Anticancer *Versus* Antigrowth Activities of Three Analogs of the Growth-inhibitory Peptide: Relevance to Physicochemical Properties

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Abstract. *A 34-amino acid peptide has been synthesized based on an amino acid sequence from the third domain of native full-length alpha-fetoprotein, which has been shown to have both antigrowth and anticancer activities. This peptide, known as the growth-inhibitory peptide (GIP), has two cysteine residues and demonstrates reduced antigrowth activity after long-term storage, presumably due to disulfide bond formation. The disulfide bridge problem was addressed by replacing the two naturally occurring cysteines with either glycines, alanines, or serines (to produce the G-, A- and S-peptides, respectively). The non-hydrophobic G- and S-peptides were found to exist as dimers, while the more hydrophobic C- and A-peptides formed trimers in solution under certain conditions of pH and peptide concentration. The A-peptide was already known to display anticancer activity; however, the G- and S-serine analogs have not been studied in depth since they had demonstrated low antigrowth activities in rodent uterine assays. Using both in vivo and in vitro assays, the A-, G- and S-peptides were shown to exhibit various degrees of cancer growth suppression. An in vitro culture assay, using MCF-7 breast cancer cells, demonstrated that both the G- and S-peptides showed modest cancer growth suppression, while the A- analog showed strong inhibition at doses ranging from 10^{-5} M to 10^{-7} M. In contrast, an in vivo ascites tumor study of all four peptides showed them to have notable activity in the suppression of mouse mammary tumor growth. Overall, our data indicated that physico-chemical properties, such as hydrophobicity, oligomeric state and secondary structure, contribute to the anticancer activity of both the active C- peptide and its analogs. In addition, the antigrowth rodent uterine assay was not always predictive of the anticancer potential of the peptide forms, suggesting a difference between the mechanism of peptide action in the antigrowth models and that in the anticancer assay. Notably, the antigrowth assay failed to predict the marked anticancer activity of the analogs against a mammary tumor, indicating that the growth bioassay cannot always be relied upon as a screening protocol.*

Human α-fetoprotein (HAFP), a tumor-associated fetal protein, is known to enhance both fetal and tumor growth *via* a protein kinase A-mediated pathway (1, 2). However, HAFP at low concentrations has been reported to display antigrowth properties, this activity being augmented when the protein is exposed to large molar excesses of ligands, such as steroids (3, 4). Immunochemical studies demonstrated that a conformational change in the HAFP molecule, incubated in high concentrations of estradiol, exposes a hidden epitope that is the site of a particular 34-amino acid segment (5). The 34-amino acid encrypted antigrowth segment is located in the third domain of the HAFP molecule; that domain, by itself, has an antigrowth activity similar to that of the full-length HAFP molecule. In contrast, the first and second domains of HAFP do not display such an antigrowth property (6).

The 34-amino acid segment of naturally-occurring HAFP was chemically synthesized and was found to possess both antigrowth and anticancer properties (7). The synthetic peptide, growth inhibitory peptide (GIP), has two cysteines in its amino acid sequence spaced 14 amino acids apart and, over several hours in solution, disulfide bonds form a bridged construct. At low concentrations (0.2 mg/ml), the synthetic peptide forms trimers, while at higher concentrations the peptide forms higher-order oligomers (8, 9). The trimeric form of GIP displays antigrowth activity in the suppression of estrogen-induced immature mouse uterine growth, while the dimer does not exhibit this activity. Certain fragments of the 34-mer GIP fragments

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Table I. *Physicochemical properties of the human alpha-fetoprotein (HAFP) full-length molecule, its third domain fragment, the cysteine peptide and analogs of the 34-mer growth-inhibitory peptide are displayed.*

Protein and/or peptide	Molecular weight (Daltons)	Oligomeric *active form	Mutation of AA total present	Total amino acid residues in sequence				Secondary structure			
				Cys	Ala	Gly	Ser	α Helix	β Sheets	β Turns	Random coil
1) Full length HAFP	69,000	3-domain monomer	NA	32	44	26	34	52.6	13.0	22.0	11.0
2) Third domain AFP molecule	27,000	1-domain monomer	NA	12	19	11	9	34.0	23.0	23.0	22.0
3) Cysteine-peptide (50% TFE)	3,573	Trimer	2C→2C	2	3	4	1	10.6 (31.7)	33.4 (17.6)	27.1 (29.0)	27.0 (20.7)
4) Alanine peptide (50% TFE)	3,507	Trimer	3A→5A	0	5	4	1	9.2 (38.4)	33.5 (13.7)	28.0 (28.8)	28.0 (19.3)
5) Glycine peptide (50% TFE)	3,479	Dimer	4G→6G	0	3	6	1	9.6 (28.1)	34.0 (18.7)	27.5 (29.8)	27.9 (23.9)
6) Serine peptide (50% TFE)	3,541	Dimer	1S→3S	0	3	4	3	10.3 (27.1)	34.1 (13.8)	27.1 (28.0)	29.1 (21.6)

NA = Not-applicable; TFE = trifluorethanol; AA = amino acid; ( ) = 50% trifluorethanol treatment; Cys, C = cysteine; Ala, A = alanine; Gly, G = glycine; Ser, S = serine. *Oligmeric form of the analog peptides are 34-amino acid synthetic segments; the third domain of HAFP was produced by recombinant DNA technology (6). HAFP was purified as described in (4). Note that the cysteine and three analog peptides displayed the highest percentage of β-sheet structure among the peptides, *i.e.*, mutation of the cysteine peptides to alanine contributes to a more highly hydrophobic species among the analog peptides.

have also been shown to possess various antigrowth and anticancer activities, as previously reported (10, 11). The trimer form of the peptide was previously reported to display notable anticancer properties (12).

Analogs of the GIP were chemically synthesized in which the two cysteines were replaced by either alanines, glycines, or serines, respectively, producing the A-, G- and C-analogs/peptides (9). A rodent uterine assay has previously been used as a screen to determine the potential anticancer activity of AFP-derived peptides. Here, the G- and S- peptides are reported to have shown marked anticancer activities, but they did not always conform to the antigrowth predictive pattern; rather, the anticancer activities of the analogs appeared to more closely correlate with the peptides' physicochemical properties in their linear form.

Materials and Methods

Physicochemical studies. The C- peptide and its analogs were synthesized using F-moc solid-state chemistry at the Peptide Synthesis Core facility of the Wadsworth Center, USA, using a 431A peptide synthesizer from Applied Biosystems (Foster City, CA, USA), as previously described (4, 8). High-pressure liquid chromatography (HPLC) purification was carried out using a Delta-Pak C18-reverse-phase-column (Waters Corp, Milford, MA, USA). The original C-peptide sequence was LSEDKLLACGEGA ADIIIGHLCIRHEMTPVNPGV. The physicochemical properties of the C-peptide and its analogs, together with those HAFP and the isolated third domain of HAFP, are displayed in Table I. The molecular masses of the peptides were determined by electrospray ionization mass spectrometry using a Finnegan TSQ-700 triple quadrupole Instrument (Bruker Co., Bellerica, MA, USA) (4, 8, 9). All lyophilized peptides were weighed and dissolved in pH 7.4 buffer, 0.1 M NaCl and 0.01 M sodium phosphate, prior to use. The peptides were subjected to secondary structure analysis by circular dichroism (CD) using a JASCO (model J-720) spectropolarimeter (Jasco Corp, Easton, MD, USA), as previously reported (13, 14). Trifluoroethanol (TFE)-treated peptides were further analyzed by CD for their propensity to induce α-helical and β-sheet alterations (13).

Biological studies. An *in vivo* non-cancer growth assay of immature rodent uterine growth was utilized, as previously reported (15, 16). A syngeneic mouse mammary tumor growth assay was also performed using NYLAR mice inoculated with 1.0×10^6 6WI-1 mammary adenoacanthoma (squamous) tumor cells (5). A control tumor-transplanted group received daily injections of saline, whereas the tumor-treatment groups received the C-peptide or one of three analogs (1.0 μg/day) for 12 days. Tumor growth was assessed as the increase in the total body weight and accumulation of both ascitic fluid and tumor cells in the peritoneal cavity of the host mouse. Tumor growth suppression was expressed as the percent reduction in total body weight, tumor cells and ascitic fluid as compared to the treated *versus* the control tumor-bearing mice.

Tumor cytostatic *in vitro* assays of the C-peptide and its analogs were performed as previously described (5, 12), using a non-estrogen-dependent human breast cancer MCF-7 cell line in a 6-day treatment regimen. Cell proliferation was determined using the sulforhodamine B staining method on MCF-7 cells incubated in 96-well microtiter plates (17). The growth of peptide-treated cells was expressed as the percent growth compared to non-peptide-treated MCF-7 cells.

Results and Discussion

C-peptide GIP physicochemical properties. Synthetic C-peptide was purified and characterized in a series of physicochemical steps. The biochemical studies of the C-peptide and its analogs established that purification by C-18 reverse-phase HPLC produced a peptide species having the correct molecular weights as determined by mass spectroscopy; oligomer formation was evaluated by HPLC gel filtration (4, 8). The molecular masses were those expected from the sequences, indicating that the two cysteines of the C-peptide were not oxidized. The CD in the far-UV of the peptides displayed negative maxima at 201 nm. Both computer modeling (non-solution) and the CD spectra of the peptides indicated the presence of β-sheets/turn and other disordered types of secondary structure in approximately equal proportions, with the remaining proportion comprising a small amount of α-helix, as previously reported (14). Thus, the C-peptide and its analogs are largely amphipathic β-sheet peptides.

All four peptides, as studied by gel-filtration HPLC, exhibited complex aggregation behaviors characteristic of β-sheet peptides (8). Soon after solubilization, the C-peptide formed trimers and hexamers (<1.0 mg/ml), but at high C-peptide concentrations (>8 mg/ml) the trimers clustered into higher order aggregates. Mass spectroscopy demonstrated that the dimers contained an intra-peptide disulfide bond, while the trimers had free cysteines (13). Physicochemical analysis was then performed on the three analogs (14). At low concentrations (0.2 mg/ml), the A-peptide formed trimers, while the G-peptide and S-peptide formed dimers; however, at high concentrations, all three peptides aggregated to some extent. All of the peptides, although differing in amino acid content, had similar secondary structures as determined by CD (Table I). In contrast to the relatively high α-helical content of full-length AFP (53%) and its purified third-domain fragment (34%), the C-peptide and the three analogs all displayed low amounts of α-helix (~10%) accompanied by nearly equal amounts of β-sheets/turns (45%) and disordered structure (45%), confirming that they are largely β-sheet peptides (Table I). However, at TFE concentrations of 25 to 50% the C-, A-, G- and S-peptides were converted into forms with greater α-helical content (Table I). The TFE reagent is known to convert peptides into increasingly

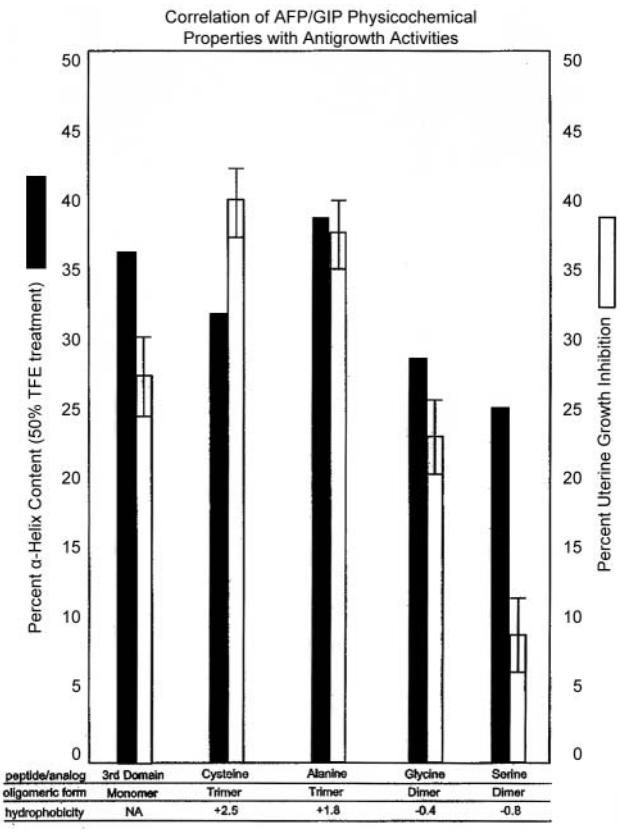

Figure 1. *The antigrowth activities in a murine uterine assay of a protein isolate from the isolated third domain from HAFP, the C- peptide and the A-, G- and S-peptides are displayed together with their percent α-helical content. Note the trend for higher α-helical propensity, trimer formation, and hydrophobicity with increased biological activity. The hydropathy index employed was derived from the data of Kyte and Doolittle (21). The peptide α-helical content shown followed 50% TFE treatment.*

helical configurations, if they possess the innate propensity for such a structure. These data suggest that the C-peptide segment probably has a higher α-helical content when it forms an intrinsic portion of HAFP than when it is in the form of a free peptide.

The A-, G- and S-peptides, unlike the original C-peptide, cannot form an internal disulfide bond and remain as trimers and/or dimers at low concentrations (<1.0 mg/ml). Dimers were found to arise either from the primary sequence of the amino acid in the S- and G-peptides, or from internal disulfide bonding in the original C-peptide. Both dimer types were found to have lowered activity in the antigrowth uterine assay, as well as in the anticancer assays described below.

The analogs: antigrowth activities. The C-peptide displayed activity as an inhibitor of normal growth in the rodent immature uterus (Figure 1). A previous report of C-peptide

suppression of E2-dependent growth in the 24-h immature mouse uterus assay had demonstrated an anti-uterotrophic activity of 35 to 40% (10). A series of physiochemical studies of the C-peptide were performed, one of which compared the oligomeric state of the peptide to its antigrowth activity. In this study (Figure 1), the complex aggregation behaviors of the various oligomers of the C-peptide revealed that the trimers were the most active species in the uterine antigrowth assay, whereas both dimers and higher-order aggregates were much less active. Carboxy-methylation and amidation modification of the two cysteine residues of the C-peptide had not previously (13) been found to impair the antigrowth properties, suggesting that participation of the two free cysteines was not crucial for this activity and that they could be mutated.

Replacement of the cysteines showed that certain substitutions resulted in dimer peptides having reduced growth-inhibitory activity in the uterine assay. The A-peptide retained the trimer configuration and displayed antigrowth properties comparable to those of the C-peptide (Figure 1). In contrast, the G-peptide, which occurred in a dimeric state, displayed only marginal growth-inhibitory capability, while the S-peptide (with its reduced hydrophobicity) also showed a much lower activity. It was interesting that the secondary structures of the three analogs were somewhat similar even in the presence of TFA, although the antigrowth activities differed among the three peptides (Table I, Figure 1). As shown in Figure 1, the secondary structure, oliogomeric form and hydrophobicity all appeared to contribute to the antigrowth activity of a given analog. In the presence of TFA, all the analogs were transformed to configurations with a higher α-helical content (*i.e.*, from 10% to 30%), while the β-sheet structure was concurrently reduced (Table I). The propensity for higher α-helical formation appeared to correlate with the higher antigrowth activities. Thus, the physicochemical properties were all contributing determinants for the antigrowth activity.

The analogs: anticancer activities. The A-peptide was subsequently subjected to *in vitro* and *in vivo* anticancer growth assays. This peptide had already been shown to display anticancer activity in several cancer cell lines (12). In the present study of the MCF-7 human breast cancer cell line, the A-peptide displayed 70% growth inhibition at high (10^{-5} M) concentrations, consistent with previous reports (5). The A-peptide analog is shown here (Figure 2) to display notable anticancer activity *in vivo* in the transplanted 6WI-1 mouse mammary tumor isograft assay. When the A-peptide was administered to mice in 1.0 μg daily doses throughout the 12-day post-transplantation period, a marked reduction in body weight gain, tumor cell numbers, and ascitic fluid production was observed (Figure 2). It is further evident from Figure 3 that hydrophobicity, oligomeric state and helical propensity tend to correlate with the degree of anticancer potency (see below). Thus, notable anticancer effects of the A-peptide were evident in both the cell culture and animal models.

The C- and A-peptides had been previously shown to be active in the suppression of several human cancer types, over a molar range extending from 10^{-5} to 10^{-7} M (6). However, only a modest growth inhibitory activity (35-45%) of the G-and S-analog peptides occurred over this same dose range. These low-to-moderate anticancer activities again confirm that hydrophobicity, oliogomeric form and α-helical propensity are indeed determinants of the anticancer potential (Table I, Figure 3). Note in Table I that substitution of alanines for the two cysteines resulted in a peptide with a total of five alanine residues, thus increasing the overall hydrophobicity of the molecule. The G-peptide contained six rather than four of the C-peptide's original glycine residues; however, glycine has a low hydropathy index. In the TFA-induced α-helical alterations, both the G- and A-peptides displayed slightly reduced α-helical content, correlating with their lower anticancer growth effects.

The assessment of estrogen-dependent uterine growth in mice has previously served as a predictive measure for further cancer studies of α-fetoprotein-derived peptides (15, 16). The C-peptide displayed antigrowth activity that diminished as the trimeric peptide was converted to dimers with intrinsic disulfide bonds (13). The dimeric G-peptide showed only marginal ($\sim$20%) activity in the mouse uterine assay (Figure 1). It might be predicted that reduced antigrowth activity would correlate with lower anticancer activity; however, the G-peptide displayed modest *in vitro* potency and notable *in vivo* activity, respectively, in the two cancer protocols. These data suggest that the mechanism of the peptide action must differ between the antigrowth and the anticancer models.

The *in vivo* anticancer system employed a highly anaplastic tumor consisting of the 6WI-1-mouse mammary adenoacanthoma cells serially transplanted into mice. The mortality of this tumor in the host mouse is 100% at 12 to 14 days. The results for the A-, G- and S-peptides showed significant reductions in both cancer cell numbers and ascitic fluid accumulation (50-70% each); the host lifespan was extended by 3 weeks or more. In this murine tumor assay, cell growth was reported to directly correlate with the volume of ascitic fluid (18). The results for the analog peptides in the ascites tumor assay tended to parallel the results of the *in vitro* rhodamine β cell proliferation assay. Thus, the results of both cancer assays were consistent with the findings that all three analog peptides have modest to marked anticancer activity. Vakharia and Mizejewski (5) had previously reported that the original C-peptide had notable activity in these same cancer assays. In summary, the G-, A- and S-peptides were all effective to some degree in the suppression of mouse

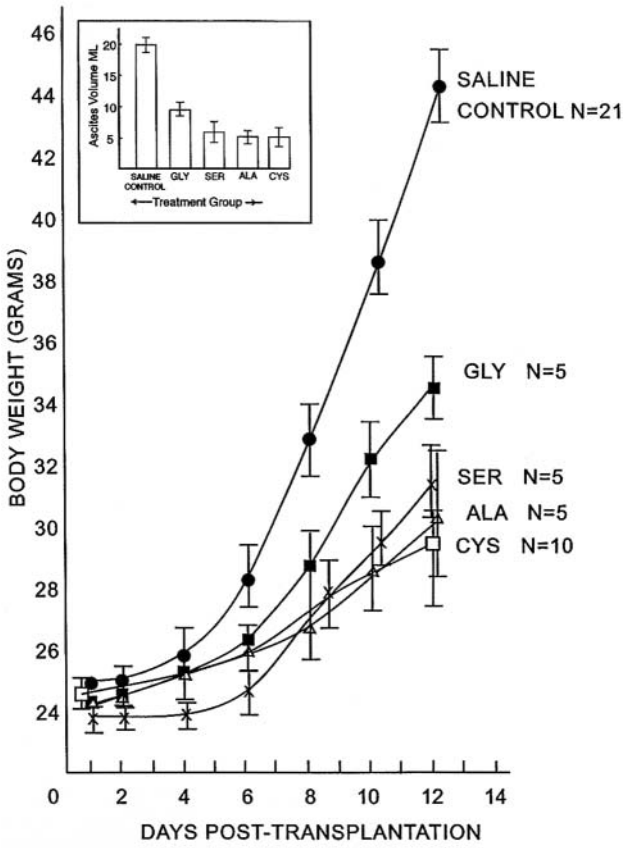

Figure 2. *Effects of the C-, A- G- and S-peptides on growth suppression of the 6WI-1 mouse mammary tumor isograft. Total body weight and ascitic fluid volume were determined as indicators of tumor growth in this murine ascites cancer model. Note that all four peptides were growth-suppressive in this in vivo cancer model.*

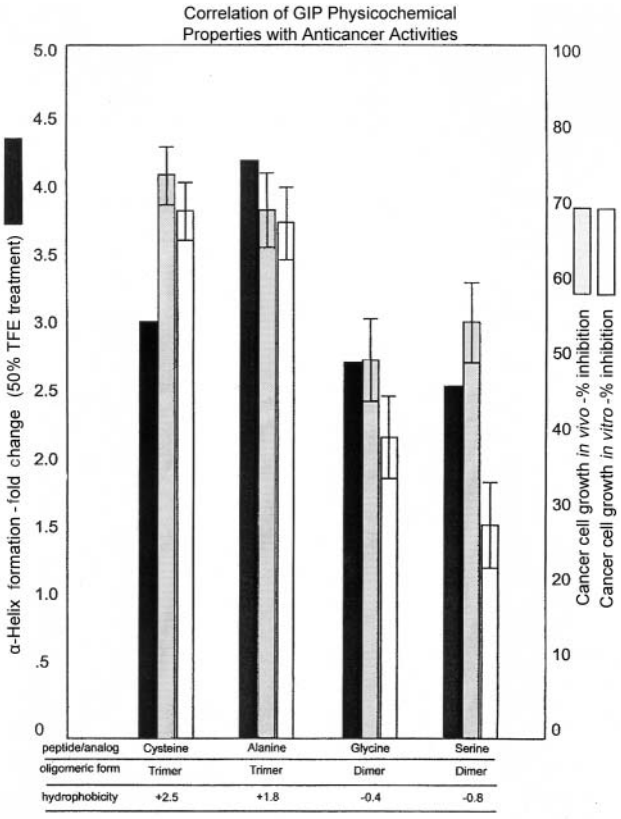

Figure 3. *Performance in two anticancer assays of the cysteine peptide and its analogs is plotted together with the peptides' respective α-helical fold-changes as a result of exposure to 50% TFA. Cancer cell growth inhibition is indicated for both the in vivo mouse ascites isograft assay (shaded bar) and the in vitro MCF-7 assay (white bar). Note that a higher propensity for α-helical formation (fold increase), greater propensity for trimer formation and increased amino acid hydrophobicity all roughly correlated with greater cancer cell growth suppression.*

mammary tumor growth. It is of interest that a decrease in the specific amino acid hydrophobicity seemed to parallel the decline in both biological activities, *i.e.*, antigrowth and anticancer (Figures 1 and 3).

Two different assays were employed to demonstrate the anticancer activities of the C-peptide and its analogs. It was found that only the C- and A-peptides seem to be candidates for further anticancer studies since: a) they showed the highest anticancer activities; b) they are derived or modified from a naturally-occurring human fetal protein; c) no toxicity has been reported for either peptide; and d) both peptides displayed notable growth suppression in the two cancer models studied. It is conceivable that exposure of the GIP segment on the HAFP molecule during pregnancy functions to halt growth in specific environments of the fetus (*i.e.*, during oxidative stress) until cell signaling pathways can be re-established. Thus, an antigrowth peptide intrinsic to the AFP molecule appears to be an innate structure to protect women from cancer and benign growths during pregnancy and in both the child-rearing and post-menopausal years, as epidemiological studies into HAFP have indicated (19, 20).

One hypothesis, to explain the diverse activities of trimers *versus* dimers, would be to assume that monomers are really the active form in both the anticancer and antigrowth assays. This might mean that trimers tend to dissociate to monomers more easily and rapidly than dimers. Since it was assumed above that both timers and dimers are intrinsically inactive, solutions that initially form trimers would be more active than solutions first forming dimers. However, in a situation where the conditions are favorable to promote equally the dissociation of both trimers and dimers (low peptide concentrations), then both oligomeric configurations would form monomers at similar rates and biological activities would be observed for both forms, as seen in the cancer assays.

In conclusion, these results continue to demonstrate that GIP and its alanine analog have considerable potential as therapeutic agents for the treatment of certain cancers. The action of all the peptides against a mammary tumor isograft (Figure 2) was notable. The substitutions of cysteine in the original peptide also helped to confer long-term storage stability to the biological activities (8, 9).

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