P192 = HAFP carboxyterminal 3rd Domain
ECM = extra-cellular matrix
*For other peptide designation, see footnotes to Table-3.

cancer cells, and occurrence of inhibition was less frequent in the mouse tumor cells (Table-7 #3). The AFP peptide was capable of cell adhesion inhibition in all major ECM proteins in both tumor cell lines. Cell adhesion inhibition was somewhat equivalent between human mouse tumor cells in collagen I, collagen IV, fibrinogen, fibronectin, and thrombospondin; however, inhibition was significantly different in laminin and vitronectin, between the two tumor cell lines [78]. Human MCF-7 cells were found to display peptide-induced inhibition to vitronectin, while mouse tumor cells failed to respond to this ECM. Likewise, mouse 6WI-1 cells demonstrated peptide-induced inhibition to laminin adhesion but MCF-7 did not respond to this ECM protein. Finally, it was observed that albumin and scrambled peptide controls were not capable of cell adhesion inhibition (see Ref. #78 for details).

H9) Competitive Uptake of GIP and HAFP by MCF-7 Whole Cell Suspensions

The uptake of GIP and HAFP by MCF-7 cells has been previously demonstrated by histological procedures [3, 20]. A receptor for HAFP on the surface of MCF-7 cells has been reported, as were methods for its study [87]. The binding/uptake of radiolabeled HAFP in the presence of various AFP peptides (P149, P187, P192) was performed (Table-7 #5). The P149 peptides, at 1.0 μg/ml, demonstrated enhanced HAFP uptake/binding compared to ^{125}I-HAFP alone. In contrast, the P192 peptide segment showed reduced binding of ^{125}I-HAFP at the same concentration; however, scrambled peptides did not inhibit or enhance (data not shown). Thus, at the same concentration, peptide P192 inhibits binding to the MCF-7 cells acting as an antagonist, while P149 enhances such binding serving as an agonist. The mechanism of the enhanced uptake of HAFP in the presence of GIP is thought to be receptor-mediated endocytosis; this is, a concentration-dependent event. Two possibilities for enhanced uptake exist. First, GIP itself could bind directly with HAFP and both be bound to the cell as a molecular complex (see Section G-4). Alternatively, P149 peptides may induce clustering of cell surface G-coupled receptors; receptor clustering is known to facilitate binding and uptake of ligands at cell surfaces. Binding of a G-coupled receptor (GPR30) to a GIP fragment has been shown to be plausible by computer modeling [88].

H10) GIP Binding Studies to the HAFP Receptor Using MCF-7 Membrane Extracts

A cell surface receptor for HAFP has been isolated and purified from the surface of human breast cancer cells (MCF-7) and is found on other cancer cell types [89]. 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. In the initial publication describing GIP in 1996, it was suggested that GIP was capable of binding to the HAFP receptor, which had previously been detected and measured on cell membranes of human breast cancers [87, 90]. However, only recently have attempts been made to confirm or refute the hypothesis that GIP utilizes the HAFP receptor to gain entrance into cells via endocytosis (Table 7 #6).

By use of microtiter plates coated with MCF-7 cell AFP receptor-enriched membrane preparations (50 x 10^5 cells/ml), biotinylated HAFP (B-AFP) was found to bind to the walls of AFP-receptor coated microtiter plates. By means of strepavidin-horseradish peroxidase-ABTS, color changes were monitored at 492-nm wavelength, and optical density (O.D.) was determined for each well. When using a B-AFP solution of 500 μg/ml, non-labeled HAFP was titrated in 3-fold dilutions from 50 μg/ml to 1.9 μg/ml and displaced labeled AFP with the non-labeled form. Linear P149, P149b, P149c, or cyclic P149 when employed as competitive binding agents, did not exhibit binding inhibition or enhancement to the MCF-7 receptor (Table 7 #6). In summary, it was clearly demonstrated that linear GIP, its fragments, and its cyclic version do not displace the binding of B-AFP to the putative HAFP receptor, whereas full-length HAFP does. Although GIP has been shown to be highly reactive at various cell surfaces, it does not appear to bind the AFP receptor-rich membrane preparations derived from MCF-7 cells (see Section H-9).

H11) A Cell Surface Level Overview of GIP Administration

At the structural level of the cell surface, especially the plasma membrane GIP was demonstrated to be highly interactive, even to the point of membrane bilayer disturbance. For example, GIP was demonstrated to inhibit platelet aggregation, red blood cell hemagglutination, as well as tumor cell adhesion. Fluorescent antibody studies employing anti-GIP antiserum clearly localized GIP at the cell surface in both punctate and diffuse patterns. Although GIP was able to inhibit the binding of HAFP to the plasma membrane of MCF-7 tumor cells, GIP itself did not bind to the canonical human AFP receptor. These data clearly assign a crucial function to GIP at the plasma membrane interface, possibly related to bilipid disturbance.

I) THE CYTOPLASMIC LEVEL

I1) Interaction of Peptide P149 with Acetylcholinesterase Enzymes

The activity of Ache (EC 3.1.1.7) was assayed by means of a Lambda 4B UV/visible spectrophotometer (Perkin-Elmer), according to a modified Elman procedure using acetylthiocholine iodide (ATC) as substrate [91]. The enzyme activity was monitored at 412 nm by following the increase of yellow color produced from thiocholine when it reacted with the dithiobisnitrobenzoate ion. The specific activity of Ache, which was obtained on the basis of the rate of hydrolysis (μmol of product/min. mg of protein or units/mg of protein), was calculated on the basis of an extinction coefficient of the yellow anion (13.6 $mM^{-1}cm^{-1}$) (92).

As shown in Table-**8** #1, the soluble Ache enzyme activity was inhibited using linear P149 peptide. The specific activity of Ache, in the presence of the P149 peptide based on the ATC hydrolysis reaction, immediately decreased at 10^{-7}M peptide concentrations, and then gradually rose but never attained the activity of the control enzyme (Table **8-1**, Fig. **10A** and **A1**). It has been shown that n-butanol, as a positive control, displays a strong inhibitory reaction [92]. These studies indicated that GIP was as potent an inhibitor as N-

Table 8. The Cytoplasmic Level: Summarized Activities of Human Alpha-Fetoprotein (HAFP) and Growth Inhibitory Peptides (P149) in Various Enzymatic and Cytoskeletal Reactions

Process/Activity Studied	Peptide Biological Response/Activity			
	Inhibition, Suppression	Enhancement Activation	No Effect	Not tested
1) Soluble Ache enzyme activity	P149 In	None	P237	P149 cy, P149b, P149a, P149c
2) Rat liver microsomal P450 Aromatase Activity	P149 In, P192	None	P237	P149cy, P149b, P149a, p149c
3) Tissue factor induced thrombin formation	P149 In	None	P237	P149cy, P149b, P149a, P149c
4) Microtubule Polymerization	None	P149 In	P237	P149cy, P149b, P149a, P149c

P192 = HAFP carboxyterminal 3rd domain
Ache = acetylcholinesterase
*For other peptide number designations, see footnotes to Table-3.

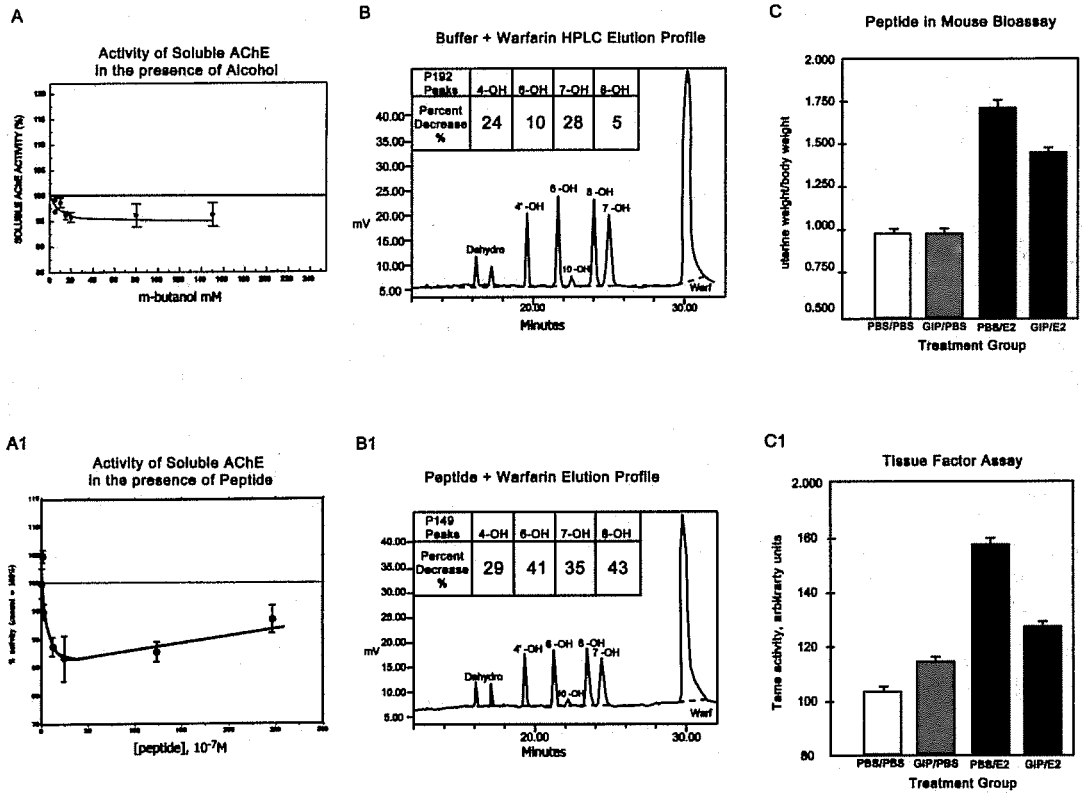

Fig. (10). The effect of GIP on three separate cytoplasmic enzymes systems is depicted. Panel-A Effect of P149 on the enzyme activity of soluble acetylcholinesterase (ACHE) is displayed. Soluble enzyme was isolated from electric organs according to the reports described in the text (11, 16, 26). Note that GIP (P149) imposes a competitive inhibitory effect on the soluble Ache enzyme even greater than the N-butanol antagonist control agent (top panel). Panel-B: The effects of the P149 peptide on Rat Warfarin Metabolism in Rat Liver Microsomes were studied. The HPLC hydroxylated product profiles are depicted in Panels B and B1. Note that P149 reduced enzymatic hydrolysis of warfarin by 29 to 43% while the P192 peptide hydrolysis inhibition of warfarin ranged from 5 to 28% (P192 not shown). Panel-C: Effect of P149 Peptide on Estrogen-induced uterine tissue factor formation is displayed. Rats, 18-20 days old, were injected with 1μg E2 in 2% alcohol in saline (0.3 ml) and peptide (P149) was injected either just before the E2 or 1 hour before the saline. The TAME activity is displayed as specific activities, cpm/μg protein/min (see text) and increases in E_2 compared to control-treated animals. However, E_2 plus peptide treated groups decreased enzyme activity by 33% compared to the E_2 only group. Panel C: The effect of GIP in that rat immature uterine assay significantly displays growth suppression in uterine tissues (30-40%) at 24 hours. Panel C1: Tissue factor/thrombin-induced esteropeptidase is clearly reduced in the uteri of rats by injection of P149 1.0 hr prior to E2 administration and measured at 3 hrs post injection. Note that suppression of uterine organ growth (24 hr assay) in Panel C can be predicted at 3.0 hrs by the reduction of TAME activity (Panel C1).

butanol under comparable experimental conditions. It can be proposed that P149 either 1) inhibits Ache activities directly, 2) binds to Ache substrates, 3) neutralizes non-specific and/or other broad-spectrum esterases (from the tissue extract) that compete with the Ache enzyme, or 4) serves as a decoy enzyme (binds without activation).

I2) Effects of P149 peptide on Rat Warfarin Metabolism by Rat Liver Microsomes

The P149 peptide showed sequence similarity to the cytochrome P450 (aromatase) enzymes; one of the target substrates of this enzyme family is warfarin. A high-pressure liquid chromatographic (HPLC) assay of warfarin and all its known metabolites was employed as a model to analyze the effects of GIP on microsomal P450 aromatase enzyme and its substrate, warfarin [93]. A HPLC analysis of warfarin and its diastereoisomeric alcohols and 4`-, 6-, 7-, 8- and benzylic hydroxylated metabolites was accomplished using a C18 reverse-phase column.

Rat liver microsomal aromatase extracts were prepared by modified methods from a previously published report [93]. A typical HPLC separation of the synthetic metabolites of warfarin exposed to microsomal P450 enzymes is illustrated in (Fig. **10** (Panels B and B1), Table **8 #2**). A comparison of the HPLC elution profile of the synthetic warfarin metabolites with that of the products of the microsomal metabolism of warfarin indicates that P149 peptide, and to a much lesser extent P192, was capable of reducing the hydroxylated metabolites of the warfarin substrate in the rat microsomal extracts. The P149 peptide significantly reduced all of the hydroxylated (4-OH, 6-OH, 7-OH, 8-OH) products as shown in the post-elution profile (Panel B), while the P192 control peptide affected only the 6-and 8-OH products, while slightly influencing the 4-and 7-OH elution profiles (Table **8** #2). The P149 peptide suppressed the warfarin hydroxylated product turnover from 29-41%, (Panel-B), while the control P192 peptide suppressed such turnover from 5-28% (not shown). These results suggested that GIP either 1) inhibits P-450 enzymes directly, 2) binds to P450 substrates, or 3) serves as a decoy enzyme for the substrate. It is of interest that rat AFP binds warfarin [94].

I3) Effect of P149 Peptide on Estrogen-induced Uterine Tissue Factor-Thrombin Induction

The immature rat uterus responds to a single injection of estrogen with immediate increases in wet weight and protein content (3-5 hr), nucleic acid and protein synthesis, and the initiation of two waves of cell division at 15 and 24 hr [95]. An increase in prothrombin-associated proteins is one of the early responses and occurs within 3 hr of estrogen administration, coinciding with an imbibition of water and an accompanying influx of plasma proteins into uterine tissues [96, 97]. Thus, the administration of 0.5 μg 17β-estradiol (E_2) to immature rats initiates a series of biochemical changes in the uterus that lead to cell hypertrophy and hyperplasia within 24 hr [98]. An early step is the binding of E_2 to nuclear receptors; such responses occur within a few hours after binding, and include synthesis of specific proteins and increased specific activity of certain coagulation-related enzymes [99]. The present enzyme of interest is a cytoplasmic estrogen-dependent arginine esteropeptidase.

Tissue factor (TF), a ubiquitous protein present in high concentrations in placenta and to a lesser extent in brain, lung, and kidney [100], was identified as a procoagulant in the immature rat uterus were it activates prothrombin and Factor X to clot normal plasma. TF is an integral membrane glycoprotein that binds blood clotting factors VII and VIIa. The complex TF:VIIa catalyzes the activation of blood clotting factor X to Xa; the latter participates in the prothrombinase reaction that yields thrombin (8, 9), a key enzyme in clot formation (fibrinogenesis). An arginine-dependent esteropeptidase serves as the key enzyme in this latter reaction. It is of interest that GIP displays amino acid sequence similarity to blood factors VIII, IX, and XIII (Table **1**).

The tissue factor-induced enzyme activator (esteropeptidase) was quantitated using a modification of the assay described for the esterase enzyme assay, named the tosyl-L-arginine-O-methyl ester (TAME) assay [99]. The effect of the P149 on activity in the TAME esterase assay has been previously described [16] and is listed in Table 8 #3 and Fig 10C and C1. TAME activity in cytosols from E2-treated animals was similar to that of the untreated control uterine tissues. However, in the E2-treated animal cytosols, P149 peptide administration reduced TAME activity by 33%. Addition of the peptide (100 ng/assay) directly into the assay tubes had no influence on TAME activity.

I4) GIP Influence on Intracellular Aggregation/ Polymerization of Intracellular Cytoskeleton Proteins

Microtubules have been implicated in the uptake, secretion, and degradation of fetal proteins in the liver (23) and in hepatoblastomas [101] [102]. The microtubules comprise a family of filamentous proteins that form the cytoskeletal framework for mammalian cells [103]. Microtubules such as α- and β-tubulins, gephyrins, and ankyrin contribute to spindle formation during mitotic and meiotic cell division [104] [105]. Drugs such as taxol (from the bark of the yew tree) interfere with spindle formation at the onset of cell mitosis, by enhancing tubulin polymerization, which is required for cell proliferation [106]. Taxol has served as an effective cancer therapeutic agent in clinical trials. Compounds that mimic and/or bind to microtubule-associated anchor proteins and enhance polymerization might also be involved. Microtubule–associated proteins (MAPs) are chemical agents that either enhance or inhibit such polymerization. Finally, chemicals that affect intracellular cytoskeletal filaments can also influence the formation of focal adhesions at the cell membrane crucial for angiogenesis.

Microtubules in the non-dividing cells are stabilized and stored in bundles of polymerized subunits in the cytoplasm (Fig. **11**). Agents such as taxol function to enhance tubulin polymerization, thus removing tubulin from spindle formation in preparation for cell division. Polymerized microtubules are unavailable for the formation of mitotic spindles; hence, cell proliferation is prevented. When AFP-peptide is added to a purified preparation of α-tubulin (from chick embryo extracts) and subjected to measurements of specific viscosity (Fig. **11**), peptide P149 (1.0 μg/ml) was found to enhance α-tubulin polymerization by 3-fold over control

(tubulin only) solutions [3]. Other control protein/peptides did not demonstrate this property and behaved similarly to tubulin alone (Table **8** #4). These data would suggest that AFP-peptide either binds α-tubulin or serves as a microtubule-associated protein to initiate a polymerization reaction on α-tubulin *in vitro* (see Cytoskeletal-related study in Section G-1).

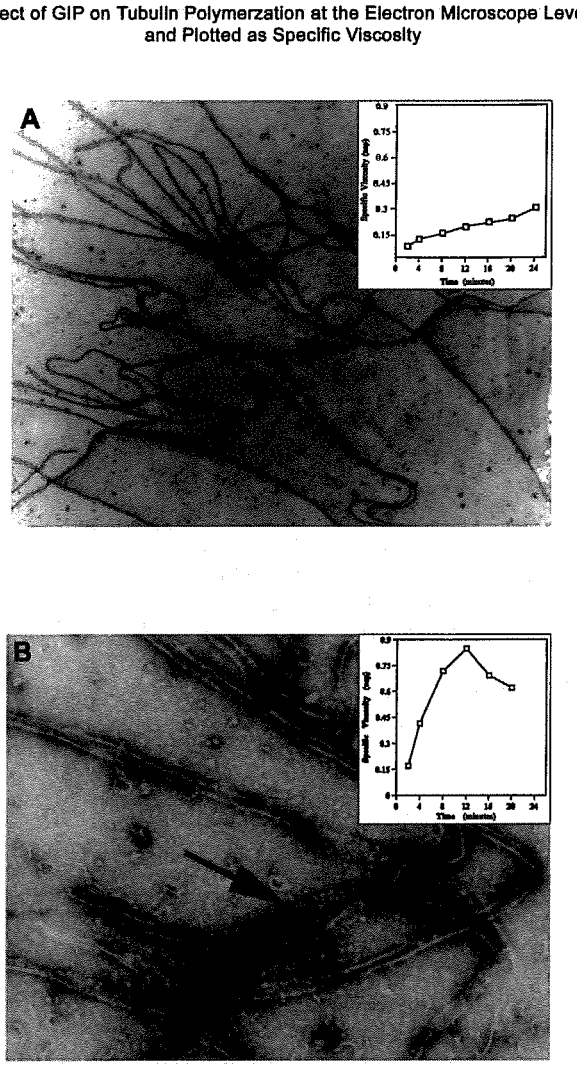

Fig. (11). Alpha-tubulin polymerization is depicted as the single stranded (Panel-A) to the 13-stranded protofilament conversion (Panel-B) using electron microscopy (see arrows). Electron micrographs were prepared by Dr. George Smith (Union College) with magnification of: A = 24,000X; B = 156,000X. Inset Panel-A: Specific viscosity readings of non-activated alpha-tubulin alone, Inset Panel-B: Specific viscosity readings of alpha-tubulin in the presence of 1000 ng/ml of P149 peptide. Note the 3-fold increase in tubulin polymerization when P149 is added to the reaction vessel.

I5) A Cytoplasm Level Overview of GIP Treatment

At the structural level of the cytoplasm, GIP was determined to be a modulator of certain cytoplasmic-associated enzyme and cytoskeleton events. GIP robustly inhibited soluble Ache enzyme activity comparable to known alcohol inhibitors such as n-butanol. Furthermore, GIP interacted with microsomal P450 aromatases possibly serving as a substitute or competitive substrate. In the formation of Tissue Factor (TF) induced thrombin, GIP was capable of inhibiting 33% of the thrombin inductive capability of TF. Finally, GIP was shown to increase by 5-fold the α-tubulin polymerization reaction in a taxol-like fashion. Overall, present data indicates that GIP has a notable impact on cyplasmic reactions involving enzymes and the cytoskeleton microtubule system.

J) THE NUCLEAR LEVEL

J1). Estrogen Receptor (ER) Binding to GIP and Its Fragments

To determine whether the estrogen-sensitive growth suppression of GIP might be due to the interference with the estrogen receptor-E2 interaction, the affinities of these peptides for ERα were determined [18, 25]. Results of the ER-binding assay demonstrated that P149 (IC_{50} = 8 x 10^{-6} M) and P149A (IC_{50} = 9 x 10^{-5} M) competed with ^3H-E_2 for ERα binding, whereas P149B and P149C did not compete (Table-9). This result indicated that the major ERα binding site in P149 resides on the P149a amino acid fragment and that these peptides, similarly to antiestrogens such as tamoxifen, interfere with the ER-ligand interaction. The binding GIPs exhibited 1000-fold lower affinity for ERα than E_2 (IC_{50} = 3 x 10^{-9} M) or tamoxifen (IC_{50} = 5 x 10^{-8} M). Various other peptides, including two different natural full-length AFP preparations, served as controls and exhibited no binding to ERα (Table **9**). Peptide H-1, a modified P149 with amino acid substitutions in the P149B region, did not compete for ERα-binding, suggesting that an AA substitution of VYASKV for IIIGHL in P149 may have altered the conformational state, thus preventing the P149A fragment of P149 to bind ERα. Additional ER-binding experiments were performed to analyze the simultaneous effect of two peptide fragments on ER-binding, and to determine whether the ER-binding property of P149 could be reconstituted by mixing the three fragments. When equimolar concentrations of two peptide fragments were allowed to compete with ^3H-E_2 for ERα binding, P149C neither interfered with ERα-binding of P149A, nor did it disrupt the enhanced ^3H-E_2 –binding observed with P149B. However, the presence of P149B neutralized the ability of P149A to bind ERα. Furthermore, the equimolar amounts of P149A, P149B, and P149C did not exhibit the ER-binding property of either P149 or P149A.

The P149b peptide exhibited enhanced binding of ^3H-E2 in the assay. To understand the mechanism for increased binding of ^3H-E_2 in reaction mixtures containing ERα and P149B and to confirm that P149B did indeed bind E_2, the ability of P149B and other peptides to bind ^3H-E_2 in the presence or absence of ERα or full-length AFP was examined. P149B, but neither P149 nor a P192 control peptide, bound ^3H-E_2 (Table **9**). The concentrations giving comparable binding of ^3H-E_2 to ERα and P149B were 1.25 x 10^{-9} M and 1.6 x 10^{-9} M, respectively. In contrast, full-length native AFP alone did not bind ^3H-E_2.

J2). The Effects of GIP on the Transcriptional and Proliferative Effects Due to Estrogen

Estrogen acts to promote DNA synthesis in the MCF-7 human breast cancer cell line via its interaction with high

Table 9. Nuclear Level: Estrogen Receptor and Estradiol Binding to Full Length Human alpha-fetoprotein (HAFP). Growth Inhibitory Peptide, and Various Control Peptides Compared to the Human Estrogen Nuclear Receptor (ERα). Note that HAFP and P149c Does Not (P149) Participate In Any Binding Reactions

Binding Ligands Employed	Human Estrogen Receptor Alpha	Growth Inhibitory Peptides					Human Albumin	Human Alpha-fetoprotein		
		P149	P149 (H-1)	P149a	P149b	P149c	P263	Native HAFP 1-590 Amino Acids	NH_2-HAFP Amino-Terminus (H-2)	Carboxy-terminal HAFP (P192)
Human Estrogen Receptor α	NA	Binding ($8x10^{-6}$M)	Non Binding	Binding ($5x10^{-8}$M)	Non-Binding	Non-binding	Non-binding	Non-binding	Non-binding	Non-binding
17-Beta Estradiol	Binding ($1x10^{-9}$M)	Non-Binding	Non-Binding	Non-Binding	Binding ($2x10^{-4}$M)	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding
Tamoxifen	Binding ($5x10^{-8}$M)	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding	Non-Binding

NA = nonapplicable
*For peptide number #designations, see footnotes to Table-3.
H-1, VYASKV was substituted for IIIGHL on the 34-mer GIP
H-2, (denotes) amino acids #8 to 39 on the full length HAFP molecule with a sequence of EYGIASILDSYQCTAEILADLATIFFAQFVQ

levels of estrogen receptor. Previous reports have indicated that estrogen, acting through the estrogen receptor, is capable of inducing the mitogen-activated protein kinase (MAPK) cytoplasmic signaling cascade [107]. In the present study, AFP-derived peptides were used to determine the influence of such kinase cascades on the growth factor induced and estrogen-mediated mitogenesis. In a second study using ER-mediated transcription, cells were lysed after 24 hr of stimulation, and luciferase activity was determined using a dual-luciferase reporter assay system. Thus, the first assay examined the effects of the peptide on cell cycle progression in MCF-7 cells treated with estrogen, and EGF, IGF-1, or bFGF growth factor. The second assay examined the effect of the peptide on the ability of estrogen to stimulate ER-mediated transcription in MCF-7 cells. The P149 peptide had no effect on either system. Thus, no dose response occurred, and only a trend toward E2-mediated enhancement by P149 was observed.

It was evident that GIP showed only a trend toward enhancement of E2-induced transcription in two different assays described in the "nucleus" section above as well as the contact inhibition assay in Section G-3. Both were systems in which GIP was added to cells in culture. As shown in Section H-7, fluorescent studies of GIP in cultured MCF-7 cells resulted in cytoplasmic and perinuclear localization, without nuclear membrane penetration and translocation. However, at the onset of mitosis, nuclear membranes are dissolved *in vivo*, allowing free access to the nuclear DNA transcription machinery. Since GIP has been reported to slightly enhance contact inhibition during E2-induced MCF-7 cell proliferation (Table 6 #3), it is possible that P149 peptides are in contact with nuclear components at early prophase stages in cells that were not arrested in G1/S. The slight trend of E2-induced cell proliferation enhancement (Section G-3) was observed in cell culture experiments, but peptide concentrations greater than 10^{-7} M were required. The slight enhancement could also have been due to the sequence identity of GIP with High Mobility Group proteins which are known to enhance binding of ER to the ERE [24, 108]. In previous reports utilizing full-length HAFP, E2-induced growth enhancement could only be achieved using freeze/thaw denatured proteins [109] [110]; however, such trials were not performed using GIP preparations. It is of interest that E2-occupied ER has been shown to inhibit cell proliferation [111], and that overexpression of the ERα in endometrial cells can result in growth inhibition and anti-angiogenesis [112].

J3) A Nucleus Level Overview of GIP Treatment

At the structural level of the nucleus, GIP displayed significant competitive activity with the steroid nuclear estrogen receptor, but had little if any effect on estrogen-mediated/growth factor-induced mitogenesis and estrogen-induced transcription events. This is perhaps due to failure of GIP to engage in cytoplasmic-nuclear transport. At the steroid receptor level, the 34-mer GIP and its P149a fragment were capable of binding to the α-estrogen receptor (ER), while the P149b fragment (alone) bound the estradiol molecule itself. Interestingly, the P149c was devoid of any binding activity. However, the P149a fragment bound the ER at 5 x 10^{-4} M concentration, while the entire 34-mer GIP bound the ER at 8 x 10^{-6} M. It is evident from these results that while GIP can readily interact with the ER, GIP cannot directly influence or modulate estrogen-induced transcription and mitogenesis.

CONCLUSIONS

As stated in the Introduction, the biological activities of the GIP were surveyed and presented on organizational planes from the organism to the subcellular level including the cytoplasmic and nuclear compartments. The biological activity at each level was assessed based on an enzyme, hormone, or an event in which a response (qualitative or

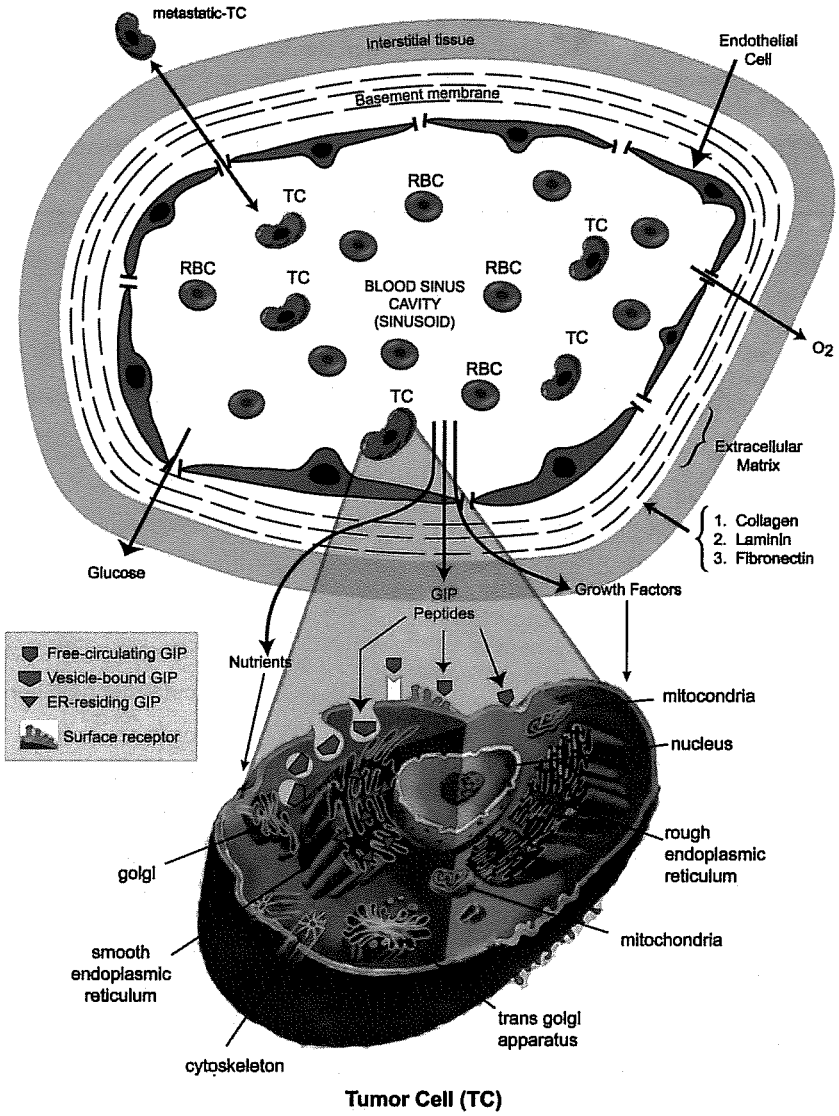

Fig. (12). The mechanism of decoy ligand endocytosis of the Growth Inhibitory peptide (GIP) is depicted. Metastatic cancer cells from a blood sinus (top half of picture) exit through intraendothelial gaps to surrounding tissues. Free circulating GIP binds to an unknown cancer cell receptor (G-coupled?) as a decoy (mimic) ligand and is endocytosed but does not activate the receptor to transduce a signal to the cell interior. A cut-away view of the epithelial metastatic cell is shown. Labels indicate tumor cells organelles. Note that the GIP is targeted to the endoplasmic reticulum (ER) surrounding the nucleus (perinuclear location) as described in reference #20. A candidate G-coupled receptor could be considered to be GPR30 as reported in Reference #88. The cell diagram was modified and redrawn from Toxicology Mechanism & Methods, 13:1, 2003.

quantitative) was recorded. The boundaries of each level are not rigid; the potential for overlap makes it sometimes difficult to distinguish one functional level from another (i.e., tissue vs. organ effect). Thus, certain conclusions concerning GIP may overlap organizational planes. At the organismal level, GIP, placed in the surrounding environment or injected into an animal, reduced both growth and differentiation in at least two assays, i.e., frog metamorphosis and brine shrimp ecdysis. In both assays, the event was either suppressed, reduced, displayed a cessation of events, or maintained a status quo condition. At the organ level, GIP administration could affect an entire uterus or liver by damping down a growth spurt induced by a hormone or a surgical procedure. Interestingly, GIP was strongly anti-angiogenic, affecting the chick fetal allantoic blood and tumor vasculature. At the cell level, exposure to GIP could influence cell shape, migration/spreading, and cell-to-cell contact inhibition. Cellular localization of the GIP further demonstrated that the peptide is detectable at the cell surface, in the cytoplasm, and immediately surrounding the nucleus. Finally, GIP could affect apoptosis, and could slightly enhance β-and T-cell immune responses, but did not engage in immunosuppression.

At the cell surface, the P149 peptide showed the greatest biological activity, by inhibiting platelet aggregation, erythrocyte hemagglutination, tumor cell adhesion, uptake of HAFP, however, it did not affect competitive binding of HAFP to its cell surface receptor. In comparison, at the cyto-

plasmic level, GIP interfered with several enzymes including acetylcholinesterases, cytochrome P450 aromatases, and a tissue factor/thrombin-induced esteropeptidase. Also, GIP enhanced the polymerization (hence, stabilization) of α-tubulin, a major component of the cytoplasmic microtubular network. Finally, in the nucleus, GIP was shown to bind the estrogen receptor but did not affect estrogen receptor transcription, and failed to influence growth factor mediated E2-induced cell proliferation.

In summary, it is difficult to rank the vast multitude of biological activities affected by the P149 peptide. It can readily be observed that the peptide activity is not limited by phylum, class, genus, or species. GIP was found to be active in cells derived from organisms ranging from crustaceans to mammals, encompassing frogs, birds, mice, rats, and humans. The P149 peptide is not limited to cell or hormone specificity; it encompasses growth suppression in multiple models, such as tadpole metamorphosis, insulin-injected chick embryos, and E2-induced toxicity in fetal mice. GIP was also involved in suppressing several cytoplasmic enzyme activities. It was at the cell membrane that GIP demonstrated its most diverse functionality, by interfering with a variety of cell surface events. Whatever the mechanism of action is, it probably affects a basic function of many cell types throughout the animal kingdom. Recent data based on immunohistochemical findings have led to the hypothesis that GIP serves as a decoy peptide ligand for a presumed family of G-coupled receptors [20] and [3]. A proposed model of GIP by a G-coupled cell surface receptor is depicted in Fig-12 (see legend). Although the present working hypothesis is still focused on decoy peptide receptor-mediated endocytosis, only further research will elucidate the precise mode of action of GIP.

FUTURE DIRECTIONS

Both biological and biochemical research endeavors, based on the physiological activities of GIP, are currently in progress. Future directions of present research efforts focus on 1) mechanism of action of GIP growth arrest, and 2) GIP therapeutic applications to cancer encompassing the development of peptide-drug delivery systems and *in vivo* storage/stability studies. Investigational attention is also being directed to the nature of the peptide-to-cell surface interaction and on cell-free systems which constitute the cytoplasmic milieu. Finally, the search for additional molecular targets of the GIP molecule and its fragments are being pursued via Genbank identity/similarity matching, computational identity searches (BLAST programs), and computer modeling of protein/peptide interactions.

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ABBREVIATIONS

AA	=	Amino acids
Ache	=	Acetylcholinesterase
AchR	=	Acetylcholine receptor
ADA	=	Arachidonic acid
AMP	=	Adenosine monophosphate
ATP	=	Adenosine tripphosphate
bcl	=	Apoptosis rescue protein
bFGF	=	Basic flibroblast growth factor
Con-A	=	Concanavalin-A lectin
EGF	=	Epidermal growth factor
ECM	=	Extracellular matrix
EGF	=	Epidermal growth factor
EPR	=	Endoplasmic reticulum
ER	=	Estrogen receptor
FTZ-F1	=	AFP transcription factor
FOS/Jun	=	Myc, Ras, oncogene products
Hox	=	Homeodomain protein
IGF	=	Insulin growth factor
PRP	=	Platelet-rich plasma
P187	=	Asp → Asn mutant form of P149
P192	=	Sequence on HAFP AA#510-550
P263	=	Albumin homolog sequence of P149
P237	=	Scrambled form of P149
TNF	=	Tumor necrosis factor.

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