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ORIGINAL ARTICLE

Computational structure–activity study directs synthesis of novel antitumor enkephalin analogs

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Abstract The capability of a Support Vector Machines QSAR model to predict the antiproliferative ability of small peptides was evaluated by screening a virtual library of enkephalin-like analogs modified by incorporation of the (*R*,*S*)-(1-adamantyl)glycine (Aaa) residue. From an initial set of 390 compounds, the peptides, Tyr-Aaa-Gly-Phe-Met (**2**), Tyr-Aaa-Gly-Phe-Phe (**3**), Phe-Aaa-Gly-Phe-Phe (**4**) and Phe-Aaa-Gly-Phe-Met (**5**) were selected, synthesized and their antitumor activity was tested and compared to that of Met-enkephalin (**1**). The antiproliferative activity correlated with the computational prediction and with the foldamer-forming ability of the studied peptides. The most active compounds were the hydrophobic peptides, Phe-Aaa-Gly-Phe-Phe (**4**) and Phe-Aaa-Gly-Phe-Met (**5**), having a greater propensity to adopt folded structures than the other peptides.

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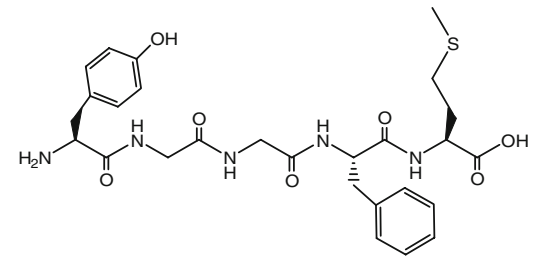
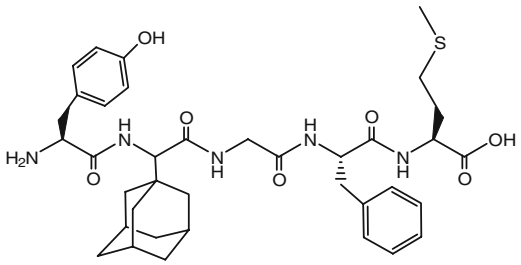
Keywords Enkephalin analogs · (1-Adamantyl)glycine · QSAR study · CD spectroscopy · In vitro antiproliferative activity · Peptide synthesis

Introduction

The native opioid growth factor (OGF), Met-enkephalin (Tyr-Gly-Gly-Phe-Met, **1**), not only plays a role in cell proliferation and tissue organization during development, cellular renewal, wound healing, and angiogenesis, but also in cancer (Zagon et al. 2002; Smith et al. 2004). To obtain peptides with improved or novel activity profiles, modifications using low molecular weight lipophilic adamantane building blocks are reported (Miyazaki et al. 2008; Karle et al. 2008; Basarić et al. 2007; Wanka et al. 2007). The adamantyl groups provide the desired membrane permeability and conformational constraint for efficient transport in lipid membranes. The susceptibility of the modified peptides to enzymatic degradation may also decrease. Recently, we designed and synthesized new analogs of Met-enkephalin (**1**) by substituting either Gly^2 or Gly^2-Gly^3 in **1** and/or in its shorter *N*-terminal fragments by unnatural adamantane-derived amino acids (Horvat et al. 2006). Exclusively, the peptide analogs containing $C^{\alpha\alpha}$-dialkylated glycine or C^{α}-alkylated glycine residues showed in vitro antitumor activity in the cell lines HEp-2, HBL, SW-620 and Caco-2. In particular, the pentapeptide Tyr-(*R,S*)-Aaa-Gly-Phe-Met (**2**) [Aaa = (*R,S*)-(1-adamantyl)glycine] (Horvat et al. 2006) (Fig. 1) showed the most potent tumoricidal activity by inducing apoptosis in the cell lines HEp-2 and SW-620. Since highly enriched diastereomers [(*S*)- and (*R*)-Aaa]-**2** showed lower antiproliferative effect than the racemic one, the obtained data suggested synergism of [(*S*)- and (*R*)-Aaa]-**2** when investigated together as a racemate.



Tyr-Gly-Gly-Phe-Met (1)



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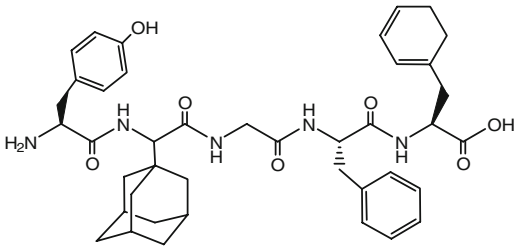
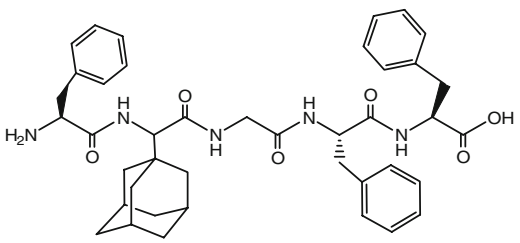
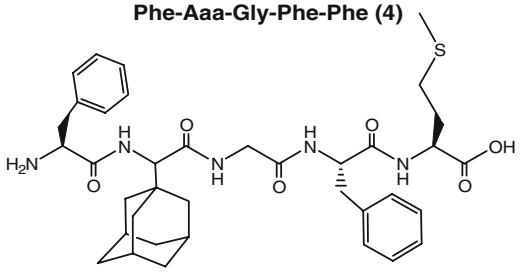


Tyr-Aaa-Gly-Phe-Phe (3)



Phe-Aaa-Gly-Phe-Phe (4)



Phe-Aaa-Gly-Phe-Met (5)

Fig. 1 Structures of pentapeptides **1–5**

It is of relevance to understand how modifications in the Met-enkephalin peptide sequence may lead to better performing drugs and such understanding is possible through mathematical approaches such as QSAR.

In this paper, we have undertaken a computational QSAR study using human tumor cell line cytostatic activity data from 22 previously synthesized and published peptide compounds structurally related to Met-enkephalin containing unnatural amino acids (Horvat et al. 2006). The Support Vector Machines (SVM) algorithm was employed to derive a model for regression of the compound's cytostatic activity against a set of 1,330 molecular descriptors. We used this model to screen a virtual library of 390 enkephalin-like tri-, tetra-, and pentapeptides modified by incorporation of the unnatural amino acid residue, (*R,S*)-(1-adamantyl)glycine (Aaa). Three highly scoring novel compounds were selected for synthesis and testing of cytostatic activity on three tumor cell lines.

Methods and materials

Construction of dataset

Human tumor cell line cytostatic activity data from 22 previously synthesized and published compounds (Horvat et al. 2006) related to Met-enkephalin derivatives were collected. This included measurements of growth inhibition at concentrations of 10^{-6} to 10^{-3} M on the HEp-2, HeLa, HBL, HT-29, SW-620, Caco-2, and MCF-7 cell lines (28 measurements in total, per compound). To obtain an overall estimate of the cytostatic activity of each compound, we used principal component analysis, as implemented in the Weka 3.5.6 package (Witten and Frank 2005). The first principal component (PC1) captured the main source of variation in the cytostatic activity measurements, and using PC1 alone described 44.7% of the variance in the original 28 measurements. PC1 was computed as a sum of weighted standardized cytostatic activity measurements at different concentrations, making it a dimensionless quantity. Here, the HBL and MCF-7 cell lines contributed less to PC1 (data not shown), which was probably not due to a genuine difference in biological response of these two cell lines, but was a consequence of missing data; 8 out of 22 compounds did not have cytostatic activity measurements on HBL and MCF-7. The full dataset, including the compounds' structures, individual cytostatic activity measurements and PC1, and molecular descriptors (see below) is available from the authors' web site at http://anticancer.irb.hr and in the Supplementary material (only structures and activities).

Modeling cytostatic activity from structure

The 22 enkephalin-like compounds were submitted to the E-Dragon web service (Tetko et al. 2005), that predicts a probable three-dimensional structure of the molecules

using CORINA (Sadowski et al. 1994) and then computes 1,666 molecular descriptors per compound. CORINA employs a rule-based system shown to be quite accurate in prediction of 3D structures (Sadowski et al. 1994). After removal of descriptors invariant within our data, 1,330 descriptors remained. The SVM algorithm (Burges 1998) for regression (ε-SVR variety) was applied to this data, as implemented in the LibSVM software (Chang and Lin 2001) adapted for the Weka 3.5.6 data mining environment (El-Manzalawy and Honavar 2005). As per recommendation by the LibSVM authors (Hsu et al. 2008), we utilized a radial basis function kernel, and optimized the SVM's metaparameters "*c*" and "*gamma*" in an exhaustive search for a combination yielding the best root-mean-square error (RMSE) estimated on crossvalidation. *Gamma* was varied exponentially from 2^{-15} to 2^{3} in steps of 2^{1}, and *c* was varied from 2^{-5} to 2^{15} in steps of 2^{1}. Five runs of fourfold crossvalidation were used to estimate the RMSE. The optimal values of the parameters were found to be $gamma = 2^{-8}$ and $c = 2^{6}$. The final estimate of the trained model's performance on unseen data was performed using leave-one-out crossvalidation and by computing the correlation coefficient between the predicted and actual values, which was $q = 0.734$. This indicates that the model has reasonable utility for screening a virtual library of compounds.

Construction and screening of the virtual library

A virtual library of 390 compounds was constructed, encompassing enkephalin-like tri-, tetra-, and pentapeptides modified with the (*R,S*)-(1-adamantyl)glycine (Aaa) residue. Our previous results clearly showed that all of the peptides structurally related to Met-enkephalin containing the Aaa residue exhibited increased cytostatic activity in vitro (Horvat et al. 2006). The E-Dragon web service (Tetko et al. 2005) was used to compute the 1,330 relevant descriptors, and applied the trained SVM model to the virtual library to screen for novel potentially active compounds. We used Marvin 5.1.1 (ChemAxon, http://www.chemaxon.com) to visualize, browse, and otherwise handle the molecules in the virtual library. The structures of the peptides in the virtual library, their molecular descriptors, and the predicted cytostatic activities are available from the authors' web site at http://anticancer.irb.hr and in the Supplementary material (only structures and activities). To verify that our activity predictions are not biased by choice of a specific algorithm for generation of the peptides' 3D conformations (CORINA), we have re-trained the SVM model and screened the virtual library with the 3D structures generated by the FROG server (Bohme Leite et al. 2007) that couples a Monte Carlo sampling procedure of the conformational space to a MMFF force field for scoring the energy of the conformations. After optimization of the SVM metaparameters, we obtain a leave-one-out crossvalidated Pearson's correlation coefficient of $q = 0.741$, comparable to $q = 0.734$ with CORINA. The predictions for the virtual library were generally similar to the original ones with CORINA, Pearson's $r = 0.532$ (Fig. 1 in Supporting material). The differences that do exist are likely attributable to the high conformational flexibility of peptides which may prove challenging to the 3D structure prediction approaches such as CORINA and FROG that were primarily designed for smaller drug-like organic molecules.

CD measurements

Circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter in the 280–185 nm range in a 0.2 mm path length quartz cell at room temperature. The sample concentrations ranged between 0.2 and 0.4 mg/ml. CD spectra were taken in 2,2,2-trifluoroethanol (TFE; Aldrich, NMR grade) and in a mixture of water and TFE in a ratio of 1:1. The following parameters were used: spectral band width 1 nm, response time 0.5 s, data pitch 0.1 nm, scanning speed 50 nm/min, accumulation 5. The CD spectral parameters were expressed as molar CD ($\Delta\varepsilon$).

Proliferation assay

The colon carcinoma (SW-620), breast carcinoma (MCF-7), and cervical carcinoma (HeLa) cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. The growth inhibition activity was assessed as described previously, according to the slightly modified procedure of the National Cancer Institute, Developmental Therapeutics Program (Horvat et al. 2009). Briefly, the cells were inoculated onto standard 96-well microtiter plates on day 0. Test agents were then added in five consecutive tenfold dilutions (10^{-8} to 10^{-4} mol/l) and incubated for a further 72 h. Working dilutions were freshly prepared on the day of testing. After 72 h of incubation, the cell growth rate was evaluated by performing the MTT assay (Horvat et al. 2009) that detects dehydrogenase activity in viable cells. The absorbance (OD, optical density) was measured on a microplate reader at 570 nm. Each test point was performed in quadruplicate in three individual experiments. The results are expressed as IC_{50}, which is the concentration necessary for 50% of inhibition. The IC_{50} values for each compound were calculated from dose–response curves using linear regression analysis by fitting the test concentrations that give PG (percentage of growth) values above and below the

reference value (i.e., 50%). If, however, for a given cell line all of the tested concentrations produced PGs exceeding the respective reference level of effect (e.g., PG value of 50), then the highest tested concentration was assigned as the default measurement value. In the screening data report, the default values are denoted by a ">" sign preceding the number (for details see Supplementary material). Alternatively, if the PGs only slightly exceeded the reference level of effect, the result was extrapolated. Each result is a mean value from three separate experiments.

Synthesis and purification of peptides

Met-enkephalin (**1**) was purchased from Bachem AG (Bubendorf, Switzerland). (*R,S*)-(1-adamantyl)glycine (Aaa), Boc-Tyr(Boc)-Aaa-OH, and Boc-Phe-Aaa-OH were prepared according to the procedure described in the literature (Horvat et al. 2006).

The peptides **2–5** were synthesized manually from *C*- to *N*-terminal by the solid-phase Fmoc method on a commercially available preloaded Fmoc-Met or Fmoc-Phe Wang resin (Bachem, *p*-alkoxybenzyl alcohol resin, 200–400 mesh, 0.8 mmol/g loading) on a 0.05 mmol scale. The consecutive steps in the solid-phase peptide synthesis performed in each cycle were: (a) deprotection of the Fmoc group by two treatments (1 and 30 min) with 20% piperidine in DMF (v/v), (b) coupling by applying HBTU/HOBt/DIPEA activation and a threefold excess of the appropriate Fmoc-amino acid for 30 min or, in the last step, by applying the dipeptide building blocks Boc-Tyr(Boc)-Aaa-OH or Boc-Phe-Aaa-OH (prepared by solution methods) for 3 h, (c) removal of the peptides from the resin by treatment with a mixture of TFA:TIS:H_2O in the ratio 9.5:0.25:0.25 (v/v/v) for 1 h. Successive deprotection and coupling steps were monitored by positive and negative Kaiser (ninhydrin) test, respectively. The peptides were obtained as a filtrate in TFA and precipitated with cold dry diisopropyl ether. Analysis and purification of crude peptides were achieved by RP HPLC performed on a Varian Pro Star 230 HPLC system using a Eurospher 100 reversed-phase C-18 semipreparative (250 × 8 mm ID, 5 µm) (flow rate 1.0 ml/min) or analytical (150 × 4.5 mm ID, 5 µm) (flow rate 0.5 ml/min) column under isocratic conditions using 57% MeOH/0.1% TFA for peptides **2** and **3** or 61% MeOH/0.1% TFA for peptides **4** and **5**. UV detection was performed at 254 and 215 nm using a Varian Pro Star 335 photodiode-array detector. The trifluoroacetate ion present after preparative HPLC was removed using an SPE cartridge. The cartridge was first eluted with water and then with MeOH to recover the peptide compounds. The effluent was evaporated and the residue dissolved in water and lyophilized. Peptides **2–5** were at least 95% pure as assessed by analytical RP HPLC. The spectroscopic data of compounds **2–5** are provided in the Supplementary material.

Results and discussion

We gathered human tumor cell line cytostatic activity data from 22 previously synthesized and published compounds (Horvat et al. 2006) related to Met-enkephalin derivatives, and used the data to train a regression model that would predict antitumor activity from a large number of molecular descriptors.

The method employed for this QSAR effort was the SVM regression. SVMs have desirable properties of robustness to noise and high generalization power in difficult situations with few training examples, and have consequently become widely popular in other sciences, such as computational biology (Noble 2004). Examples of previous applications of SVMs in QSAR studies include a classification problem involving dihydrofolate reductase inhibition by pyrimidines (Burbidge et al. 2001) and large-scale virtual screening of molecular databases for inhibitors of multiple enzymes (Jorissen and Gilson 2005); for a review, see Ivanciuc (2007).

The SVM regression model trained on our 22-compound dataset was estimated to predict activity of unknown molecules with reasonable accuracy; Pearson's correlation coefficient on leave-one-out crossvalidation was $q = 0.734$. Finally, we applied the regression model to a virtual library of 390 enkephalin-like tri-, tetra-, and pentapeptides modified by incorporation of (*R,S*)-(1-adamantyl)glycine (Aaa) residues. The top ten peptides ranked by their predicted activity (dimensionless, see "Methods and materials" for details), and their predicted log *P* (hydrophobicity) and log *S* (aqueous solubility) values are given in Table 1.

Peptides **2–5** (listed in Table 1) were synthesized using a commercial Wang resin already anchored with either the Met or the Phe residues, by the Fmoc technique using piperidine deprotection and HBTU/HOBt/DIPEA as coupling reagents. After removal from the resin with the usual TFA/TIS/water cleavage procedure, the products were purified by semipreparative RP HPLC. The prepared peptides were very sparingly soluble in water.

All the newly synthesized compounds afforded appropriate mass spectrometry data. The NMR spectra were consistent with the assigned structures and are provided in the Supplementary material.

The biological activities of peptides **2–5** (as hydrochloride salts), and Met-enkephalin (OGF, **1**) as a reference compound, were evaluated by determining their capacity to inhibit the proliferation of three tumor cell lines. In our

Table 1 Top-rated peptides as obtained by computational cytostatic activity prediction, chosen from a virtual library of 390 enkephalin-like peptides containing the (*R,S*)-(1-adamantyl)glycine (Aaa) residue

Peptide	Predicted activity[a]	log P[b]	log S[b]
Tyr-Aaa-Gly-Phe-Met (**2**)[c]	−5.77	0.65	−5.36
Phe-Aaa-Gly-Phe-Met (**5**)	−5.37	0.91	−5.62
Phe-Aaa-Gly-Phe-Phe (**4**)	−5.07	1.32	−5.83
Tyr-Aaa-Gly-Phe-Phe (**3**)	−4.20	1.10	−5.65
Trp-Aaa-Gly-Phe-Met	−4.18	1.14	−5.79
Tyr-Aaa-Gly-Phe-Gly	−3.53	0.40	−5.02
Trp-Aaa-Gly-Phe-Phe	−3.50	1.58	−5.90
Phe-Aaa-Gly	−3.28	0.29	−4.68
Phe-Gly-Gly-Phe-Phe	−3.27	0.01	−5.23
Phe-Gly-Aaa-Gly	−3.25	−0.12	−4.47

[a] The predicted activity is dimensionless; more negative values signify stronger cytostatic activity

[b] log P (hydrophobicity) and log S (aqueous solubility) values are given as predicted by the E-Dragon web service (Tetko et al. 2005)

[c] Horvat et al. (2006)

previous paper (Horvat et al. 2006), we demonstrated that the pentapeptide, Tyr-Aaa-Gly-Phe-Met (**2**), showed the most potent antiproliferative activity, therefore we checked its activity in three cell lines (SW-620, HeLa, and MCF-7), along with the activity of three novel analogs of peptide **2**, having different amino acids at position 1 (Phe-Aaa-Gly-Phe-Met, **5**), 1 and 5 (Phe-Aaa-Gly-Phe-Phe, **4**), or at position 5 (Tyr-Aaa-Gly-Phe-Phe, **3**) (Table 2; Fig. 2). The reference compound, OGF (Met-enkephalin, **1**) did not exert any inhibitory activity at all in the tested concentration range, which is in accordance with the previously published results (Horvat et al. 2006; Horvat et al. 2009). In this study, peptide **2** showed slightly more pronounced activity (probably due to its better solubility) than previously reported. However, compounds **4** and **5** inhibited the growth of all cell lines at slightly lower concentrations on

Table 2 In vitro inhibition of the growth of tumor cells treated with Met-enkephalin (OGF, **1**) and Aaa-containing peptides **2-5**

Peptide	IC_{50} (μM)[a]		
	SW-620	MCF-7	HeLa
Tyr-Gly-Gly-Phe-Met (**1**)	>100	>100	>100
Tyr-Aaa-Gly-Phe-Met (**2**)	270 ± 90[b]	55 ± 33	≥100
Tyr-Aaa-Gly-Phe-Phe (**3**)	73 ± 25	>100	>100
Phe-Aaa-Gly-Phe-Phe (**4**)	47 ± 39	69 ± 33	59 ± 38
Phe-Aaa-Gly-Phe-Met (**5**)	60 ± 32	64 ± 36	94 ± 69

[a] IC_{50}, the concentration that causes a 50% reduction of cell proliferation

[b] The result is extrapolated

average, indicating somewhat better activity. This can be seen from the concentration–response graphs, showing that up to 20% of inhibition was obtained even at 10 μM concentration of **5**. Contrary to this, compound **3** was less active, pointing to the influence of a change in hydrophobicity of the amino acid at position 1. Interestingly, the SW-620 cell line was the most sensitive cell line, as it was the case in our previous study (Horvat et al. 2006).

It was already established that replacement of Tyr residue at position 1 with a Phe residue, in an attempt to increase the hydrophobicity of a series of pentapeptides, resulted in a significant increase in the Caco-2 cell permeability of these peptides (Knipp et al. 1997). Moreover, the same authors found that other amino acid alterations that induce a β-turn spatial conformation have an effect on the peptides' physico-chemical characteristics, primarily hydrophobicity, and consequently increase the membrane permeability of the peptides.

Therefore, to gain further understanding of structure–activity relationship for peptides **1–5**, their CD curves were measured. The CD spectra of peptides **1–5** in TFE and TFE-water (1:1) mixtures are presented in Fig. 3 (for CD spectral parameters see Table 3 in the Supplementary material). The CD spectra in TFE (Fig. 3a) of Met-enkephalin (**1**) and its derivatives containing (*R,S*)-(1-adamantyl)glycine (Aaa) in position two are determined by the backbone conformation and the aromatic chromophores of the amino acids, Tyr and/or Phe. The CD spectrum of Met-enkephalin (**1**) in TFE indicated the presence of an ensemble of conformers rather than one or two prevailing ones, similarly to the structurally related Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) discussed in one of our earlier papers (Vass et al. 2008). The characteristic features of the spectra of peptides **1–3** with Tyr in position one were a positive ^1L_a band between 227 and 223 nm, and a positive ^1B_b band below 195 nm. Peptides **4** and **5** did not contain Tyr, in their spectra the ^1L_a band was blue-shifted to ~218 nm, while the ^1B_b band was red-shifted and appeared with decreased intensity.

The negative band below 205 nm in the spectra of peptides **2**, **4**, and **5** reflects the presence of more ordered structures and the spectral effect of the interaction between the aromatic side chains and the steric constrain of (*R,S*)-Aaa in position two. However, the type of folded structure is difficult to predict because of the dominance of the CD contribution of the aromatic chromophores.

The CD spectra were also measured in TFE-water 1:1 mixture (Fig. 3b). The spectra of **2**, **4**, and **5** did not show the negative band below 205 nm as a sign of decreased population of folded conformers.

By monitoring the spectral behavior of the studied compounds, it can be suggested that **2, 4, 5** have similar populations of conformers which are more fixed through

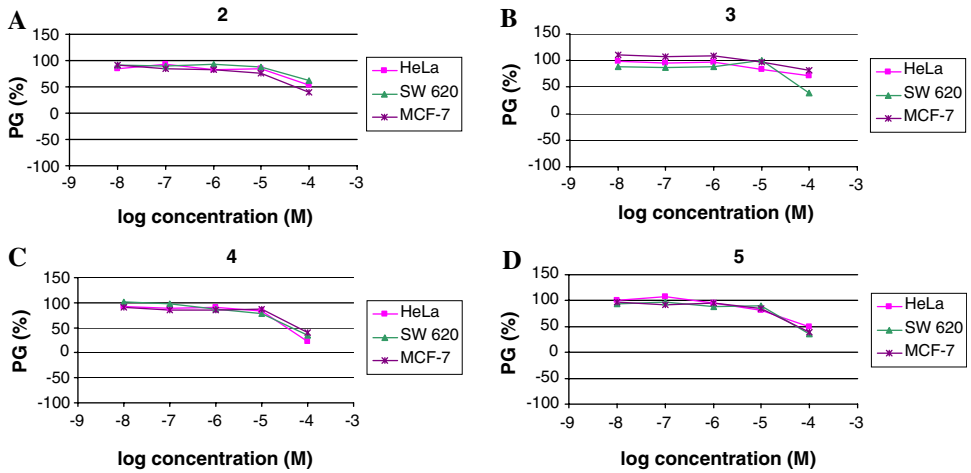


Fig. 2 Dose–response profiles for peptides **2**–**5**. *PG* percentage of growth

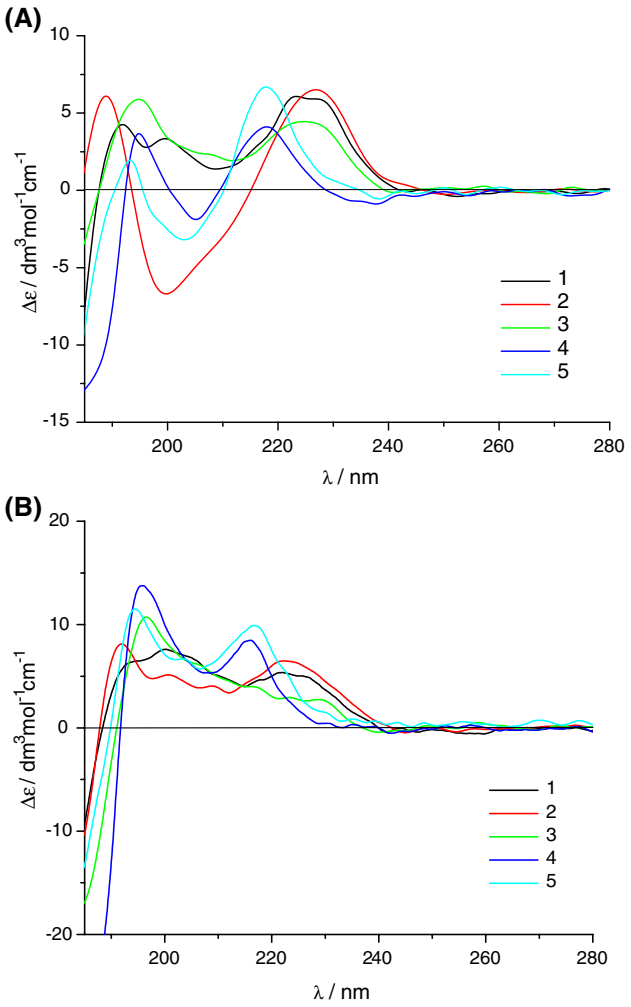


Fig. 3 The far-UV region circular dichroism spectra of peptides **1**–**5** in TFE (**a**) and in a TFE-water (1:1) mixture (**b**). The spectra were obtained at room temperature and expressed as molar CD ($\Delta\varepsilon$/ dm^3 mol^{-1} cm^{-1}) from 185 to 280 nm

the possible interactions (salt bridge and/or aromatic side chain interaction modified by the steric hindrance of the adamantyl group) than the conformations of peptides **1**, **3**. It should be recognized that adamantyl amino acids are γ-turn inducers for peptides (Kuroda et al. 1997). In addition, it was also demonstrated by theoretical (Bisetty et al. 2006) and experimental studies (Albericio et al. 2008), that some other non-natural cage amino acids, containing rigid trishomocubane moiety, were strong β-turn inducers in medium-sized peptides.

Apparently, the results of antiproliferative activity are in accordance with our conformation study using CD, whereby the spectrum of Met-enkephalin (**1**) in TFE differed significantly from those of its adamantylated derivative **2** indicating a higher population of ordered (likely folded) conformers than of the extended structure, while **1** showed a CD spectrum similar in shape to that of Phe^5-analog **3**. On the other hand, the CD spectra of compounds **4** and **5** were similar, also showing spectral features compatible with the presence of folded structures. Accordingly, the peptides with higher population of folded conformers exerted better biological activity.

Conclusion

To conclude, a computational QSAR model was used to predict the antiproliferative ability of small peptides by screening a virtual library of enkephalin-like analogs modified by incorporation of the (*R,S*)-(1-adamantyl)glycine (Aaa) residue. From an initial set of 390 compounds, peptides **2**–**5** were selected, synthesized and screened for cytostatic activity. The results clearly revealed antiproliferative activity of compounds **2**–**5** on three tumor cell lines. The most active compounds were the hydrophobic peptides, Phe-Aaa-Gly-Phe-Phe (**4**) and Phe-Aaa-Gly-Phe-Met (**5**). The antiproliferative activity can be correlated with the lipophilicity and foldamer-forming ability of the studied peptides, as determined by CD spectroscopy. The results suggest that the applied SVM model can be used to screen for novel potentially active compounds with reasonable

confidence. In addition, the results confirm the previous observation (Knipp et al. 1997) that the solution conformation has an effect on the physico-chemical characteristics and consequently, the biological activity of the peptides. We could speculate that the membrane permeability of the compounds is relevant for the magnitude of their cytostatic effect on tumor cell lines.

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