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Synthesis, analysis and biological evaluation of new RGD mimetics

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The amino acid sequence L-arginyl-glycyl-L-aspartic acid (RGD) is a part of many extracellular proteins. It is a specific recognition site by integrins. Some synthetic RGD analogues bind specifically with integrin receptors on the cell membrane, which are over-expressed on the surface of various malignant human tumour and angiogenic endothelial cells. These peptides exert dual role by: inhibiting proliferation and migration of tumour cells and on the other hand by inhibiting angiogenesis. In recent years, many RGD cytotoxic agents have been developed, that showed promising results *in vitro* and *in vivo*. Herein we present the synthesis, analysis and biological evaluation of two new RGD analogues, modified in position 1 with Arg mimetics (Agb or Agp). Our pilot studies on their cytotoxicity were presented in comparison to parent RGD as standard.

Key words: RGD; biologically active peptides; cytotoxicity

INTRODUCTION

One of the major problems in cancer chemotherapy is poor selectivity of anticancer agents to cancer versus normal cells. Although cancer cells share many common characteristics with normal cells, certain receptors are over-expressed on their surface. Among receptors over-expressed on tumour cells, integrins are particularly attractive pharmacological targets. These heterodimeric transmembrane cell adhesion glycoproteins have a fundamental role in increasing migration, invasion, proliferation and survival of tumour cells. In addition, integrins have been linked to tumour angiogenesis, which is an essential process for tumour growth and metastasis [1].

The discovery of the minimal peptide sequence RGD, which plays a prominent role in cell adhesion via integrin interaction, has led to a large increase in biomedical and biomaterials research on this motif. Various RGD-containing peptides have been increasingly developed for adapting to versatile applications including tumour imaging and therapy, drug delivery vector, targeted gene transfer, and biomaterial or tissue engineering [2, 3].

Although RGD analogues have been approved for clinical use, their application is still ineffective because of their low bioavailability. This is largely due to the metabolic instability of this class of compounds in the presence of proteases and peptidases.

We gave our contribution in the field by developing several RGD peptide analogues with enhanced cytotoxic activity [4].

In the present report we describe the synthesis, analysis and biological evaluation of novel RGD mimetics, in which the arginine residue was replaced with Agb and Agp (Fig. 1), two of its structural analogues.

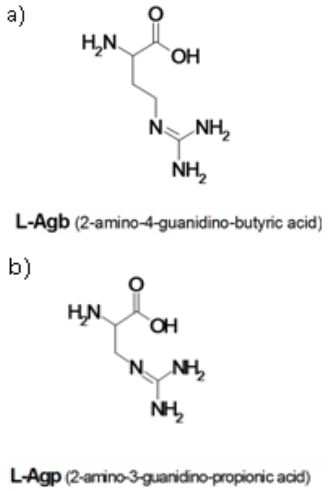

Fig 1. Arginine mimetics: a) L-Agb (2-amino-4-guanidino butyric acid); b) L-Agp (2-amino-3-guanidine-pronionic acid)

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EXPERIMENTAL

Peptide synthesis and analysis

Resins and Fmoc-amino acids used in peptide synthesis were purchased from Merck (Darmstadt, Germany) and Iris Biotech GMBH (Germany). Solvents of dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Merck (Darmstadt, Germany). Electrophoretic experiments were performed using a BeckmanP/ACE (Beckman Coulter Inc., Pasadena, CA, USA).

Synthesis of all peptides was performed by the conventional and manual stepwise Fmoc solid-phase synthesis on 2-chlorotrityl chloride resin with substitution, 1.4 mmol/g. The coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, 3 mol excess of N-hydroxybenzotriazole (HOBt), 3 mol excess of diisopropylcarbodiimide (DIC), and 5 mol excess of diisopropylamine (DIPEA) in dimethylformamide (DMF). Completion of coupling reactions were monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine in DMF.

The peptides were cleaved from the resin and the final deprotection was done in a cocktail containing trifluoroacetic acid (TFA), triisopropylsilane (TIPS), thioanisole, and water (92.5: 2.5: 2.5: 2.5). The crude peptides were precipitated into cold petroleum ether/diisopropyl ether (50:50). Then, the precipitate was dissolved in 10% CH_3COOH and desalted by gel filtration on a Sephadex G25. The chemical purity of peptides was characterized by RP-HPLC and capillary electrophoresis**.**

Cell cultures

The HepG2 cells (human liver hepatocellular carcinoma cell line) were cultured in Dulbecco Modified Eagle's medium (DMEM) (Gibco, Austria) supplemented with 10% fetal bovine serum (Gibco, Austria), 100 U/ml penicillin (Lonza, Belgium) and 0.1 mg/ml streptomycin (Lonza, Belgium) under a humidified 5% CO_2 atmosphere at 37°C. Plastic flasks supplied by Greiner, Germany, were used to grow the cells. Cells were trypsinized using Trypsin-EDTA (FlowLab, Australia) when they reached approximately 80% confluence. For experiments the cells in exponential phase of growth after treatment with Trypsin-EDTA were seeded into 96-well plates (Greiner, Germany) in a concentration $1.5x10^5$ cells/ml. 24 hours incubation post seeding (under a humidified 5% CO_2 atmosphere at 37°C) allowed the cells to attach to the wells.

Cytotoxicity assay – MTT test

The cultivated cells were treated with RGD and RGD-analogues (AgbGD and AgpGD) for cytotoxic effect in a wide concentration range (2 - 0.0039 mM). Untreated cells were used as controls. Empty wells were blank controls. Cytotoxicity was measured by colorimetric assay based on tetrasolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Chemical Co.).

The MTT assay is based on the protocol first described by Mossman [5]. In this assay, living cells reduce the yellow MTT to insoluble purple formazan crystals. The peptides were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in samples did not affect the viability of the cells. The assay was performed on HepG2 cell line.

The assay was performed 72 hours after treatment with the amino acid analogues. For this purpose, MTT solution was prepared at 5 mg/ml in PBS and was filtered through a 0.2 μm filter. Then 1 ml of MTT solution was added to 15 ml DMEM and 100 μl of this solution were added into each well, including the cell free blank wells. Then the plates were further incubated for 3 hours to allow MTT to be metabolized. After lysis buffer (DMSO: ethanol (1:1)) was added, optical density (OD) was determined at a wavelength of 540 nm and a reference wavelength of 620 nm by ELIZA plate reader (TECAN, Sunrise TM, Grodig/Sazburg, Austria).

Cell cytotoxicity determined by MTT assay was expressed as per cent of dead cells:

% cytotoxicity = (1 – (OD sample – OD blank control) / (OD control – OD blank control)) x 100.

RESULTS AND DISCUSSION

In our lab a series of arginine analogues have been designed and synthesized and their cytotoxic potential has been studied. Guanidinium group of Arg is crucial for various bioactivities as the regulation of structure and function of proteins. There is evidence that if arginine is replaced with a homologue comprising one or two methylene groups less (Agb or Agp) (Fig. 1), there is an improvement in stability of proteins to enzymatic degradation [6].

As mentioned above we obtained two new RGD mimetics that contain the sequence Xaa-GD (Fig. 2), where Xaa is structural analogue of Arg with

shortened side chain: L-Agb (2-amino-4-guanidino butyric acid) and L-Agp (2-amino-3-guanidine-pronionic acid).

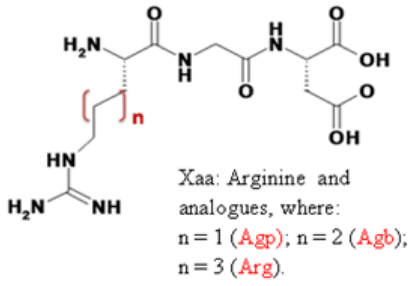

Fig. 2 Arginine and analogues.

A target peptide is assembled on a 2-chlorotrityl chloride resin by standard Fmoc-SPPS. The N-terminal amino group of the peptides was protected with a Fmoc group, and the side guanidine groups of arginine mimetics with Boc group. After final deprotection from the resin the peptides were purified with simple gel filtration on a Sephadex G25. The obtained purity characterized by RP-HPLC and capillary electrophoresis, was 87-94% (Fig. 3).

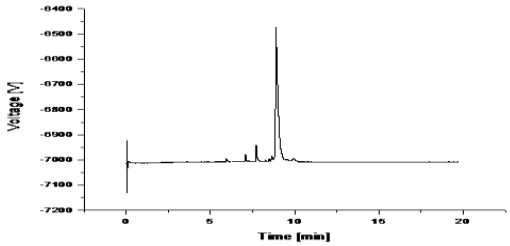

Fig 3. Electrophoregramme of AgbGD, running buffer: 20 mMTris, 5mM H_3PO_4, 50 mM SDS, pH 7; Effective capillary length: 290/400 mm

Next we investigated the cytotoxic activities of RGD and its newly synthesized analogues on HepG2 tumour cell line using MTT analysis. All peptides were tested for cytotoxic effect in a wide concentration range (2 mM - 0.0039 mM) (Fig 4).

The modification of Arg by shortening of its side chain (in the case of Agb or Agp) didn't show significant increase of the cytotoxic effect of the compounds in comparison to parent RGD molecule.

We also compared the cytotoxic activity of these analogues with RGD-mimetics modified at the C-terminus, previously synthesized and reported by us [7]. It was shown that our new peptides have weaker cytotoxic effect in comparison to the methyl ester (RGD-OMe). The cytotoxic activity of some of the synthesized peptides are demonstrated by half maximal inhibitory concentrations (IC50 values), shown in Table 1.

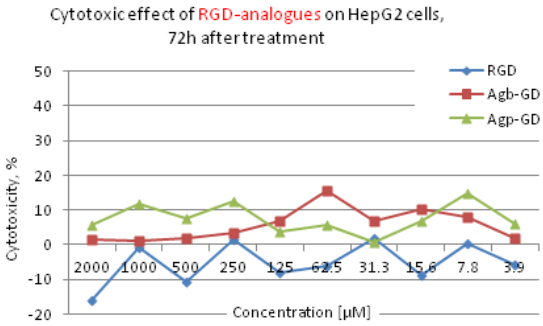

Fig. 4. Cytotoxic effect of novel RGD-analogues on HepG2 cells after 72 h treatment in concentrations from 2 mM to 0.0039 mM.

Table 1. Comparative table of cytotoxic effect of the RGD and its analogues (RGD-OMe, Agb-GD and Agp-GD), in 3T3 and HepG2 cells after 72 h of treatment [7].

Mean IC50 values (mM), after 72h		
Compounds	Cell lines	
	3T3	HepG2
RGD*	>2mM	>2mM
RGD-OMe*	0.758±0.0504 mM	0.524±0.0766 mM
Agb-GD	_	>2mM
Agp-GD	_	>2mM

In conclusion, the modification in the carboxylic group of RGD peptide even with simple esterification (RGD-OMe) leads to the highest cytotoxic effect on HepG2 cells [7] in comparison with parent RGD molecule and newly synthesized Agb-GD and Agp-GD analogues.

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СИНТЕЗ, АНАЛИЗ И БИОЛОГИЧНА ОЦЕНКА НА НОВИ RGD МИМЕТИЦИ

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(Резюме)

Аминокиселинната последователност L-аргинил-глицил-L-аспаргинова киселина (RGD) е част от много протеини от екстрацелуларния матрикс и е специфично място за разпознаване от интегрини. Някои синтетични RGD аналози се свързват специфично с интегринови рецептори върху клетъчната мембрана, които са свръхекспресирани при различни злокачествени човешки туморни и ангиогенни ендотелни клетки. Тези пептиди упражняват двойна роля чрез инхибиране на пролиферацията и миграцията на туморни клетки, и от друга страна инхибиране на ангиогенезата. През последните години са разработени много RGD цитотоксични агенти, които показват обещаващи резултати *ин витро* и *ин виво*. В настоящия доклад ние представяме синтез, анализ и първоначална биологична оценка на два нови RGD аналога, модифицирани в 1-ва позиция с Arg миметици (Agb или Agp). Тяхната цитотоксичност е сравнена с тази на изходния RGD пептид, използван като стандарт.