



Interferon alpha and rapamycin inhibit the growth of pheochromocytoma PC12 line *in vitro*

Interferon alfa i rapamycyna hamują wzrost linii komórkowej pheochromocytoma PC12 w warunkach *in vitro*

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Abstract

Introduction: Pheochromocytomas are benign or malignant neuroendocrine tumours. The unsatisfactory efficacy of the traditional therapeutic methods for patients with metastatic disease results in a continuing search for more effective and targeted agents. Due to the increased vascularisation of these tumours, inhibitors of angiogenesis could be potentially a new group of drugs in pheochromocytoma/paraganglioma therapy.

Material and methods: The aim of this study was to evaluate the influence of angiomodulators: VEGF (vascular endothelial growth factor) and five endogenous and exogenous antiangiogenic compounds (endostatin; IFN- α [interferon alpha]; rapamycin — mTOR [mammalian target of rapamycin] inhibitor; JV1-36 and SU5416 (semaxinib)) on the growth of rat pheochromocytoma PC12 cell line.

Results: IFN- α (10^5 U/mL) strongly inhibited PC12 growth in a 72 h culture, increasing apoptosis and arresting the cell cycle. Rapamycin in a wide range of concentrations (10^{-5} to 10^{-8} M) induced a slight inhibitory effect on PC12 viability and decreased cell proliferation at the concentration of 10^{-5} M. VEGF, endostatin and JV1-36 did not influence the growth of PC12.

Conclusions: The study has shown for the first time that IFN- α inhibited the growth of pheochromocytoma PC12 line and confirmed the inhibitory action of rapamycin on these cells. The results suggest that IFN- α and mTOR inhibitors could be potentially effective in the therapy of malignant pheochromocytoma, and encourage further study in this field.

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Key words: pheochromocytoma, interferon alpha, rapamycin, antiangiogenic factors, PC12 line

Streszczenie

Wstęp: Guzy chromochłonne (pheochromocytoma/paraganglioma) należą do łagodnych lub złośliwych nowotworów neuroendokrynych. Wobec niezadowalającej skuteczności standardowych sposobów leczenia pacjentów z rozсіяną postacią choroby, wciąż poszukuje się nowych metod terapii, w tym możliwości skutecznego leczenia celowanego. W związku ze wzmożonym unaczynieniem tych nowotworów, preparaty o działaniu antyangiogennym mogą potencjalnie stanowić nową grupę leków stosowanych w leczeniu pheochromocytoma/paraganglioma.

Materiał i metody: W badaniu oceniano wpływ różnych angiomodulatorów: VEGF (naczyniowo-środbłonkowy czynnik wzrostu) oraz pięciu endo- i egzogennych czynników antyangiogennych (endostatyna; IFN- α [interferon alfa]; rapamycyna — inhibitor szlaku mTOR [mammalian target of rapamycin]; JV1-36 and SU5416 [semaxinib]) na wzrost szczurzej linii pheochromocytoma PC12.

Wyniki: IFN- α (10^5 U/mL) silnie hamował wzrost komórek PC12 w hodowli 72 h, nasilając apoptozę i hamując cykl komórkowy. Rapamycyna w szerokim zakresie stężeń (10^{-5} to 10^{-8} M) nieznacznie zmniejszała żywotność komórek PC12, a w stężeniu 10^{-5} M także hamowała ich proliferację. VEGF, endostatyna oraz JV1-36 nie wpływały na wzrost linii PC12.

Wnioski: W badaniu po raz pierwszy wykazano, że IFN- α hamuje wzrost linii komórkowej pheochromocytoma PC12, a także potwierdzono hamujący wpływ rapamycyny wobec tej linii komórkowej. Uzyskane wyniki sugerują zatem, że IFN- α oraz inhibitory szlaku mTOR mogą być potencjalnie skuteczne w leczeniu złośliwych postaci guzów chromochłonnych i zachęcają do dalszych badań w tym kierunku. (*Endokrynol Pol* 2013; 64 (5): 368–374)

Słowa kluczowe: pheochromocytoma, interferon alpha, rapamycyna, czynniki antyangiogenne, linia PC12

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Introduction

Pheochromocytoma and paraganglioma are numbered among neuroendocrine tumours (NETs). They can occur

sporadically or as a part of familial syndrome, and can be benign or malignant tumours [1]. Gene mutations often observed in these tumours are part of two distinct molecular pathways: cluster 1 (mutations of *VHL*, *SDHB*



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and *SDHD*) leading to overexpression of angiogenic factors; and cluster 2 (mutations of *RET*, *NF1*, *TMEM127*, and *MAX*) associated with abnormal activation of kinase signalling pathways such as PI3kinase/AKT, mTOR1/p70s6K and RAS/RAF/ERK, leading to abnormal cell growth and lack of apoptosis capacity* [2]. These signalling alterations promote tumourigenesis, as hyperactivity of mTOR pathway is a well established factor leading to different neoplasms [3] and increased angiogenesis has been shown to be associated with the development of metastases, poor prognosis and reduced survival in many tumours [4]. The invasiveness of pheochromocytoma also correlated with the number of tumour blood vessels and increased VEGF (vascular endothelial growth factor) immunoreactivity [5]. The importance of VEGF for the angiogenesis of pheochromocytomas has also been demonstrated in *in vitro* studies [6]. Moreover, malignant pheochromocytomas are characterised by increased expression of several other proangiogenic factors [7]. The traditional therapeutic methods (surgery, ¹³¹I-MIBG (metaiodobenzylguanidine), chemotherapy) for patients with metastatic pheochromocytoma/paraganglioma are limited and often ineffective [2, 8, 9]. Thus, better understanding of specific molecular pathways alteration in the development of pheochromocytoma/paraganglioma might lead to molecular-targeted therapy, using antiangiogenic agents and mTOR inhibitors.

Therefore, this paper aims to study the direct influence of proangiogenic (VEGF) and antiangiogenic (rapamycin, interferon alpha, endostatin, JV1-36, SU5416) compounds on the growth of rat pheochromocytoma PC12 cell line. The PC12 cell, a clonal cell line derived from a pheochromocytoma of the rat adrenal medulla, seems to be an excellent experimental model for studying the influence of exogenous angiogenic modulators on the growth and functional activity of neuroendocrine adrenal chromaffin cells.

Rapamycin, a naturally occurring macrolide antibiotic, was the first known inhibitor of PI3K/AKT/mTOR-pathway, which was initially discovered as an antifungal drug and immunosuppressive agent. The antiangiogenic and antitumour activity of rapamycin was found later [3, 10] and gave rise to the use of mTOR inhibition in cancer treatment, which has proved to be effective in a number of neoplasms [3], including neuroendocrine tumours [11]. In 2011, the FDA (Food and Drug Administration) approved an mTOR inhibitor, everolimus, for the treatment of progressive pancreatic neuroendocrine tumours (PNETs) (NCI; <http://www.cancer.gov/cancertopics/druginfo/fda-everolimus>).

JV1-36 belongs to GHRH (growth hormone releasing hormone) antagonists, which have been shown to exert antiproliferative effects in different tumours. The mechanism of GHRH antagonist action is complex and apart from inhibition of IGFs (insulin-like growth factors) production, the suppression of VEGF and bFGF (basic fibroblast growth factor) expression in neoplasms is suggested [12].

SU5416 (semaxinib), a 3-substituted indolinone compound, was the first VEGFR (vascular endothelial growth factor receptor) tyrosine kinase inhibitor tested clinically as antiangiogenic treatment in cancer [13].

Endostatin is a proteolytic fragment of collagen XVII. It is an endogenous inhibitor of angiogenesis which also exerts an antineoplastic action [14, 15]. Although endostatin suppressed the growth of pancreatic neuroendocrine tumour in transgenic RT2 (RIP-Tag2) mice [16], the clinical study did not confirm the effectiveness of endostatin in PNET treatment [17].

Interferon alpha (IFN- α) is a pleiotropic cytokine with proven antitumour activity. The antineoplastic effect of IFN- α is triggered in direct (prolongation of the cell cycle time and enhancement of apoptosis in malignant cells), and indirect (interaction with other cytokines, immunomodulatory and antiangiogenic influence), mechanisms [18]. IFN- α as an angiogenesis inhibitor was first reported in 1980 [19] and then was confirmed by other authors [18, 20]. The clinical use of IFN- α includes treatment of renal cell carcinoma, melanoma, neuroendocrine tumours and some other neoplasms [18].

Material and methods

Cell line and culture condition

The rat pheochromocytoma cell line PC12 obtained from the American Type Culture Collection (ATCC) was used in the experiments. The cells were routinely grown as a monolayer in a humidified incubator at 37°C with 5% CO₂ in F12-K medium (ATCC), supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin solution (Sigma), 15% horse serum (ATCC) and 2.5% fetal bovine serum (Biochrom). The cells were passaged every seven days with 0.05% trypsin/0.02% EDTA (Trypsin-EDTA, Sigma) and the medium was changed every 3–4 days.

Substances

The following substances were used in the experiments: mouse IFN- α (Sigma), rapamycin (Sigma), JV1-36 (Bachem), SU5416 (Sigma), mouse endostatin (Sigma), mouse VEGF (Sigma).

*(VHL — von Hippel Lindau, SDHB and SDHD — subunits B and D of the succinate dehydrogenase (SDH); RET — rearranged during transfection; NF1 — Neurofibromatosis type I; TMEM127 — transmembrane protein 127; MAX — MYC associated factor X; PI3kinase/AKT — phosphatidylinositol 3-kinase/protein kinase B; mTOR1/p70s6K — mammalian target of rapamycin/p70 ribosomal S6 kinase; RAS/RAF/ERK — cascade of extracellular signal-regulated kinase)

IFN- α , JV1-36, endostatin and VEGF were diluted in serum free culture medium and added to the appropriate wells at the final concentrations as indicated in figures. The control group in these experiments received medium.

Rapamycin was dissolved in 96% ethanol and serum free culture medium (5% of 96% ethanol in stock solution) and added to the appropriate wells at the final concentrations as indicated in figures. The appropriate volume of serum free culture medium and 96% ethanol at the final concentration identical with the solvent concentration in 10⁻⁵M group was added to the control wells.

SU5416 was dissolved in DMSO and serum free culture medium (10% of DMSO in stock solution) and added to the appropriate wells at the final concentrations as indicated in figures. The appropriate volume of serum free culture medium and DMSO at the final concentration identical with the solvent concentration in every examined group was added to the control wells.

The action of all the agents in a wide range of concentrations was determined using an MTT colorimetric assay. Following this, based on the results of the MTT study, some concentrations of the substances were further assessed using the BrdU incorporation method, cell cycle analysis and apoptosis examination. However, the MTT method was not used to estimate the influence of SU5416 on PC12 cell growth because of the potential interference of this yellow-dyeing compound with the manufacturer's procedures.

Cell viability/cytotoxicity study/Mosmann method

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was used as an indicator of cell viability/cytotoxicity. PC12 cells were seeded at a density of 10 × 10³ cells/well into 96-well microplates in 180 μ L/well of complete culture medium. After preincubation (24 h), the cells were cultured for a further 72 h in the presence of various concentrations of the examined substances (20 μ L: IFN- α , rapamycin, JV1-36, endostatin, VEGF). Cell viability was assessed using the EZ4U assay kit (The 4th Generation Non Radioactive Cell Proliferation & Cytotoxicity Assay, Biomedica Gesellschaft mbH, Biomedica Gruppe, Austria) according to the manufacturer's instructions. The optical density (OD) of each sample was measured by a microplate reader at 450 nm.

Cell proliferation study

Colorimetric immunoassay based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis was used for the quantification of PC12 cell proliferation. PC12 cells were seeded at a density of 10 × 10³ cells/well into 96-well microplates in 90 μ L/well of complete culture medium. After pre-

incubation (24 h), the cells were cultured for a further 72 h in the presence of various concentrations of the examined substances (10 μ L: IFN- α , rapamycin, JV1-36, SU5416, endostatin, VEGF). Cell proliferation was assessed using the BrdU assay (Cell proliferation ELISA, BrdU, Roche) following the producer's instructions. The optical density (OD) of each sample was measured by a microplate reader at 450 nm.

Cell cycle analysis

PC12 cells were seeded at a density of 3 × 10⁵ cells/well into six-well microplates in 1,350 μ L/well of complete culture medium. After preincubation (24 h), the cells were cultured for a further 72 h in the presence of various concentrations of the examined substances (150 μ L: IFN- α , rapamycin). After 72 h of incubation, cells were harvested by gentle trypsinisation, centrifuged, resuspended in 100 μ L of phosphate-buffered calcium and magnesium free saline (PBS), and fixed in 70% ice-cold ethanol. After centrifugation at 4°C, the cells were finally suspended in PBS containing 75 μ mol/dm³ propidium iodide (PI) and 50 IU Kunitz/mL of DNase-free RNase (Sigma Aldrich). After 30 min of incubation, the cell counts were taken with an LSR II Flow Cytometer (Becton Dickinson). The percentage of cells in G1, S and G2/M phases of the cell cycle and the percentage of cells undergoing apoptosis were determined with FlowJo analytical software.

Apoptosis induction assay

PC12 cells were seeded at a density of 3 × 10⁵ cells/well into six-well microplates (NUNC, Denmark) in 1,350 μ L/well of complete culture medium. After preincubation (24 h), the cells were cultured for a further 72 h in the presence of various concentrations of the examined substances (150 μ L: IFN- α , rapamycin). Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membranes of apoptotic cells. Fluorescein isothiocyanate (FITC) Annexin-V Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, USA) was used for the differentiation of apoptotic and necrotic cells. Briefly, after 72 h of incubation, cells were harvested by gentle trypsinisation and washed with cold PBS. The cells were resuspended in 1 mL of 1 × binding buffer. One hundred microlitres were transferred to a 5 mL culture tube, and 5 μ L of Annexin V-FITC and 5 μ L of propidium iodide (PI) were added. Cells were vortexed and incubated for 15 min in the dark at room temperature. Next, 400 μ L of 1 × binding buffer was added to each tube. Flow cytometric analysis was performed immediately after staining. Annexin-V/PI fluorescence was analysed for each sample; the fluorescence of 10,000 cells was gated and counted using FlowJo software. Early apoptotic cells were identified as PI negative, FITC Annexin V positive, whereas cells in the late stages of apoptosis were positive for both annexin V and PI.

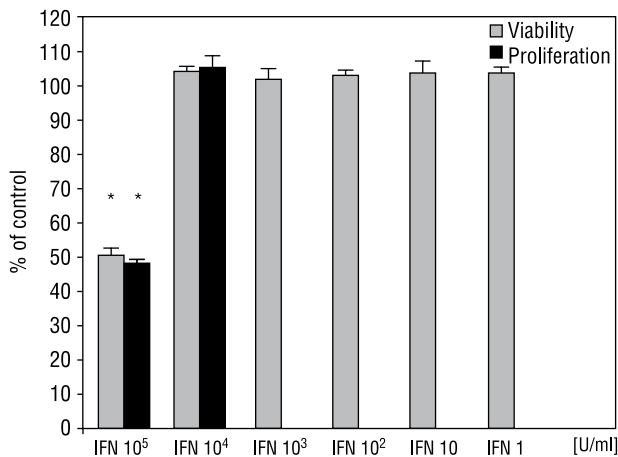


Figure 1. The effect of interferon alpha (IFN) on the growth of the PC12 pheochromocytoma cell line assessed by Mosmann and BrDU incorporation methods in 72 h culture. $\bar{X} \pm S.E.M.$, * $p < 0.05$ v. control

Rycina 1. Wpływ interferonu α (IFN) na wzrost komórek linii guza chromochłonnego PC12 oceniany metodą Mosmanna i wbudowywania BrDU w hodowli 72 h. $\bar{X} \pm S.E.M.$, * $p < 0.05$ v. kontrola

Table I. The effect of interferon alpha (IFN 10⁵ U/mL) and rapamycin (RAP 10⁻⁵ M) on the apoptosis of PC12 cells in 72 h culture

Tabela I. Wpływ interferonu α (IFN 10⁵ U/mL) i rapamycyny (RAP 10⁻⁵ M) na apoptozę komórek linii guza chromochłonnego PC12 w hodowli 72 h

	Viability	Apoptosis	Necrosis
Control	91.7%	6.1%	2.1%
RAP	88.8%	8.0%	3.2%
IFN	79.3%	17.1%	3.6%

Statistical analysis

The results of cell viability and cell proliferation are expressed as the average percentage of the optical density (OD) of the control groups. Comparisons of individual groups were evaluated by analysis of variance (one-way ANOVA) following LSD test (Least Significant Difference). Differences were considered significant if $p < 0.05$. The results of cell cycle and apoptosis analysis are expressed as mean percentage of number of cells in G1, S and G2 phases or as percentage of apoptotic cells, respectively.

Results

IFN- α at the highest concentration of 10⁵ U/mL strongly inhibited PC12 growth in 72 h culture, up to 50% of the control group in MTT method (Fig. 1) and up to 47% of the control group in BrdU incorpo-

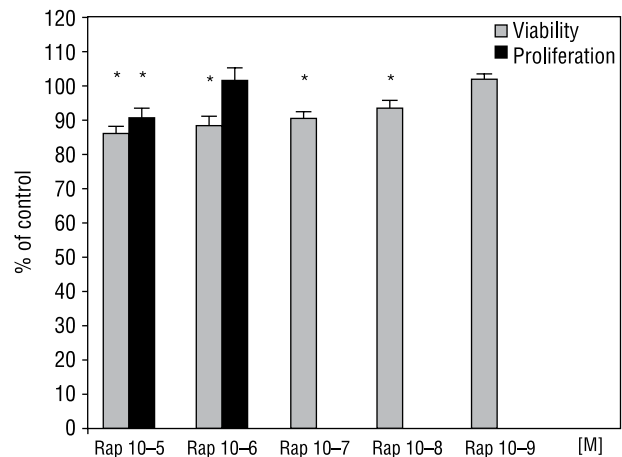


Figure 2. The effect of rapamycin (Rap) on the growth of the PC12 pheochromocytoma cell line assessed by Mosmann and BrDU incorporation methods in 72 h culture. $\bar{X} \pm S.E.M.$, * $p < 0.05$ v. control

Rycina 2. Wpływ rapamycyny (Rap) na wzrost komórek linii guza chromochłonnego PC12 oceniany metodą Mosmanna i wbudowywania BrDU w hodowli 72 h. $\bar{X} \pm S.E.M.$, * $p < 0.05$ v. kontrola

Table II. The effect of interferon alpha (IFN 10⁵ U/mL) and rapamycin (RAP 10⁻⁵ M) on the cell cycle of PC12 cells in 72 h culture

Tabela II. Wpływ interferonu α (IFN 10⁵ U/mL) i rapamycyny (RAP 10⁻⁵ M) na cykl komórkowy linii guza chromochłonnego PC12 w hodowli 72 h

	Control	RAP	IFN
G1	63.5%	66.4%	72.3%
S	21.7%	18.3%	8.9%
G2	12.6%	15.5%	19.1%

ration method (Fig. 1). At this concentration, IFN- α increased apoptosis up to 17.1% of PC12 cell (2.7% cells in early apoptosis and 14.4% cells in late apoptosis) v. 6.1% of cells in the control group (1.7% cells in early apoptosis and 4.4% cells in late apoptosis) (Fig. 3 and Table I). IFN- α at the concentration of 10⁵ U/mL induced G0/G1 phase arrest (Fig. 4 and Table II).

Rapamycin at many concentrations (10⁻⁵ to 10⁻⁸ M) slightly inhibited PC12 cell viability (up to 86–93% of the control group) (Fig. 2). However, in the cell proliferation assay, only the highest concentration of rapamycin (10⁻⁵ M) was shown to be active (90% of the control group) (Fig. 2). Rapamycin did not induce apoptosis or influence cell cycle in a statistically dependent manner (Figs. 3–4, Tables I–II).

SU5416 at the concentration of 10⁻⁵ M inhibited BrdU incorporation in PC12 cells up to 86% of the control group (Fig. 5).

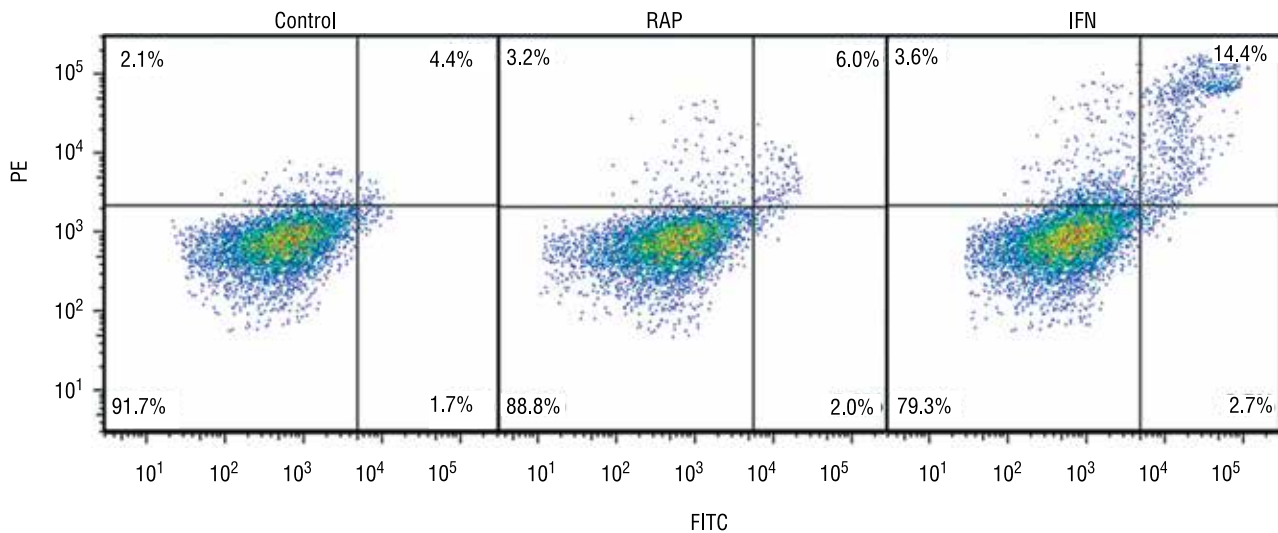


Figure 3. The effect of interferon alpha (IFN 10^5 U/mL) and rapamycin (RAP 10^{-5} M) on the apoptosis of PC12 cells in 72 h culture

Rycina 3. Wpływ interferonu α (IFN 10^5 U/mL) i rapamycyny (RAP 10^{-5} M) na apoptozę komórek linii guza chromochłonnego PC12 w hodowli 72 h

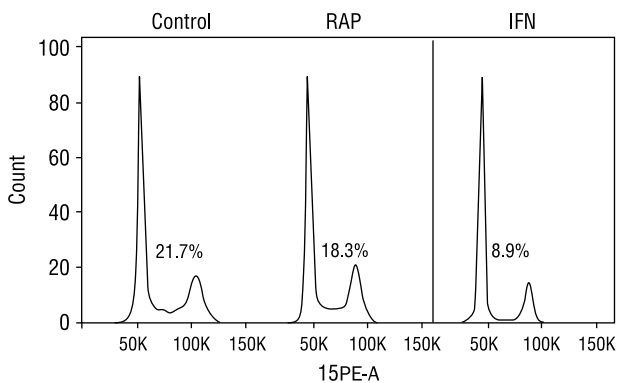


Figure 4. The effect of interferon alpha (IFN 10^5 U/mL) and rapamycin (RAP 10^{-5} M) on cell cycle phase distribution of PC12 cells in 72 h culture

Rycina 4. Wpływ interferonu α (IFN 10^5 U/mL) i rapamycyny (RAP 10^{-5} M) na cykl komórkowy linii guza chromochłonnego PC12 w hodowli 72 h

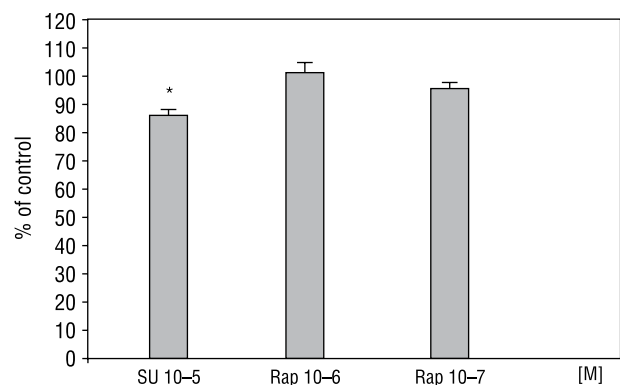


Figure 5. The effect of SU5416 on the growth of the PC12 pheochromocytoma cell line assessed by BrDU incorporation method in 72h culture. $X \pm S.E.M.$, * $p < 0.05$ vs. control

Rycina 5. Wpływ SU5416 na wzrost komórek linii guza chromochłonnego PC12 oceniany metodą wbudowywania BrDU w hodowli 72 h. $X \pm S.E.M.$, * $p < 0.05$ v. kontrola

From all studied concentrations of JV1-36 (10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M), only the highest concentration slightly (up to 92% of the control group), but statistically insignificantly, inhibited the growth of PC12 cells in Mosmann method (data not shown).

Endostatin (at the concentrations of 30; 15; 7.5; 3.75; 1.875; 0.9375 $\mu\text{g}/\text{mL}$) and VEGF (at the concentrations of 100; 50; 25; 12.5; 6.25; 3.125; 1.5625 ng/mL) did not evoke inhibition of PC12 growth (data not shown).

Discussion

This paper aims to study the influence of interferon a mTOR inhibitor (rapamycin), VEGF receptor antago-

nist (SU5416), GHRH receptor antagonist (JV1-36) and endostatin on pheochromocytoma growth, as these factors have been shown to reduce vascularisation and cell proliferation of different solid tumours. Moreover, the presence of specific molecular pathway alteration in pheochromocytoma/paraganglioma additionally suggests the potential effectiveness of antiangiogenic agents and mTOR inhibitors in these tumours. Since all efforts to establish the cell lines derived from human pheochromocytomas and paragangliomas have been unsuccessful (the cells survive but do not proliferate *in vitro*) [21], a rat pheochromocytoma PC12 cell line was chosen for our studies. PC12 line has been used for studying the mechanism of actions of different growth

and trophic factors for many years. It was revealed that NGF (nerve growth factor) and bFGF caused differentiation (neurite outgrowth), whereas EGF (epidermal growth factor) stimulated the proliferation of PC12 cells. These effects were dependent on the MAPK (mitogen activated protein kinases) and/or mTOR signalling pathways [22, 23]. PC12 cells have also been shown to synthesise several growth factors, e.g. NGF, bFGF, VEGF [5, 24, 25], as well as to possess NGF, EGF, FGF and VEGF receptors [26–28].

Although IFN- α is an alternative line treatment in some neuroendocrine tumours [29], it has not been used in pheochromocytoma therapy so far [2, 8, 9]. The influence of IFN- α on pheochromocytoma has not been estimated in experimental conditions either. Thus, we have shown for the first time that IFN- α at the concentration of 10^5 U/mL caused strong pheochromocytoma PC12 cell growth inhibition, inducing apoptosis as well as arresting the cell cycle in G0/G1 phase. The results confirmed the complex action of IFN- α , which potentially included prolongation of the cell cycle time [30], enhancement of apoptosis [31] and modulation of expression of FGF-2 [32] and VEGF in different malignant cells [33].

Similarly, the slight inhibitory effect of rapamycin (10^{-5} – 10^{-8} M) on PC12 cell growth observed in our study was probably also evoked by multitargeted activity of this drug. Rapamycin, as an mTOR inhibitor, has been shown to block the signalling pathways for many growth factors [3], including those whose receptors are expressed on PC12 cells (VEGF, bFGF, NGF, EGF). Our results remain in agreement with other studies, in which rapamycin at concentrations of 10^{-5} – 10^{-9} M inhibited PC12 cell proliferation in 48 h culture [34].

These facts encourage further studies in this field and suggest that mTOR inhibitors could be not only an alternative treatment in PNETs (everolimus - approved by FDA), but possibly also in malignant pheochromocytoma [35].

The application of VEGF receptor inhibitors in the therapy of different neoplasms gave divergent results. Although the administration of SU5416 resulted in reduction of the growth of different xenografted tumours in mice, it did not inhibit the growth of a variety of neoplastic cells *in vitro* [36]. In our study, SU5416 at the concentration of 10^{-5} M slightly inhibited the proliferation of PC12 cells. Similarly, sunitinib (a derivative compound of SU5416) markedly induced apoptosis of PC12 cells, initially exerting its apoptotic effect through the inhibition of VEGFR-2, followed by reduction of its downstream effectors (including AKT/mTOR/S6K1) and leading to inhibition of the antiapoptotic molecule Bcl-2 and activation of the proapoptotic molecule BAD [37]. Moreover, sunitinib also gave encouraging results in preliminary studies in patients with malignant pheochromocytoma

[8]. Thus, our results together with other studies suggest that tyrosine kinase inhibitors are a promising group of agents in the therapy of these tumours.

It is interesting that despite the expression of VEGF receptors on PC12 cells, VEGF used in a wide range of concentrations did not affect PC12 cell growth in our study. This is in accordance with the study of other authors, in which no functional effects of VEGF on PC12 cell proliferation or on neurite formation were observed [28]. On the other hand, it has been confirmed that angiogenesis and tumour size in xenotransplanted PC12 cells in mice are VEGF-dependent and inhibited by treatment of anti-VEGF antibodies [5]. These facts suggest that VEGF did not affect PC12 cell growth directly, but indirectly contributed to the growth of xenografted PC12 tumour *in vivo* by stimulation of angiogenesis. These observations can also explain the fact that in our experiments endostatin, which acts mainly by blocking VEGF/VEGFR signalling [38], does not have any effect on the growth of PC12 cell line. Moreover, other authors did not show a direct binding of endostatin to the PC12 cell surface [28, 39], suggesting the absence of receptors for endostatin in this cell line.

Although GHRHR (GHRH receptor) has been shown to be present in human benign (higher expression) and malignant (lower expression) pheochromocytomas, it has not been demonstrated in PC12 cells [40]. This could explain the lack of antiproliferative activity of JV1-36 in our experiments. However, the effectiveness of GHRH inhibitors is still worthy of being assessed in different pheochromocytoma/paraganglioma models.

In conclusion, although PC12 pheochromocytoma cells express receptors which could be potentially sensitive to antiangiogenic treatment, many agents evaluated in this study did not act directly as anti-mitogenic factors on PC12 cell growth *in vitro*. IFN- α mTOR and tyrosine kinase inhibitors as multitarget drugs turn out to be more effective than agents selectively blocking only pathways typical for angiogenic factors, and they seem to be drugs of sufficient interest to justify further studies.

Thus, considering the unsatisfactory efficacy of the traditional therapeutic methods in patients with metastatic pheochromocytoma, the results of our preclinical studies indicate that antiangiogenic agents and mTOR inhibitors might comprise a new therapeutic option in clinical use.

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