Tumor-Cell-Targeted Methionine-enkephalin Analogues Containing Unnatural Amino Acids: Design, Synthesis, and in Vitro Antitumor Activity

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A series of new peptides (**8**−**25**) containing different unnatural amino acids of the adamantane type (**1**−**6**), was synthesized. Possible cytotoxic activity on human cervical adenocarcinoma (HeLa), larynx carcinoma (HEp-2), colon carcinomas (HT-29, Caco-2), poorly differentiated cells from lymph node metastasis of colon carcinoma (SW-620), mammary gland adenocarcinoma (MCF-7), and melanoma (HBL) cells were tested by the MTT assay. The results were compared with the effect of methionine-enkephalin (Tyr-Gly-Gly-Phe-Met, or opioid growth factor, OGF), and its shorter N-terminal fragments. Peptide analogues containing $C^{\alpha\alpha}$-dialkylated glycine (Aaa1, **1**) or C^{α}-alkylated glycine (Aaa2, **2**) amino acid residues showed antitumor activity against melanoma, larynx carcinoma, colon carcinomas, and colon metastasis cell lines in vitro. The pentapeptide Tyr-(*R*,*S*)-Aaa2-Gly-Phe-Met (**18**) was the most effective analogue especially against the most antitumor drug-resistant cell lines HEp-2 and SW-620. Apoptosis as a mode of cell death was confirmed in these tumor cells after exposure to pentapeptide **18**.

Introduction

The development of peptide drugs for the treatment of cancer has undergone dramatic changes in recent years and new types of peptides and peptidomimetics have been designed to interact with the tumor cell at specific sites. The spectrum of new targets include control of cell proliferation, the pathways for transmission of positive and negative signals from the cell surface to the nucleus, the control of angiogenesis, and complex processes that determine apoptosis and DNA repair.[1]

Opioid peptides act as cell growth factors, in addition to regulating neurotransmission/neuromodulation in the nervous system. The native opioid growth factor (OGF), methionine-enkephalin (Tyr-Gly-Gly-Phe-Met, **7**), plays a role in cell proliferation and tissue organization during development, cellular renewal, wound healing, and angiogenesis, but also in cancer.[2,3] OGF acts as an inhibitor of cell division and does so in a receptor-mediated fashion.[4] The receptor (OGFr) has been cloned and sequenced in humans, rat, and mouse and its molecular and protein structure has no resemblance to that of classical opioid receptors. The action of OGF is constitutive, stereospecific, reversible, and noncytotoxic. At least in cancer cells, OGF does not utilize pathways of cell survival (apoptosis, necrosis).[5] OGF, however, suffers from drawbacks which include rapid degradation in biological fluids wherein the active compound very rapidly hydrolyzes into inactive form(s).[6]

To obtain more selective peptides with improved or novel activity profiles toward malignant diseases, modifications with lipophilic moieties may be of particular benefit to passive or active cellular absorption by membrane penetration or attachment. The susceptibility of the modified peptides to enzymatic degradation may also decrease. Hydrophobization of peptides may be attempted by two approaches. The first is peptide backbone modification to include hydrophobic residue; the second would be covalent conjugation with a hydrophobic moiety, for example, a lipid or a polymeric tail. Increasing hydrophobicity by incorporation of nonnatural amino acids opens up the possibility for greater chemical diversity, analogous to small-molecule medicinal chemistry approaches for developing high-affinity, high-specificity molecular recognition.[7]

In this paper, we report the synthesis of a series of new synthetic pentapeptide analogues of methionine-enkephalin, its structurally related dipeptide, tripeptide, tetrapeptide, and hexapeptide derivatives containing a variety of unnatural amino acids of the adamantane type, and their cytostatic activity toward malignant diseases.

Chemistry. The adamantane-containing amino acids **1**−**6** (shown in Figure 1) were prepared by applying modified literature procedures.[8−17] The resultant unnatural amino acid hydrochlorides were characterized by NMR spectroscopy to confirm structure and purity. Amino acid **2** [(*R*,*S*)-(1-adamantyl)-glycine, Aaa2] was used in the synthesis of peptides in its racemic form. Resolution of **2** was carried out by a published procedure;[10] however, this approach resulted in enantiomerically enriched (*R*)-Aaa2 and (*S*)-Aaa2 enantiomers.

Based on the fundamental sequence of methionine-enkephalin **7**, 18 new peptide analogues (**8**−**25**) containing unnatural amino acids **1**−**6** were prepared. Peptides **8**−**22** were designed by substituting either glycine (position 2) or glycylglycine (positions 2 and 3) in **7** or in its shorter N-terminal fragments by adamantane-derived amino acids **1**−**6**, while hexapeptides **23**−**25** were obtained by extending the C-terminal region of the fundamental structure of methionine-enkephalin. Peptides **8**−**25** were synthesized by solution-phase methods via the stepwise or fragment condensation approaches summarized in Figure 2. The crude products were purified by RP-HPLC. Separation by RP-HPLC to chiral peptides **9**/**10** and **15**/**16** containing individual enantiomers of the Aaa2 residue was successful. In contrast, the diastereoisomeric pentapeptides **18** exhibited no difference in retention and could thus not be separated under the RP-HPLC conditions studied. The configurations of the Aaa2

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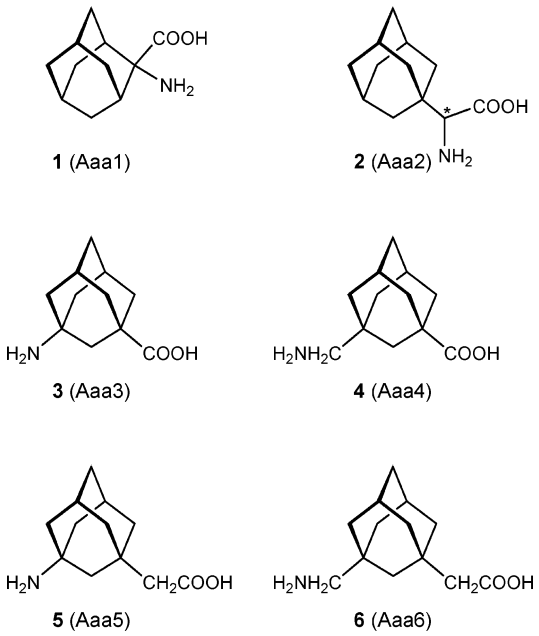

Figure 1. Structure of the adamantane-derived unnatural amino acids **1**−**6**.

amino acid residues in pure diastereoisomers **9** and **10** as well as in **15** and **16** were assigned unambiguously by hydrolysis of the corresponding peptide, isolation of the pure enantiomer of amino acid **2**, and comparison of the obtained $[\alpha]_D$ values with literature data.[9,11] TLC and HPLC analysis on chiral supports were also used for identification of the individual enantiomers of Aaa2. According to analytical HPLC with a Chiral-AGP stationary phase, the diastereoisomeric mixture **18** was slightly enriched in the (*R*)-Aaa2-enantiomer ($ee_{[(R)-Aaa2]-\mathbf{18}} = 20\%$). Attempts to separate the diastereomers of **18** by fractional crystallization resulted in diastereomerically enriched pentapeptides **18a** ($ee_{[(S)-Aaa2]-\mathbf{18}} = 70\%$) and **18b** ($ee_{[(R)-Aaa2]-\mathbf{18}} = 72\%$).

For screening of cellular uptake, fluorescein-labeled peptides were prepared from methionine-enkephalin (FITC-**7**) and pentapeptide **18**. FITC-**18** diastereoisomers were separated by RP-HPLC to pure fluorescein-peptides containing individual enantiomers of Aaa2 residue, FITC-[(*S*)-Aaa2]-**18** and FITC-[(*R*)-Aaa2]-**18**. The final compounds were characterized by analytical RP-HPLC, mass spectrometry, and elemental analyses. The physicochemical characteristics of the obtained peptides are summarized in Table 1. A detailed NMR analysis of the target compounds is given in the Supporting Information.

To investigate the possibility of a correlation between peptide hydrophobicity and biological activity in cancer cells, the hydrophobic characteristics of peptides **8**−**25** were investigated by comparing their retention times in RP-HPLC (Table 1). The incorporation of bulky adamantane-containing amino acid moieties increased the retention times, reflecting the more hydrophobic character of all analogues in comparison with Tyr-Gly-Gly-Phe-Met (**7**) and its shorter N-terminal dipeptide and tripeptide fragments. The major hydrophobic contribution of the Aaa1 and Aaa2 residues was evident when the retention times of pentapeptides **17** or **18** are compared with that of methionine-enkephalin (**7**), the differences in the retention times being about 19 and 15 min, respectively. The retention times of tetrapeptides **19**−**22** with unnatural amino acids **3**−**6** as isostere replacements of the Gly-Gly dipeptide still indicated a significant increase in hydrophobicity, but the replacement of Gly[2] in methionine-enkephalin by either Aaa1 or Aaa2 caused a greater change in hydrophobicity. The differences in retention behavior of hexapeptides **23**−**25** confirmed that adamantane-containing amino acid **1** is the most hydrophobic among the nonnatural amino acids studied.

Biological Results and Discussion

In Vitro Antitumor Activity. The effect of methionine-enkephalin (OGF, **7**) as well as peptide compounds **8**−**25**, containing unnatural amino acids, on cell proliferation was tested on the following malignant tumor cell lines derived from different tissues: human cervical adenocarcinoma cells (HeLa), human larynx carcinoma cells (HEp-2), human colon carcinoma cells (HT-29, Caco-2), poorly differentiated cells from lymph node metastasis of colon carcinoma (SW-620), human mammary gland adenocarcinoma cells (MCF-7), human melanoma cells (HBL), and human normal fibroblasts (WI38). Peptides **8**−**25** showed great variation in antiproliferative effect on tumor cell lines, depending on the cell line as well as on the structure and dose of the compound tested. In general, the novel peptides displayed moderate (20−50% inhibition) to no or weak (0−20% inhibition) cytotoxic potency on the tumor cell lines examined. Only the peptides containing unnatural amino acids of type Aaa1 (**1**) and Aaa2 (**2**) exhibited some selective cytotoxicities, whereas analogues containing unnatural amino acids **3**−**6** as isostere replacements of the Gly-Gly dipeptide did not show any anticancer activity. As presented in Table 2, compared to opioid growth factor **7** and untreated cells, peptide analogues **8**−**10**, **15**−**18**, and **23** showed increased antitumor activities against HEp-2, HBL, SW-620, and Caco-2 cell lines in vitro. In the applied concentration of 10^{-6}−10^{-3} M, peptides **8**−**10** and **15**−**18** demonstrated statistically significant stronger growth inhibition effects on SW-620 and HEp2 cells (see Supporting Information). The antiproliferative effects of these compounds on HBL and Caco-2 cell growth were weaker. The most antitumor drug-resistant cell lines HEp-2 and SW-620 were especially sensitive to the mixture of diastereoisomers Tyr-(*R*,*S*)-Aaa2-Gly-Phe-Met (**18**) (Figure 3). In both cell lines, growth was statistically significantly suppressed by **18** in comparison to methionine-enkephalin (**7**) and untreated cells. Diastereomeric tripeptides **15** and **16**, also containing the Aaa2 residue, displayed moderate inhibitory effects only on the growth of HEp-2 cells. Among the peptides containing the Aaa1 residue, dipeptide **8** was cytotoxic against the SW-620 cells and C-terminally extended hexapeptide **23** significantly inhibited the growth of HEp-2 and Caco-2 cell lines (Table 2). The cytotoxicity of novel peptides on human fibroblasts in the applied concentrations of 10^{-6}−10^{-3} M was not observed.

On the basis of the results of previously published studies,[18−21] marked inhibitory activity of pentapeptide **7** against tumor cells growth was expected. However, under the conditions of this study, OGF displayed mild or no suppression of cell proliferation, depending on the tumor cell line tested (Table 2). This disagreement may be the result of fundamental differences in the experimental procedure used in previous reports,[4,21] in which all media and compounds were replaced daily. Experiments employing smaller fragments of methionine-enkephalin (**7**), such as Tyr-Gly and Tyr-Gly-Gly, revealed no influence on the tested tumor cells even at concentrations as great as 10^{-3} M.

To check whether, due to different chirality of the Aaa2 residue, each diastereomer of pentapeptide **18** will have different activity compared to racemic **18**, the antiproliferative effect of highly enriched diastereomers (ee $\sim$ 70%) in either the (*S*)-Aaa2- (**18a**) or the (*R*)-Aaa2-enantiomer (**18b**) on HEp-2 and SW-620 tumor cell lines was studied. It was definitely shown (Figure 3) that peptides **18a** and **18b** do not differ substantially

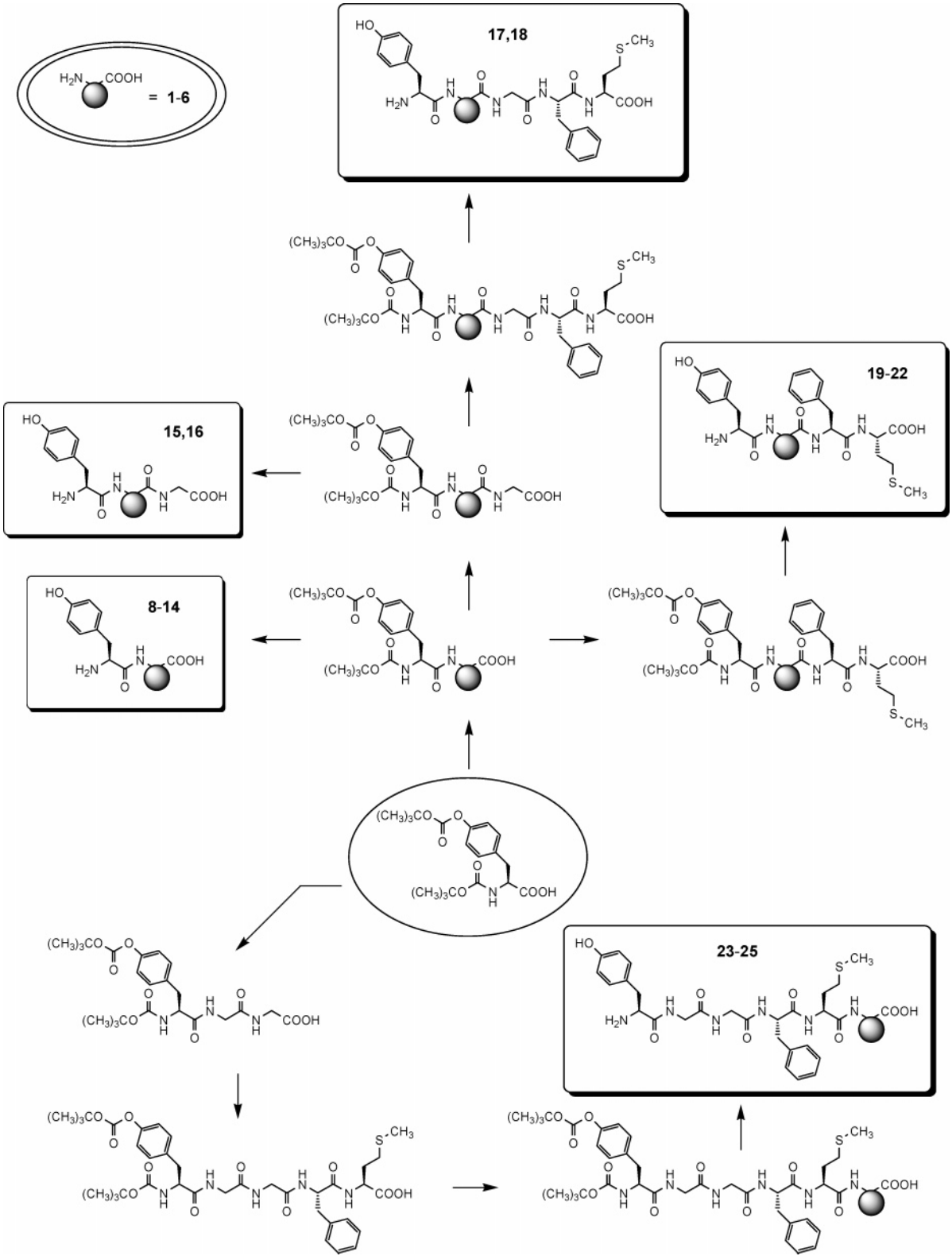

Figure 2. Synthetic pathways to peptides **8**−**25**.

in their ability to inhibit the growth of the examined cell lines. In fact, they appeared to be less effective than the racemic compound **18**. The obtained data suggest a synergistic effect between [(*S*)- and [(*R*)-Aaa2]-**18** when investigated together as a racemate. Hence, the racemic mixture **18** represents the optimal formulation for the future investigation of this molecule in terms of cytotoxic properties. In addition, the diastereomeric tripeptides **15** and **16**, containing either *S*- or *R*-Aaa2 showed similar, cell-selective, cytotoxic effects on tumor cell lines irrespective of Aaa2 chirality.

Previous studies[2] have demonstrated that ligands selective for known opioid receptors such as μ, δ, and κ, expressed on the cell membranes of normal and malignant tissues, did not affect cell growth either in vivo or in vitro. Only native opioid peptide methionine-enkephalin (**7**), also termed opioid growth factor (OGF), has been identified as a negative growth regulator.

Table 1. Retention Times, Melting Points, and Optical Rotation Data of Peptides **8**−**25**

compd	structure	t_R[a] (min)	mp (°C)	$[\alpha]_D$,[b] deg
8	Tyr-Aaa1	12.81	275−280	+40
9	Tyr-(*S*)-Aaa2	12.46	180−185	+8
10	Tyr-(*R*)-Aaa2	14.05	190−195	+19
11	Tyr-Aaa3	8.79	155−158	+55
12	Tyr-Aaa4	9.03	192−195	+22
13	Tyr-Aaa5	9.76	190−195	+45
14	Tyr-Aaa6	10.66	195−200	+20
15	Tyr-(*S*)-Aaa2-Gly	9.53	200−205	+12
16	Tyr-(*R*)-Aaa2-Gly	10.79	210−215	+21
17	Tyr-Aaa1-Gly-Phe-Met	26.70	200−205	+1
18[c]	Tyr-Aaa2-Gly-Phe-Met	23.03	194−210	+2
19	Tyr-Aaa3-Phe-Met	16.51	205−208	+17
20	Tyr-Aaa4-Phe-Met	18.35	205−208	+6
21	Tyr-Aaa5-Phe-Met	17.80	202−208	+11
22	Tyr-Aaa6-Phe-Met	19.93	212−215	+9
23	Tyr-Gly-Gly-Phe-Met-Aaa1	31.92	212−215	+4
24	Tyr-Gly-Gly-Phe-DL-Met-Aaa4	23.06	198−204	+28
25	Tyr-Gly-Gly-Phe-DL-Met-Aaa6	27.34	199−203	+24

[a] Eluent 50.5% MeOH/0.1% TFA. Retention time of methionine-enkephalin (**7**) under identical conditions, 8.06 min; Tyr-Gly-Gly and Tyr-Gly, no retention. [b] In methanol ($c = 1$). [c] $ee_{[(R)-Aaa2]-\mathbf{18}} = 20\%$.

Table 2. Growth Inhibitory Effects of Methionine-enkephalin (OGF, **7**) and Selected Novel Peptides on Human Tumor Cells

compd	concn (M)	inhibition[a] (%) of cell line						
		HEp-2	HeLa	HBL	HT-29	SW-620	CaCo-2	MCF-7
OGF, **7**	10^{-6}	5.2	2.3	0.4	14.0	18.9	4.2	3.6
OGF, **7**	10^{-5}	4.5	11.4	1.0	15.9	12.7	6.4	4.1
OGF, **7**	10^{-4}	2.4	12.0	4.5	16.1	15.3	8.4	7.6
OGF, **7**	10^{-3}	3.3	22.3	10.8	19.3	19.1	3.2	15.4
8	10^{-6}	14.2		6.5	9.8	12.9	3.5	
8	10^{-5}	19.7	10.0	14.0	1.6	16.5	22.7	2.4
8	10^{-4}	24.9	8.2	7.5	5.4	28.1	18.9	
8	10^{-3}	30.4	36.1	14.0	26.5	58.2	26.4	7.5
9	10^{-6}	6.2	7.5		4.9	10.5	3.7	
9	10^{-5}	6.4	6.1	3.9	8.9	21.9	16.5	1.1
9	10^{-4}	8.2	3.1		3.4	11.8	8.4	1.2
9	10^{-3}	21.2	14.8	7.3	20.8	17.9	14.5	17.8
10	10^{-6}	8.5	7.2	3.0	0.7	10.7	22.2	1.6
10	10^{-5}	4.9	17.1	4.7	8.3	10.0	21.4	2.6
10	10^{-4}	1.9	11.0	2.7	10.5	9.4	17.5	7.7
10	10^{-3}	16.2	20.5	7.3	21.8	18.7	16.0	18.7
15	10^{-6}	2.7	9.8	1.6	5.4	13.6	2.2	
15	10^{-5}	2.0	7.3	2.9	7.4	20.4	4.2	
15	10^{-4}	18.8	9.9	5.9	9.5	14.4	6.4	
15	10^{-3}	26.7	28.2	17.9	15.7	23.4	6.2	21.2
16	10^{-6}	4.6	11.0		7.6	17.0	8.1	
16	10^{-5}	4.9	11.7	1.2	3.8	13.5	6.9	
16	10^{-4}	12.6	11.6	3.3	12.4	13.5	8.1	
16	10^{-3}	26.4	28.2	19.4	22.4	25.3	8.6	15.6
17	10^{-6}	1.0	4.9	0.8	3.3	12.2	3.0	
17	10^{-5}	10.1	10.2	1.4	11.0	15.5	8.4	
17	10^{-4}	16.8	13.3	2.4	7.9	24.7	18.5	
17	10^{-3}	36.1	28.0	18.3	23.7	29.0	10.6	22.8
18	10^{-6}	13.7	8.5	2.4	16.2	26.4	17.7	
18	10^{-5}	25.8	9.5	4.5	6.6	13.1	10.9	
18	10^{-4}	34.7	15.4	11.4	5.1	14.6	12.1	9.6
18	10^{-3}	44.5	24.1	23.2	14.5	38.4	8.1	29.9
23	10^{-6}			4.1				2.2
23	10^{-5}	3.9		70.7			11.08	7.1
23	10^{-4}							6.9
23	10^{-3}	45.0	9.3	34.4	9.2		47.0	25.9

[a] Tumor cell growth inhibition was calculated relative to growth of control (untreated) cells and shown as a percentage; when no number is shown, there was no inhibition.

OGF, presumably transported actively or passively from the extracellular milieu, interacts with a receptor (OGFr) residing on the outer nuclear envelope. Peptide and receptor form a complex that appears to be associated with karyopherin and is translocated to the inner nuclear matrix. In view of such biochemical action and the fact that OGF receptors have been found in a wide variety of human normal and transformed cells,[2] it is reasonable to assume that critical for the magnitude of change in tumor cell number, invoked in this study by the addition of either Aaa1- or Aaa2-modified peptides, will be peptide interaction with membranes and peptide transport through the cell membrane. This transport has been suggested to be largely dependent on the hydrophobic properties of the peptide, its conformation, and molecular size as well as on the ability of the peptide to change conformation upon lipid−peptide interaction.[22] In this regard, our results showed that the high hydrophobicity of pentapeptides **17** and **18** and hexapeptide **23** was well correlated with their antiproliferative activity.

To verify the cell-penetrating properties of peptides **7** and **18** experimentally, fluorescein-labeled methionine-enkephalin (FITC-**7**) as well as diastereomers containing individual enantiomers of the Aaa2 residue, FITC-[(*S*)-Aaa2]-**18** and FITC-[(*R*)-Aaa2]-**18**, were prepared. Fluorescence micrographs presented in Figure 4A (a−d) showed that labeled **7** and FITC-[(*S*)-Aaa2]-**18** rapidly penetrate to the cellular interior of HEp-2 cells. Microscopy of HEp-2 and SW-620 cells treated with FITC-[(*S*)-Aaa2]-**18** for 6 h showed both labeled peptides localized mainly on the outer nuclear membrane of the tumor cells (see Supporting Information). In contrast, the FITC-(*R*)-analogue of compound **18** neither entered the cells nor bound to their outer membranes, even after prolonged incubation time [Figure 4A (e,f)].

Induction of active cell death, apoptosis, by antitumor drugs is vital to the treatment of most tumors. In this study, the number of annexin V positive cells was increased in SW-620 cells treated by Aaa2-containing pentapeptide **18** in comparison to the control, untreated cells. Simultaneous staining with pro-

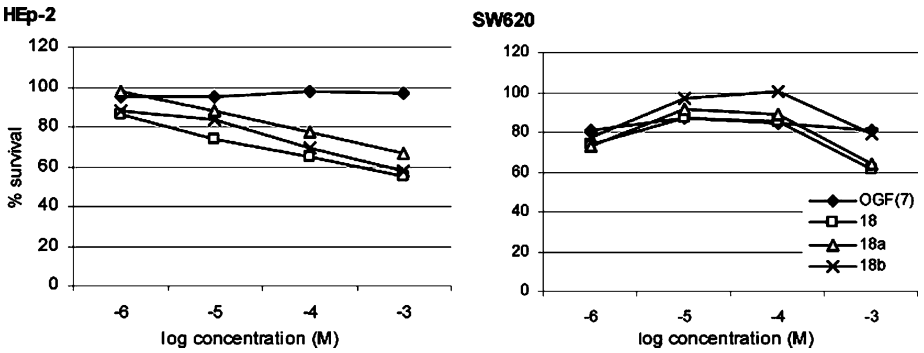

Figure 3. Growth inhibitory effect of opioid growth factor (OGF, **7**) and Aaa2-containing pentapeptides **18**, **18a**, and **18b** on HEp-2 and SW-620 tumor cells.

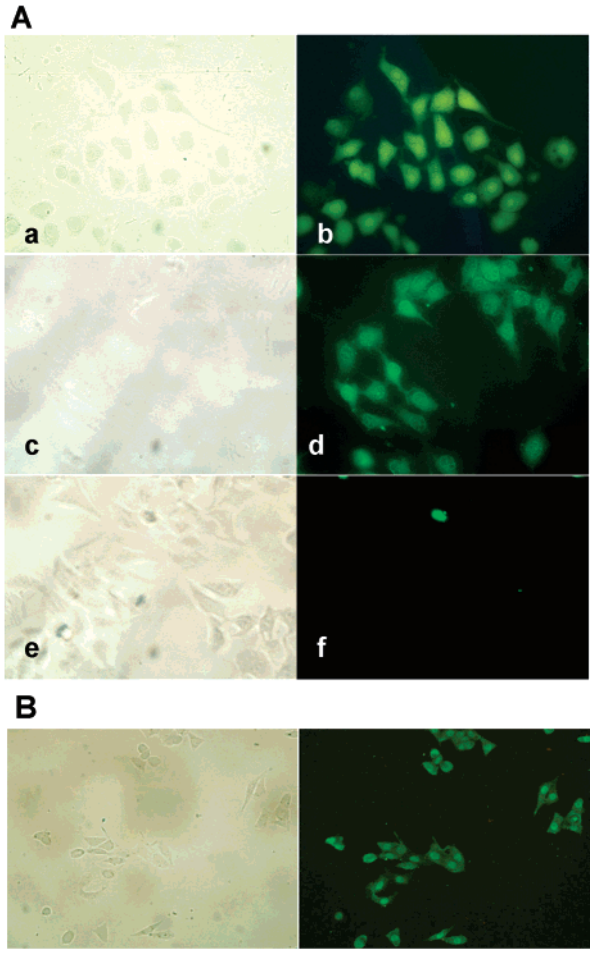

Figure 4. (A) Fluorescence micrographs of HEp-2 cells stained with 10 μM FITC-**7** (a, b), 10 μM FITC-[(*S*)-Aaa2]-**18** (c, d), and FITC-[(*R*)-Aaa2]-**18** (e, f). The cells were incubated with the test compounds for 30 min and washed with PBS prior to observation (×400). The slides were analyzed by use of filters for FITC detection (b, d, f). (B) Fluorescence microscopic analysis of apoptosis in HEp-2 cells. DNA strand breaks were detected by use of the In Situ Cell Death Detection Kit, Fluorescein, after incubation with pentapeptide **18** (10 μM) for 1 h (37 °C, 5% CO_2). Slides were analyzed under an Axioskop2 MOT microscope with 450−490 nm excitation filter and 520 nm barrier filter.

pidium iodide allowed discrimination of the necrotic cells (red) from the apoptotic cells (green) (see Supporting Information). At the same time, SW-620 cells exposed to methionine-enkephalin (**7**) did not express apoptotic features. Similar observation has been also previously reported for methionine-enkephalin.[5] By use of the TUNEL method as a relatively late marker of the apoptotic process, apoptosis as a mode of cell death was confirmed in HEp-2 (Figure 4B) as well as in SW-620 cells exposed to racemic pentapeptide **18** (see Supporting Information).

Conclusion

In the present study, we designed and synthesized 18 new analogues of the endogenous peptide methionine-enkephalin (**7**) by substituting either Gly^2 or Gly^2-Gly^3 in **7** and/or in its shorter N-terminal fragments. Instead of Gly^2 or Gly^2-Gly^3, adamantane-derived amino acids **1**−**6** were incorporated while the hexapeptide derivatives were obtained by extending the C-terminal region of the fundamental structure of methionine-enkephalin by unnatural amino acids **1**, **4**, or **6**. Only the peptide analogues containing $C^{\alpha\alpha}$-dialkylated glycine (Aaa1, **1**) or C^{α}-alkylated glycine (Aaa2, **2**) residues showed antitumor activity against HEp-2, HBL, SW-620, and Caco-2 cell lines in vitro. In particular, the pentapeptide Tyr-(*R*,*S*)-Aaa2-Gly-Phe-Met (**18**) showed the most potent tumoricidal activity by inducing apoptosis in HEp-2 and SW-620 cell lines.

The obtained results indicate that in the investigated structural analogues of methionine-enkephalin (**7**), such as **18**, increased hydrophobic interaction with membranes and transport through the cell membrane may play major roles. Therefore, pentapeptide **18** is the most promising candidate for development of new anticancer agents.

To understand the biological function of peptide **18**, further studies are intended concerning apoptosis/necrosis, conformation−activity relationships, and other systems related to binding of **18** to either OGFr or classical opioid receptors.

Experimental Section

General Methods. Melting points were determined on a Tottoli (Büchi) apparatus and are uncorrected. Optical rotations were measured at room temperature on an Optical Activity LTD automatic AA-10 polarimeter. NMR spectra were recorded on a Bruker AV 600 spectrometer, operating at 150.91 MHz for ^{13}C and 600.13 MHz for ^{1}H nuclei. The spectra were measured in DMSO-d_6 solutions, unless otherwise specified. Chemical shifts in parts per million (ppm) were referenced to tetramethylsilane (TMS). Spectra were assigned on the basis of 2D homonuclear (correlated spectroscopy, COSY) and heteronuclear multiple quantum coherence (HMQC) and multiple bond correlation (HMBC) experiments. Mass spectra were recorded on a ThermoFinnigan Deca ion trap mass spectrometer operating in electrospray ionization (ESI) mode. High-performance liquid chromatography (HPLC) was performed on a Varian Pro Star 230 or HP 1090 HPLC systems with a Eurospher 100 reversed-phase C-18 semipreparative (250 × 8 mm i.d., 5 μm) column (flow rate 1.0 mL/min) or analytical (250 × 4 mm i.d., 5 μm) column (flow rate 0.5 mL/min) eluted isocratically with different concentrations of MeOH in 0.1% aqueous trifluoroacetic acid (TFA). Chiral HPLC separations were performed on a chiral CrownPak CR(+) (150 × 4 mm i.d., 5 μm) column eluted with 15% MeOH in 0.1 M $HClO_4$ (flow rate 0.8 mL/min) or on a Chiral-AGP (100 × 4 mm i.d., 5 μm) column eluted with 15% MeOH in 0.05 M phosphate buffer (pH 7.0) (flow rate 0.7 mL/min). UV detection was performed at 280 and 215 nm on a HP 1090 diode-array detector. Enantiomeric resolution of amino acids by TLC was performed on Chiralplate.

Chemistry. Methionine-enkephalin (**7**) and Tyr-Gly-Gly were purchased from Sigma Chemical Co. Tyr-Gly was from Bachem AG.

Synthesis of Unnatural Amino Acids 1−6. Modified literature procedures were used for the synthesis of 2-aminoadamantane-2-carboxylic acid[8] (Aaa1, **1**), (*R*,*S*)-(1-adamantyl)glycine[9] (Aaa2, **2**), 3-aminoadamantane-1-carboxylic acid[12,13] (Aaa3, **3**), 3-(aminomethyl)adamantane-1-carboxylic acid[15,16] (Aaa4, **4**), 3-aminoadamantane-1-methylcarboxylic acid[14] (Aaa5, **5**) and 3-(aminomethyl)-adamantane-1-methylcarboxylic acid[15−17] (Aaa6, **6**).

Partial resolution of an analytical sample of (*R*,*S*)-(1-adamantyl)-glycine (**2**) was achieved through the esterification of amino acid Aaa2 to the corresponding methyl ester followed by preparation of diastereomeric salts with (+)-dibenzoyltartaric acid, fractional crystallization, and hydrolysis to enantiomerically enriched (*R*)- and (*S*)-Aaa2 as described by Krasutskii et al.[10] HPLC retention times on chiral CrownPak CR(+) column: for (*R*)-**2**, 35.58 min; for (*S*)-**2**, 41.37 min.

Details of the syntheses and NMR analyses for amino acids **1**−**6** and their intermediates are available in the Supporting Information.

Synthesis of Peptides 8−25. The peptides were synthesized in solution by either a stepwise or a fragment condensation approach (Figure 2). The N-terminal amino and hydroxyl groups of the tyrosine moiety were *tert*-butoxycarbonyl- (Boc-) protected. The coupling reactions were carried out by BOP/HOBt, EDC/HOBt, HATU/HOBt, or mixed anhydride activation protocols. All the

intermediates were used in the next step after chromatographic purification. After removal of the N-terminal Boc groups with TFA/H_2O (9:1), the crude peptides were purified by semipreparative RP-HPLC. Diastereomeric mixtures of dipeptides **9**/**10** and tripeptides **15**/**16** were successfully separated by RP-HPLC due to the stronger retention of one isomer on the reversed-phase column. Pentapeptide **18**, containing a racemic Aaa2 residue, could not be separated into individual isomers by RP-HPLC. Determination of diastereomeric purity was achieved by HPLC analysis on a Chiral-AGP column. Retention time for [(*S*)-Aaa2]-**18** was 5.6 min; for [(*R*)-Aaa2]-**18**, 8.4 min. Fractional crystallization of **18** ($ee_{[(R)-Aaa2]-\mathbf{18}}$ = 20%) resulted in diastereomerically enriched pentapeptides **18a** ($ee_{[(S)-Aaa2]-\mathbf{18}}$ = 70%) and **18b** ($ee_{[(R)-Aaa2]-\mathbf{18}}$ = 72%). Assignment of the *S*/*R* configurational features of the Aaa2 residue in **18a** and **18b** was based on differences in the chemical shifts of the H-$\delta\delta'$ protons in the tyrosine aromatic ring and the S-CH_3 protons of the methionine residue in the ^{1}H NMR spectra of the two diastereoisomers, and on comparison with the corresponding protons in the NMR spectra of the enantiomerically pure compounds **9**, **10**, **15**, and **16**. Additional confirmation of the stereochemical assignment of the Aaa2 residue in **18a** and **18b** was obtained by analysis of the corresponding hydrolysates on Chiralplate (for details see Supporting Information).

Peptides **8**−**25** were at least 95% pure as assessed by analytical RP-HPLC. Molecular structures were confirmed by NMR, MS, and elemental analyses. For bioactivity assays, the organic salts present after preparative HPLC were removed by use of an octadecylsilica solid-phase extraction (SPE) cartridge. The cartridge was eluted with water to remove the salt. The peptide compounds were then recovered with methanol. The effluent was evaporated, dissolved in water, and lyophilized.

The structures, RP-HPLC retention times, melting points, and optical rotation data of the novel peptide compounds **8**−**25** are listed in Table 1.

Stereochemical Assignment of the Aaa2-residue Configuration in Peptides 9, 10, 15, and 16. Hydrolysis of dipeptide **9** in 6 N HCl and separation of the obtained amino acids on an SPE cartridge afforded enantiomerically pure (*S*)-(1-adamantyl)glycine [(*S*)-**2**], $[\alpha]_D$ +15° (*c* 0.53, MeOH) [lit.:[11] (*S*)-**2**·HCl $[\alpha]_D$ +16° (*c* 0.50, MeOH)]. Chiral TLC (eluent $CH_3CN-MeOH-H_2O$, 4:1:1): (*S*)-**2** R_f 0.58.

Following the same procedure, hydrolysis of dipeptide **10** afforded enantiomerically pure (*R*)-(1-adamantyl)glycine hydrochloride [(*R*)-**2**·HCl], $[\alpha]_D$ −14° (*c* 0.52, MeOH) [lit.:[9] $[\alpha]_D$ −18° (*c* 0.67, MeOH)]. Chiral TLC (eluent $CH_3CN-MeOH-H_2O$, 4:1:1): (*R*)-**2** R_f 0.34.

The same procedure was applied for the stereochemical assignment of the configuration of the Aaa2 residue in tripeptides **15** [Tyr-(*S*)-Aaa2-Gly] and **16** [Tyr-(*R*)-Aaa2-Gly].

Synthesis of Fluorescein-Labeled Peptides. Methionine-enkephalin (**7**) or Tyr-(*R*,*S*)-Aaa2-Gly-Phe-Met (**18**) was reacted with fluorescein-5-isothiocyanate (FITC isomer I) by using modified procedures for labeling of small peptides.[23] The FITC-peptides were purified by gel chromatography and/or RP-HPLC, affording pure FITC-**7** as well as the labeled diastereomers of **18** containing the individual enantiomers of Aaa2, that is, FITC-[(*S*)-Aaa2]-**18** and FITC-[(*R*)-Aaa2]-**18**.

Details of the synthesis for all peptide compounds and their intermediates, NMR and MS data, as well as elemental analyses are included in the Supporting Information.

Antitumor Activity Assays. Human cervical adenocarcinoma cells (HeLa), human larynx carcinoma cells (HEp-2), human colon carcinoma cells (HT-29, Caco-2), poorly differentiated cells from lymph node metastasis of colon carcinoma (SW-620), human mammary gland adenocarcinoma cells (MCF-7), human melanoma cells (HBL), and human fibroblasts (WI38) were obtained from ATCC. The cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with 2 mM glutamine, 100 units of penicillin, and 0.1 mg of streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The trypan blue dye exclusion method was used to assess cell viability. Cytotoxic effects on tumor cell growth were determined by the MTT assay.[24] Compounds were dissolved in high-purity water with 10% DMSO and diluted into working concentration. The concentration of DMSO was too small to affect the growth as confirmed in the control experiments. All working dilutions ($10^{-3}-10^{-6}$ M) were prepared immediately before each experiment in phosphate-buffered saline (PBS). At day 0 of the experiment, tumor cells, 2×10^4 cells/mL, were plated onto 96-microwell plates. Twenty-four hours later, the medium was replaced with fresh medium containing defined concentrations of the compounds investigated and the cells were treated for an additional 72 h. Control cells (without any compound) were growing under the same conditions. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay. Data are expressed as means of three individual experiments conducted in triplicate. The percentage of growth of the cell lines was calculated from the following equation:

$$\text{percentage of growth} = (A_{\text{peptide}} - A_{\text{blanck}})/(A_{\text{control}} - A_{\text{blanck}}) \times 100$$

where blanck denotes medium without cells, containing the peptide and MTT.

Cellular Uptake. To detect cellular uptake and intracellular distribution of pentapeptide **18** and methionine-enkephalin (**7**), as a control substance, SW-620 and HEp-2 cells (1×10^5 cells/chamber slide) were incubated with 10 μM fluorescein-labeled methionine-enkephalin (FITC-**7**) or with 10 μM FITC-[(*S*)-Aaa2]-**18** or FITC-[(*R*)-Aaa2]-**18**, for 30 min and 6 h. After incubation, cells were rinsed with PBS and analyzed under an Axioskop2 MOT microscope with excitation filter 450−490 nm and barrier filter 520 nm for FITC detection.

Detection of Apoptosis. Detection of apoptotic cells was performed by use of an Annexin-V-FLUOS Staining Kit according to manufacturer's recommendations. SW-620 cells (1×10^5 cells/chamber slide) were incubated with either Tyr-Gly-Gly-Phe-Met (**7**) or Tyr-(*R*,*S*)-Aaa2-Gly-Phe-Met (**18**) (10 μM) for 1 h and analyzed under a fluorescent microscope.

DNA strand breaks in treated SW-620 and HEp-2 cells (1×10^5 cells/chamber slide) were detected by use of the In Situ Cell Death Detection Kit, Fluorescein, after incubation with pentapeptide **18** (10 μM) for 1 h (37 °C, 5% CO_2). Positive controls were incubated with DNase I (10 μg/mL) for 10 min. Slides were prepared for fluorescence microscopy according to the manufacturer's instructions (air-dried cell samples are fixed for 1 h at room temperature; slides were rinsed with PBS and incubated in permeabilization solution for 2 min on ice; cells were washed twice with PBS and incubated with TUNEL reaction mixture for 1 h at 37 °C in a humidified atmosphere in the dark; slides were rinsed 3× with PBS) and then analyzed under an Axioskop2 MOT microscope with a 450−490 nm excitation filter and a 520 nm barrier filter.

Statistics. The original results of MTT tests were subjected to statistical analysis. The Kolmogorov-Smirnov test, a normality distribution test was applied. Differences between groups were assessed by a nonparametric Kruskal−Wallis test ($p < 0.05$). Statistical analyses were performed with Statistica 6 package for Windows.

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Supporting Information Available: Details of amino acid and peptide syntheses with ^{1}H and ^{13}C NMR spectroscopic data and tables listing HPLC retention times, purities, and MS and elemental analysis data; in vitro antitumor activities of selected novel peptides; and cellular uptake and intracellular distribution and detection of

apoptosis for peptide **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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