

Synthesis and study of cytotoxic effect of novel AVPI-RGD hybrid peptides

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Received September 30, 2016 ; Revised February 18, 2017

Targeting critical apoptosis regulators is a promising strategy for development of new classes of anticancer drugs. Herein, we focused on synthesis and study of novel bi-functional AVPI-RGD hybrid peptides. Despite of, being functionally different motifs of separate proteins, AVPI and RGD peptides are still both known for their pro-apoptotic potential and therefore interesting objects of pharmacology design. Herein, we report for hybrid molecules and their monomeric subunits. Fmoc solid phase peptide strategy (SPPS) was preferred as synthesis method, whereas proline and arginine residues were subjected to modifications. Cytotoxic potential of molecules was examined by initial screening over two cell lines – HepG2 and MDA-MB-231 cells – by MTT colorimetric assay. It was found that almost all tested compounds had weak or none cytotoxic effect when they were used as single agents. However, we showed that AVPI-RGD hybrids exert comparatively higher cytotoxic effect than individual AVPI and AVHyPI peptides .

Ke ywords: AVPI, RGD, apoptosis, SPPS

INTRODUCTION

Resistance to apoptosis is an important hallmark of cancer cells and partly reason for their resistance to conventional anti-cancer treatment. Vast number of IAP-inhibitors, Smac-mimetics, AVPI-, and RGD- mimetics, have been synthesized over the past decade, as new promising agents for overwhelming higher apoptotic thresholds of cancer cells, as some of them have entered clinical trials [1].

Inhibitors of apoptosis proteins (IAP) are important regulators of processes - cell death and survival. At this time, 8 different IAP proteins are known in mammals. Three of them (cIAP-1, cIAP-2, XIAP) are well recognized as regulators of apoptosis. These functions they perform either by directly inhibiting caspases (XIAP) or by interfering formation of death-receptor complexes (cIAP1, cIAP2).

Distinctive for these IAPs is the presence of N-terminal BIR (baculoviral IAP repeat) domains and C-terminal RING domain - essential for their anti-apoptotic effects. While BIR domains mediate the interaction with caspases, RING domain exhibits ubiquitin-E3 ligase activity so IAPs can promote their own as well as proteasome degradation of binded-partner molecules.

Function of cIAP-1/2 is mainly dependent on their RING domains. On the other hand, XIAP is known to bind directly and inhibiting initiator

(caspase-9) and effector (caspases-3 and -7) caspases via its BIR3 and BIR2-linker regions, respectively [2].

AVPI (Ala-Val-Pro-Ile) is a tetrapeptide sequence of the N-terminus of the mature pro-apoptotic Smac protein. AVPI itself is the major IAP-binding motif (IBM) in mammals, and fruit flies. Via its AVPI motif Smac directly interacts with BIR2 and BIR3 domains of IAPs, releasing inhibitory effect of XIAP and stimulating c-IAPs autoubiquitination and proteasomal degradation [3, 4], that ultimately re-activates apoptosis.

Smac is localized in mitochondrial intermembrane space, but it is released into the cytoplasm upon apoptotic stimuli. Several studies established inverse correlation between Smac expression levels and cancer progression so prompted the development of Smac- and/ or AVPI-mimetics as therapeutic agents [5, 6]. Some of them are reported to be efficient in the induction of apoptosis in tumorigenic cells as single agents, others - in combination with different therapeutic agents (cisplatin, doxorubicin, etoposide, TRAIL, etc) [1, 7 – 9].

The RGD (L-arginyl-glycyl-L-aspartic acid) peptide sequence is found in many proteins of extracellular matrix, as well as in intracellular proteins such as caspases. RGD is also known to interact with specific over-expressed proteins on the membrane of cancer cells (example - $\alpha\beta3$, $\alpha\beta5$ integrins). That sets RGD-peptide motif as an advantageous tumor-targeting ‘device’ for selective inhibition and elimination of cancer cells that is still

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an overwhelming problem for most of the drugs [10].

Regarding that synthesis of hybrid-peptides, combining two or more pharmacological effects is an advantageous pharmacological approach, we focused on synthesis of new AVPI-RGD hybrid molecules and examination of their cytotoxic potential.

EXPERIMENTAL

Peptide design, synthesis and analysis

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% CH₃COOH and desalted by gel filtration on a Sephadex G25.

Peptides' purity was characterized by RP-HPLC and capillary electrophoresis.

Cell cultures

Both cell lines (HepG2 and MDA-MB-231) 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% CO₂ atmosphere at 37°C. Cells were trypsinized using Trypsin-EDTA (FlowLab, Australia) when they reached approximately 80% confluence. The cells in the exponential phase of growth after treatment with Trypsin-EDTA were seeded into 96-well plates (Greiner, Germany) in a concentration of 1x10⁴ cells/well for further MTT assay.

Cytotoxicity assay - MTT test

Cell cytotoxicity was determined 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 [11]. In this assay, living cells reduce the yellow MTT to insoluble purple formazan crystals, dissolved later in lysis buffer (1:1, ethanol : DMSO).

Cell suspension (100 µl) was added to each well of 96-well plates except for blank control wells. Cells were treated 24 h later with newly synthesized peptides in a wide concentration range (2 mM - 0.004 mM), and incubated for further 72 h. Then MTT was added followed by 3-hour incubation. MTT absorbance was read by ELIZA plate reader (TECAN, Sunrise TM, Grodig/Sazburg, Austria) at a wavelength of 540 nm and a reference wavelength of 620 nm. Cell cytotoxicity determined by MTT assay was expressed as the percentage of dead cells:

$$\% \text{ cytotoxicity} = (1 - (\text{OD sample} - \text{OD blank control}) / (\text{OD control} - \text{OD blank control})) \times 100.$$

RESULTS AND DISCUSSION

We focused our work on synthesis and study of the cytotoxic effect of bifunctional AVPI-RGD hybrid peptides over two cell lines.

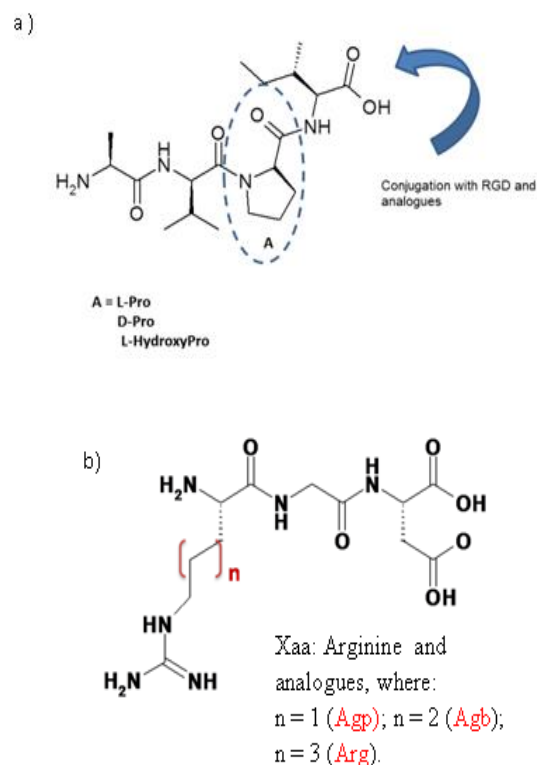


Fig. 1. Functional subunits: a) AVPI analogues; b) Tripeptide XaaGD and analogues

Regarding the data about Smac- and AVPI-mimetics and pharmacophore regions of AVPI, we decided to modify the proline at position 3 in AVPI

(Fig. 1a). We synthesized original AVPI peptide with L-Pro, and next Hydroxy-Pro (HyP) instead of proline itself. Next we prepared two new RGD analogues, containing an Arg-mimetic (Agb or Agp) with shortened side chain (with one or two –CH₂– groups respectively), in order to improve the stability and cytotoxicity of RGD molecule (Fig. 1b). Next corresponding AVPI-RGD hybrids were made. All peptides were synthesized by stepwise Fmoc solid-phase peptide strategy.

Analysis

The purity of the crude peptides characterized by RP-HPLC and capillary electrophoresis, was 87 – 97% (Fig. 2). All synthesized peptides were found to be stable in aqueous solution even after 72 hrs period and different values of pH, available physiologically.

Cytotoxicity

Next we performed initial screening for cytotoxic potential of peptides by colorimetric MTT analysis. Peptides were tested in a wide concentration range (2mM - 0.004 mM). The assay was performed on two cell lines - HepG2 cells (hepatocellular carcinoma cells) and MDA-MB-231 cells (breast cancer cells) recommended by literature data.

It was found that almost all tested compounds had weak or none cytotoxic effect when they were used as single agents (Fig. 3). Still that is in concordance with the literature data describing most of the AVPI- and Smac-mimetics as agents sensitizing cells to chemotherapeutics. Regarding that we are going to test our peptides with agents initiating apoptotic pathways, particularly TRAIL (cytotoxic death ligand) and cisplatin (triggering intrinsic pathway).

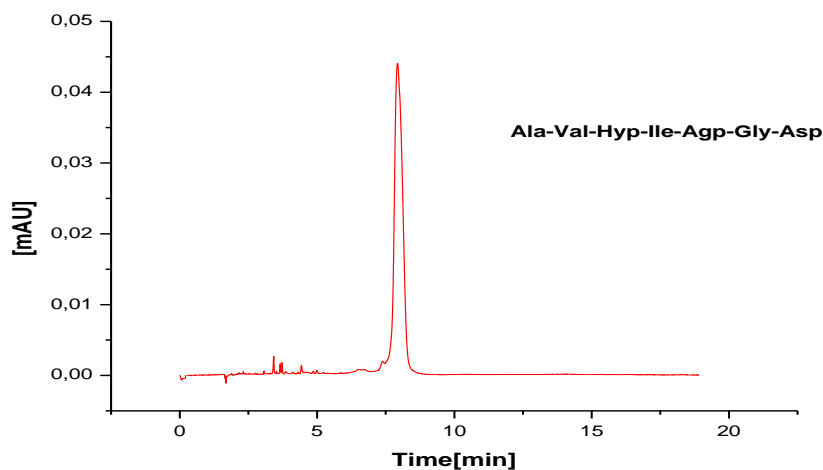


Fig. 2. Electrophoregramme of Ala-Val-HyP-Ile-Agp-Gly-Asp.

Conditions: Capillary: fused silica, 50/375 μ m, 30,4/40,6; BGE: 20 mM Tris, 5 mM H₃PO₄, 50 mM SDS; pH 8,6; U = 15 kV, I = 38 μ A; T = 23° C; 6,9 mbar, 10 s; UV 200 nm.

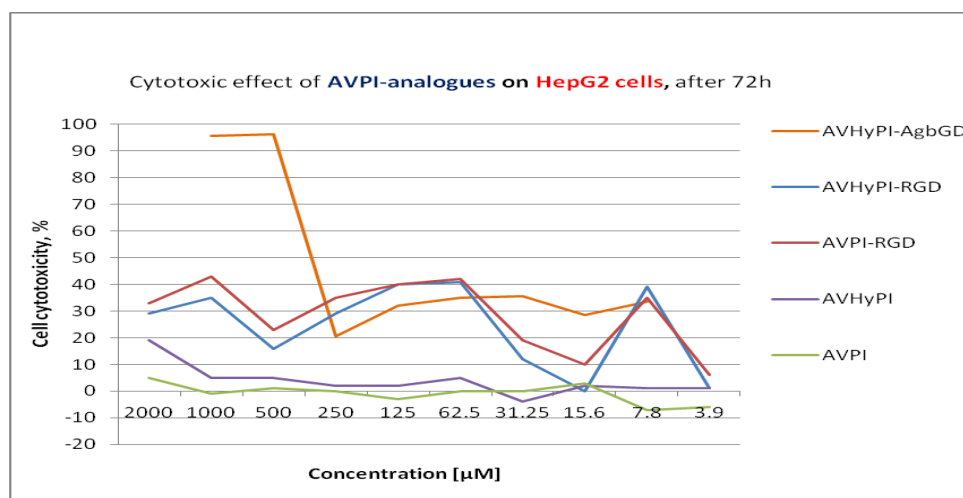


Fig. 3. Comparison of cytotoxic effect of AVPI-RGD-hybrids and AVPI / AVHyPI structural subunits on HepG2 cells after 72 h treatment (MTT-dye reduction assay).

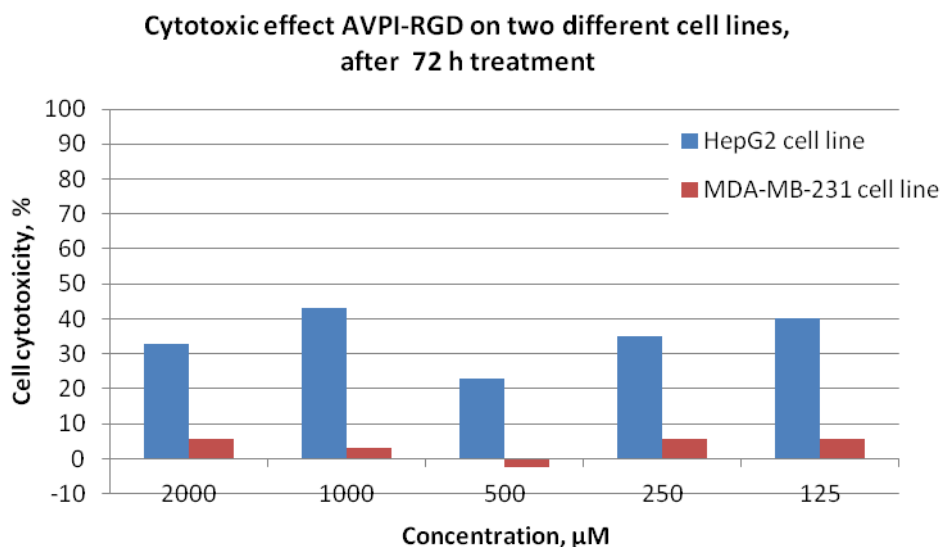


Fig. 4. Comparative graph of cytotoxic effect AVPI-RGD-hybrid over two different cell lines (HepG2 and MDA-MB-231) after 72 h treatment.

Nevertheless, we showed that AVPI-RGD hybrids exert comparatively higher cytotoxic effect than individual subunits - AVPI, AVHyPI (Fig. 3). One of the hybrids - Ala-Val-HyP-Ile-Agb-Gly-Asp (AVHyPI-AgbGD), double modified, showed higher activity, so we may speculate it is caused by the substitution of Arg amino acid in RGD with its analogue Agb.

Besides that, cytotoxic activity of peptides over both cell lines was compared, as it was shown slightly higher effect of AVPI-RGD on HepG2 cells in comparison to MDA-MB-231 cells (Fig. 4).

That pilot experiments directed us to further examination of peptides over the same and additional cell lines, in search for repeatability and selective effect over different cells. We need further combination of these peptides with other cytotoxic agents in search of sensitizing effect.

Acknowledgements: The work was supported by project no. 34 of the cooperation between ASCR and BAS in the period 2014-2016.

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СИНТЕЗ И АНАЛИЗ НА ЦИТОТОКСИЧНИЯ ПОТЕНЦИАЛ НА НОВИ AVPI-RGD ХИБРИДНИ ПЕПТИДИ

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Постъпила на 30 септември, 2016 г.; Коригирана на 18 февруари, 2017 г.

(Резюме)

Разработването на агенти, повлияващи селективно критични апоптотични регулатори в раковите клетки, е обещаваща стратегия за развитие на нови по-селективни и ефективни класове противоракови лекарства. В настоящия доклад ние се фокусирахме върху синтез и изследване на цитотоксичния потенциал на нови AVPI-RGD хибридни пептиди. AVPI- и RGD- пептидните последователности са познати със своя про-апоптотичен потенциал. Това ги превърна в интересен обект на фармакологичен дизайн през последното десетилетие. Чрез Fmoc твърдофазен пептиден подход (SPPS) ние синтезирахме AVPI-, RGD-аналози, както и съответните им конюгати. Химични модификации бяха направени в аминокиселинните остатъци на пролин и аргинин. Цитотоксичният потенциал на пептидите беше изследван върху две различни ракови клетъчни линии (HerG2, MDA-MB-231) чрез МТТ тест. При първоначалните изследвания, беше установено, че пептидите, прилагани самостоятелно, имат слаб или нямат цитотоксичен ефект. Въпреки това, показахме, че конюгатите имат висока активност в сравнение с пептидите AVPI и AVHyPI.