

## Growth factor/growth factor receptor loops in autocrine growth regulation of human prostate cancer DU145 cells

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Autocrine growth factors produced by epithelial cells mediate the development and proliferation of neoplastic human prostate tissue. Various approaches have been used to down-regulate neoplastic growth of prostate cancer using natural flavonoids, soluble receptors, pseudo-ligands, monoclonal antibodies and tyrosine kinase inhibitors (tyrphostins). Selected growth factor/growth factor receptor loops (mainly TGF $\alpha$ /EGFR and IGFs/IGFIR) have been proposed as regulators of prostate cancer cell growth. We have previously determined that blockade of IGFIR or VEGF2R signaling pathways by tyrphostin AG1024 and SU1498 inhibits autocrine growth and viability of DU145 cells *in vitro*. Recently, we compared the activity of AG1024 and SU1498 with the inhibiting effect of tyrphostin A23 (a selective inhibitor of EGFR). The results described in this paper confirm that DU145 cells do not produce IGF1 or EGF. In contrast, DU145 cells produce a great amount of VEGF, much more than TGF $\alpha$  (about 60-fold), and VEGF may be the real autocrine growth factor of the investigated cells. The results indicate that the growth of DU145 may be regulated by at least three autocrine loops: TGF $\alpha$ /EGFR, IGFII/IGFIR and VEGF/VEGFR2. Neither AG1024 nor SU1498 affected the production of TGF $\alpha$  substantially, which excludes the possibility that IGFIRs or VEGFR2 inhibitors arrest the growth of these cells by inhibition of synthesis and/or secretion of TGF $\alpha$ . The obtained data indicate that all three investigated tyrphostins (AG1024, SU1498 and A23) inhibit signal transmission by Akt (PKB), ERK(1/2), Src and STAT in a similar manner. A comparison of the effects of the investigated tyrphostins indicates that TGF $\alpha$ , IGFII and VEGF stimulate cell growth by affecting the same signaling pathway. The hypothesis was confirmed by the effect of the investigated tyrphostins on activation of EGFR. All these inhibitors decreased phosphorylation of EGFR to the same extent, and after the same time of incubation with cell culture. These results strongly suggest that stimulation of EGFR kinase is the main step in the initiation of mitogen signaling in DU145 cells, regardless of the type of ligand (TGF $\alpha$ , IGFs or VEGF) and their specific receptors.

**Keywords:** tyrphostins, DU145 cells, receptor cross-talk, autocrine growth regulation

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### INTRODUCTION

An important event in the development of the neoplastic phenotype is the induction of genes encoding growth factors and their receptor (Sporn & Roberts,

1985; Goustin *et al.*, 1986). The autocrine growth factor-mediated pathways are engaged in proliferation of human prostate cancer cells, including the DU145 cell line (Marker *et al.*, 2003; Bellezza *et al.*, 2006). Understanding the role of different growth factors in the control of autocrine growth may help to clarify the mechanism of prostate cancer progression.

The growth of human prostate cancer cells is probably dependent on several growth factors. Members of the EGF, IGF, PDGF, VEGF and FGF families and neurotrophins have been postulated as stimulators of prostate cell growth (Ferrer *et al.*, 1999; Ozen *et al.*, 2001; Montano & Djamgoz, 2004; Bellezza *et al.*, 2006; Gennigens *et al.*, 2006). Selected growth factor/growth factor receptor loops such as EGF/EGFRs, IGFs/IGFRs and FGFs/FGFRs are probably engaged in the autocrine growth regulation of prostate cancer cell lines (Ozen *et al.*, 2001; Bellezza *et al.*, 2006; Gennigens *et al.*, 2006). An increased production of transforming growth factor  $\alpha$  (TGF $\alpha$ ), ligand from EGF family, has been found in several prostate cell lines, including DU145 and synthetic inhibitors of EGFR have been shown to decrease the proliferation of different prostate cell lines (Vicentini *et al.*, 2003; Bonaccorsi *et al.*, 2004; Sgambato *et al.*, 2004). On the other hand, acquired resistance to inhibitors of EGFR has been reported clinically (Jimeno & Hidalgo, 2005) and suggested to be associated with increased signaling through the IGF1 receptor (Jones *et al.*, 2004). A high level of IGFs and VEGF has also been observed in patients with prostate cancer (Ferrer *et al.*, 1999; Gennigens *et al.*, 2006) suggesting their participation in autocrine growth regulation of the investigated cells. It has been proposed, that IGFs affect the growth of prostate cells *via* the EGF receptor and VEGF is a mediator of their motility and an angiogenic factor (Jackson *et al.*, 2002). In contrast to EGF, IGF and VEGF, the production of NGF is progressively lost in prostate cancer development (Montano & Djamgoz, 2004) and exogenous NGF inhibits the growth of DU145 cells (Sigala *et al.*, 2008).

The mechanism of autocrine growth regulation of prostate cells is far from being explained. Why does one type of cancer cells produce increased levels of several growth factors and do they activate the same or different signaling pathways?. Investigation of the molecular mechanisms of interaction between different autocrine

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Abbreviations: Akt (PKB), kinase Akt (protein kinase B); EGF, epidermal growth factor; ERK, extracellular signal-related kinase; FGF, fibroblast growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; R, receptor; STAT, signal transducers and activators of transcription; TGF $\alpha$ , transforming growth factor  $\alpha$ ; VEGF, vascular endothelial growth factor

signal pathways may explain the mechanism of neoplastic growth and resistance of cancer cells to single inhibitors (cytostatics), often observed in anticancer therapy.

A useful tool for use in the investigation of autocrine cell growth regulation are inhibitors of tyrosine kinases (Bennasroune *et al.*, 2004; Madhusudan & Ganesan, 2004; Lacal, 2006), including small-molecular compounds known as tyrphostins (tyrosine kinase inhibitors) (Levitzi & Gazit, 1995). Previous experiments indicated that tyrphostins: AG1024 and SU1498 inhibit autocrine growth of DU145 human prostate cancer cells in a dose-dependent manner (Kisielewska *et al.*, 2008). In this paper, we compare the mitogenic signal transmission initiated by different autocrine growth factor receptors of DU145 cells: EGFR, IGF1R and VEGFR2.

## MATERIALS AND METHODS

**Chemicals and reagents.** Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12), Minimal Essential Medium (MEM), bovine serum albumin (BSA), transferrin, sodium selenite ( $\text{Na}_2\text{SeO}_3$ ), Hoechst 33258, propidium iodide, MTT (thiazolyl blue tetrazolium bromide), dimethylsulfoxide (DMSO) and tyrosine kinase inhibitors: A23 (EGFR  $\text{IC}_{50}$  = 35  $\mu\text{M}$ ), AG1024 (IGF1R  $\text{IC}_{50}$  = 0.4  $\mu\text{M}$ ) and SU1498 (VEGFR2  $\text{IC}_{50}$  = 0.7  $\mu\text{M}$ ) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories GmbH (Austria). RNeasy® Mini Kit was purchased from QIAGEN (Germany). RayBio® Human EGF (TGF $\alpha$ , VEGF, IGF1) ELISA kits were bought from RayBiotech, Inc. (USA). Oligo GEArray® Human Cancer Pathway Finder™ and CASE™ (Cellular Activation of Signaling ELISA) kits for EGFR, Akt, ERK1/2, Src and STAT3 were obtained from SuperArray Bioscience Corporation (USA). X-Ray film was obtained from Agfa (Belgium). Other reagents were provided by POCh (Gliwice, Poland).

Stock solutions of the tyrphostins were prepared by dissolving the compounds in DMSO to the final concentration of 10 mM or 100 mM in the case of A23 and storing the resultant solutions at  $-20^\circ\text{C}$ . DMEM/F12 (1:1) medium was used to dilute stock solutions to working concentrations (1–10  $\mu\text{M}$ ).

**Cell culture.** DU145 (HTB-81) cells were obtained from the American Type Culture Collection (ATCC) and cultured in MEM in the presence of 10% FBS. The cells were passaged two times per week. The experiments were carried out in DMEM/F12 supplemented with BSA (0.5 mg/ml), transferrin (5  $\mu\text{g}/\text{ml}$ ) and  $\text{Na}_2\text{SeO}_3$  (2 ng/ml).

Proliferation of the investigated cells was determined by the procedure described earlier (Kisielewska *et al.*, 2008). The mean  $\pm$  S.E.M. of at least three experiments with ten replicates each was used. Statistical analysis was performed with the use of the Statistica 9 program.

**Cell cycle analysis.** To estimate the proportion of cells in various phases of the cell cycle, cellular DNA contents was measured by flow cytometry (FACS Scan from Beckton-Dickinson) as described earlier (Kisielewska *et al.*, 2008). The resultant data were analyzed with FCS Express V3 with Multicycle plug-in.

**Cellular activation of signalling ELISA (CASE).** Cells ( $1 \times 10^4$ ) were seeded in 96-well plates and cultured in DMEM containing 10% FBS for one day, followed with a period of serum-deprivation of 24 h (in DMEM/F12+), then cultured for 3 to 24 h in DMEM/F12+

supplemented with the investigated tyrphostins (at respective  $\text{IC}_{50}$  concentrations). Next the cells were fixed (4% formaldehyde) and stained with, phospho-protein or pan-protein primary antibodies (12 h at  $4^\circ\text{C}$ ). Subsequently, the cells were washed, stained with secondary antibody (60 min at room temperature), incubated with color developer (3–5 minutes at room temp.), and the plates were read at 450 nm. Thereafter, the plates were washed thrice with PBS and then stained with crystal violet. The absorbance was measured using a Tecan (Spectra Fluor Plus) multiscan plate reader.

Readings were taken to assess the total number of cells per well. The results were calculated as the ratio of 450 nm absorbance (antibody signal) and 595 nm absorbance (cell number), and normalized to non-stimulated cells.

**RNA isolation.** Total RNA was isolated from DU145 cells (up to  $6 \times 10^6$  cells) using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 30  $\mu\text{l}$  of RNase-free water and stored at  $-20^\circ\text{C}$ . The integrity of ribosomal RNA and DNA contamination was checked using denaturing formaldehyde gel electrophoresis (1%) before proceeding with the microarray analysis. Protein and phenol contamination and concentration of total RNA was assessed by determining the ratio  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$ , respectively (NanoDrop).

**Microarray analysis.** Superarray® hybridization was performed on 128 sites (2 blanks, 4 negative reference spots, 2 biotinylated artificial sequences, 6 household gene spots, and 113 human genes related to drug metabolism) on cDNA gene microarrays (Oligo GEArray® Human Cancer Pathway Finder™ OHS-033, SuperArray® Bioscience Corporation; Frederick, MD, USA). Four arrays were used to determine the difference in mRNA expression between cells treated with the three different receptor tyrosine kinase inhibitors (A23 80  $\mu\text{M}$  and SU1498 10  $\mu\text{M}$  and AG1024 10  $\mu\text{M}$ ) and untreated cells.

DU145 cells were investigated after 24 h of serum starvation followed by 30 h incubation with or without (control) the investigated compounds.

Work-up of the array was performed according to the manufacturer's protocol. Single-stranded cDNA was synthesized (from approx. 6  $\mu\text{g}$  of total RNA) and labeled with dUTP-biotin by using the TrueLabellingAMP™ 2.0 kit. The nylon membrane based DNA macroarray was hybridized overnight at  $60^\circ\text{C}$  with the biotin-labelled cDNA. The hybridized membrane was subjected to chemiluminescence detection with streptavidin-linked alkaline phosphatase and CDPstar substrate. The resulting signals were captured on X-ray film and analyzed with the TIGR Spotfinder 3.1.1 program (Saeed *et al.*, 2003). The assessed luminescence intensities were elaborated with the use of MIDAS program. Lowess smoothing and standard deviation regularization were applied to raw data (Quackenbush, 2002; Pasanen *et al.*, 2003; Saeed *et al.*, 2003).

The differences in mRNA levels for the investigated genes, between the control cells and tyrphostins-treated cells were expressed as  $\log_2(T/C)$ ; where T is luminescence intensity of a spot on an array hybridized with cDNA obtained from tyrphostin-treated cells, and C — from untreated cells.

**Quantitation of selected growth factors in cell culture medium.** DU145 cells were seeded on 24-well plates,  $8 \times 10^4$  cells per well in MEM, SP, NEAA and 10% FBS. After 24 h, the cells were serum-starved for 24 h in DMEM/F12+. Afterwards, the medium was replaced by a fresh dose of DMEM/F12+ supplemented or not with A23 (80  $\mu\text{M}$ ), AG1024 (8  $\mu\text{M}$ ), SU1498 (8

**Table 1. Percentage of DU145 cells in different cell cycle phases after incubation with tyrphostins**

AG1024, SU1498 and A23 were added at concentration equal their respective  $IC_{50}$  and  $2 \times IC_{50}$  in serum-free DMEM/F12+ for 30 and 48 h.

30 h	Cycle phase	Apoptosis			$G_1/G_2$	
		$G_1&G_0$	S	$G_2&M$		
Control		57.0	25.5	17.5	-	3.26
AG1024 $IC_{50}$		71.4	15.8	12.8	-	5.58
AG1024 10 $\mu$ M		68.9	18.6	12.5	-	5.52
SU1498 $IC_{50}$		74.1	10.6	15.3	-	4.85
SU1498 10 $\mu$ M		68.4	16.7	14.9	-	4.59
A23 $IC_{50}$		59.7	19.1	11.7	8.3	5.09
A23 80 $\mu$ M		56.1	24.4	9.8	8.7	5.74

48 h	Cycle phase	Apoptosis			$G_1/G_2$	
		$G_1&G_0$	S	$G_2&M$		
Control		60.8	21.7	17.5	-	3.47
AG1024 $IC_{50}$		72.6	14.6	12.8	-	5.68
AG1024 10 $\mu$ M		70.8	16.7	12.5	-	5.67
SU1498 $IC_{50}$		72.2	9.7	11.8	3.7	6.12
SU1498 10 $\mu$ M		67.03	14.64	12.51	4.96	5.36
A23 $IC_{50}$		52.8	22.5	10.8	13.0	4.89
A23 80 $\mu$ M		46.1	22.4	7.8	22.8	5.88

$\mu$ M), and the cells were incubated for additional 24 h or 48 h. Subsequently, samples of culture media were collected and analyzed for the presence of the investigated growth factors with the use of RayBio Human EGF,

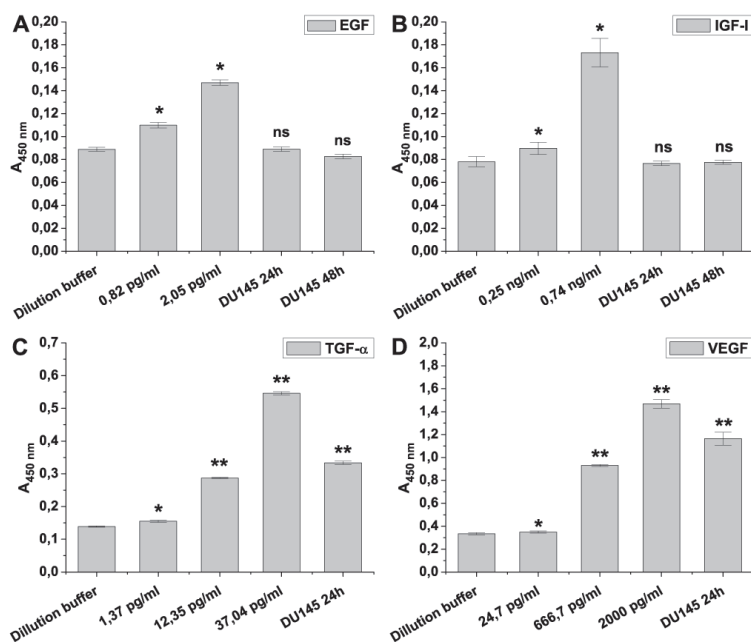
with the effect of a selective inhibitor of epidermal growth factor receptor (EGFR) — tyrphostin A23. The anti-proliferative activity of the investigated tyrphostins was determined by two colorimetric methods (MTT and CV). The influence of the tyrphostins on autocrine growth of DU145 cells was characterized by average values estimated by both methods. Tyrphostins A23, AG1024 and SU1498 inhibited growth of DU145 cells by 50% at the concentration: 35  $\mu$ M, 3.2  $\mu$ M and 3.6  $\mu$ M respectively.

TGF $\alpha$ , VEGF and IGF-I ELISA Kits following manufacturer's instruction with minor modifications. At the same time, the number of DU145 cells was estimated in each well. Conditioned media from cell cultures and standard solutions were incubated on ELISA plates overnight at 4°C. Biotin-labeled secondary antibodies were applied for 3 h in room temp. HRP-Streptavidin was added to the plates for 45 min. Finally, TMB One-Step Substrate Reagent was added to each well and incubated in the dark with gentle shaking for 30 min.

Standard curve (exponential model) was fitted to obtained data and the concentration of the secreted growth factors in culture medium was calculated. The results were expressed as pg or ng of the investigated protein produced by  $1 \times 10^6$  cells after 24 h of incubation. The effects of tyrosine kinases inhibitors were shown as the ratio of the amount of growth factors produced by the treated cells to the amount produced by the control cells.

## RESULTS

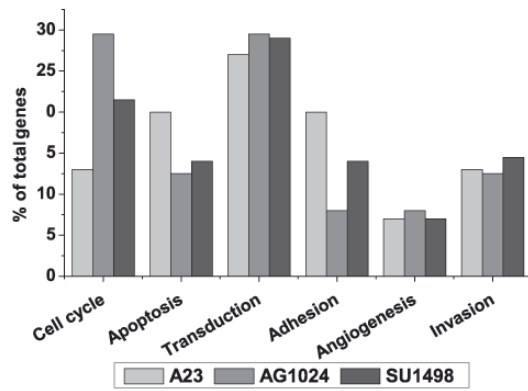
The recently reported results on the cytostatic effects of AG1024 and SU1498 on autocrine growth of DU145 cells (Kisielewska *et al.*, 2008) indicated similar inhibitory activities of the both investigated tyrphostins. The next step in our investigations was a comparison of cytostatic activity of AG1024 and SU1498



**Figure 1. Concentration of growth factors in DU145 cell culture medium** Mean by ELISA method for cell culture medium after 24 h (C, D) or 24 h and 48 h (A, B) of incubation, compared to mean values of absorbance for dilution buffer and two standard samples with lowest concentrations of EGF (A) and IGF-I (B) or three standard samples for TGF $\alpha$  (C) and VEGF (D). Differences between samples was assessed by Mann-Whitney U-test (\* $p < 0.05$ , \*\* $p < 0.001$ , ns, not significant).

These results indicate that only EGFR-induced signaling is inhibited by the specific tyrphostin (A23) in the same concentration range as the inhibition of the enzymatic activity of the kinase receptor ( $IC_{50} = 35 \mu$ M) and receptor autophosphorylation ( $IC_{50} = 40 \mu$ M). Effective cell growth inhibition by the other two investigated tyrphostins required much higher concentration of the compounds than those needed to inhibit the enzymatic activity of IGFIR and VEGFR2. However, to observe an inhibitory effect of the tyrphostin AG1024 (the effect of SU1498 was not determined) on the autophosphorylation of specific receptor ( $IC_{50} = 7 \mu$ M) required a similar concentration range as for cell proliferation of DU145 cells. It should be noted that the growth inhibition of DU145 cells by all the investigated tyrphostins was reversible (not shown).



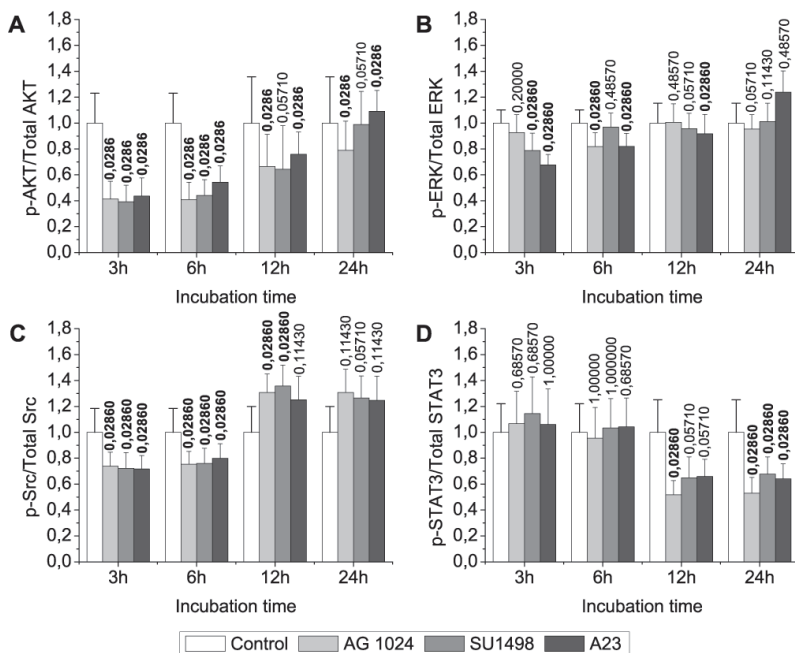


**Figure 2. Influence of investigated typhostins on expression of genes engaged in different cellular processes**

Proportion of up- or down-regulated to total analyzed genes involved in particular cell signalling pathways. DU145 cells were incubated for 30 h with AG1024, SU1498 and A23 added at concentration equal their  $IC_{50}$ .

### Influence of typhostins AG1024, SU1498 and A23 on DU145 cell cycle

The effect of typhostins AG1024 and SU1498 on the cell cycle was described earlier (Kisielewska *et al.*, 2008). They influenced the cell cycle of DU145 in a similar manner. They increased the cell number in G1 and S phases and decreased it in G2 phase in comparison with the control culture. AG1024 did not stimulate apoptosis, and the cytotoxic effect of SU1498 was very weak. In this paper, we compare the influence of AG1024 and SU1498 with the inhibitor of EGF receptor (A23) on the cell cycle of DU145 cells (tested in the same experiment). In contrast to typhostins AG1024 and SU1498, inhibition of EGF receptor by typhostin A23 was associated with a significantly increased proportion of apoptotic cells (Table 1).



**Figure 3. Effect of typhostins AG1024, SU1498 and A23 on phosphorylation of Akt (A), ERK (B), Src (C) and STAT (D) signaling proteins in DU145 cells**

The investigated typhostins were added at concentration equal their  $IC_{50}$ . Statistical significance  $< 0.05$  in bold.

### Synthesis of autocrine growth factors

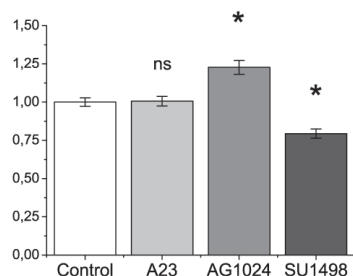
The effect of the investigated typhostins on growth and cell cycle of DU145 cells suggests one of the two possibilities: a) each of the inhibitors stimulates a separate signaling pathway or b) two or three investigated inhibitors utilize a common pathway of mitogen signaling. The results shown in Fig. 1 indicate that DU145 cultured in DMEM/F12+ produce vascular endothelial growth factor (VEGF) and TGF $\alpha$  and confirm that the cells do not produce EGF and IGF1. Moreover, the amount of VEGF secreted to the culture medium calculated per  $10^6$  cells, was about 60 times higher ( $6.35 \pm 1.05$  ng/24 h) than the amount of TGF $\alpha$  ( $118.4 \pm 3.3$  pg/24 h). The prostate cancer cell line DU145 did not secrete a detectable amount of EGF or IGF-I, even after 48 h of incubation.

### Comparison of the effect of typhostins AG1024, SU1498 and A23 on gene expression and phosphorylation of selected signaling proteins

To compare the signaling pathways of IGFIR, VEGFR2 and EGFR, we tested the effect of the investigated inhibitors on the expression of selected genes and on phosphorylation of some proteins engaged in mitogen stimulated signaling pathways.

The effect of the investigated typhostins on gene expression was assessed by cDNA microarray analysis, using Oligo GEArray® Human Cancer Pathway Finder™ OHS-033. Four arrays were used to determine differences in mRNA expression between DU145 cells treated with A23, SU1498, and AG1024 (at the concentration equal to  $2 \times IC_{50}$ ) and untreated cells after 24 h serum starvation followed by 30 h incubation with or without (control) the investigated compounds. The effect of the inhibitors on the expression of selected genes related to six different signaling pathways is shown in Fig. 2. Differences (mainly between AG1024 and A23) were observed in the response of genes engaged in regulation of the cell cycle and cell adhesion. The former could have resulted, at least partly, from the ability of the investigated typhostins to stimulate apoptosis. Only A23 (at the concentration equal to its  $IC_{50}$ ) does effectively stimulate this process (8.3% of apoptotic cells) after 30 h of incubation. The different effects on expression of genes engaged in cell adhesion may be characteristic for individual signaling pathways affected by these typhostins. In the remaining cellular processes, the differences in the levels of gene expression in response to individual inhibitors were much smaller.

Stimulation of EGFR receptor may trigger numerous intracellular signaling pathways involved in such processes as: survival (Akt/PKB), cell growth (ERK1/2; Src) and transcription (STAT). The activity of these proteins is regulated by phosphorylation/dephosphorylation. A comparison of the effects of the inhibitors of the EGFR, IGFIR and VEGFR2 tyrosine kinases on phosphorylation of the investigated proteins is shown in Fig. 3. The obtained results indicate a similar-



**Figure 4. Effects of AG1024, SU1498 and A23 on synthesis and secretion by DU145 cells**

Concentration of TGF $\alpha$  was assessed by ELISA after 30 h of incubation of DU145 cells in DMEM/F12+ supplemented or not with investigated typhostins. \*Statistical significant  $p < 0.05$ .

ity in growth factor-stimulated pathway signaling, probably with participation of the same signaling proteins. Therefore, we checked the influence of the investigated typhostins on phosphorylation of proteins involved in mitogen signaling pathway.

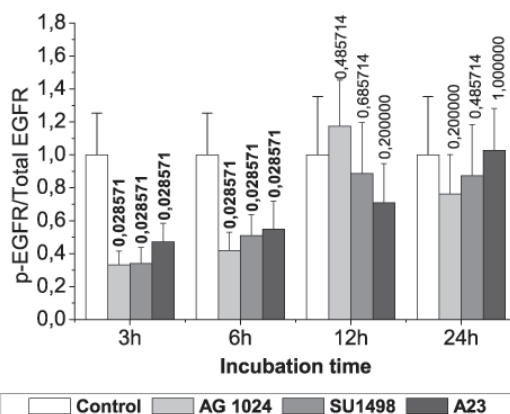
The time course of the changes in protein phosphorylation, as well as the level of inhibition for all three inhibitors was very similar. The phosphorylation of Akt was decreased to about 50% of the control value after 3 h of incubation, regardless of the kind of the investigated typhostin. It returned to the control level after 24 h of treatment with these typhostins. Similarly, phosphorylation of Src decreased to about 70% of the control level after the first 3 h of incubation with the investigated typhostins and returned to the control value after the subsequent 9 h. Also the kinetics of the inhibition of STAT3 phosphorylation did not differ between the investigated compounds. A comparison of the effect of A23, AG1024 and SU1498 on ERK phosphorylation was difficult to interpret because of the small effect of the inhibitors. However, some differences in the inhibition activity of the investigated typhostins after the first 3 h and 6 h of incubation were observed.

#### Effect of typhostins AG1024, SU1498 and A23 on TGF $\alpha$ production by DU145 cells

One of the possible indirect actions of IGFs and VEGF on autocrine growth stimulation of DU145 cells was their effect on TGF $\alpha$  production. Therefore, we determined the effect of AG1024 and SU1498 (in comparison with A23) on the level of TGF $\alpha$  in the culture medium of the investigated cells. As shown in Fig. 4, DU145 cells incubated with typhostin A23 produced the same amount of TGF $\alpha$  as untreated cells. AG1024, a selective inhibitor of IGFIR tyrosine kinase, increased the concentration of the secreted TGF $\alpha$  in the conditioned medium by about 24%. In contrast, treatment with SU1498 resulted in lowering of the production of this EGFR ligand to 74% of the control value.

#### Effect of typhostins AG1024, SU1498 and A23 on activation of EGFR

Activation of receptor tyrosine kinases is due to their autophosphorylation, stimulated by specific ligand binding with the extracellular part of the receptor. The effect of typhostins AG1024, SU1498 and A23 on phosphorylation of EGFR in DU145 cells (Fig. 5) was surprising.



**Figure 5. Influence of typhostins AG1024, SU1498 and A23 on EGFR phosphorylation**

DU145 cells were incubated with investigated compounds (added at concentration equal  $IC_{50}$ ) for 3, 6, 12 and 24 h in serum-free medium (DMEM/F12+). Statistical significance  $< 0.05$  in bold.

All the investigated inhibitors, used in respective  $IC_{50}$  concentrations, decreased the phosphorylation of EGFR to the same degree, and following the same time course.

The obtained results strongly suggest that inhibition of EGFR activation is the first (main) step in inhibition of mitogen signaling in DU145 cells, regardless of the kind of ligands (TGF $\alpha$ , IGFs or VEGF) and their specific receptors.

## DISCUSSION

Autocrine growth stimulation of DU145 cells by EGF/TGF $\alpha$  is well documented (Salomon *et al.*, 1995; Seth *et al.*, 1999; Blackledge, 2003). The results of earlier experiments (Ferrer *et al.*, 1999; Gennigens *et al.*, 2006) indicated that DU145 cells showed also overexpression of IGFII and VEGF. These findings, in connection with an increased level of IGFRI and VEGFRs, suggested the participation of IGFs and VEGFs in autocrine growth regulation of the investigated cells. There was one *in vitro* study which showed that IGFIR antibodies inhibited DNA synthesis in PC3, but not the DU145 cell lines (Kimura *et al.*, 1996); however, other investigators found that IGFIR antibody prevents proliferation of both the prostate cell lines (Maloney *et al.*, 2003). Although an association may exist between IGF1 and prostate cancer, direct causality has not been established (Meinbach & Lokeshwar, 2006). Another question is the role of VEGFR in autocrine growth regulation of prostate cells. Several investigations indicated that VEGF is mainly the angiogenic and chemotactic factor for prostate cancer cells; however, it may regulate tumor growth *in vitro* (Jackson *et al.*, 2002).

We have determined (Kisielewska *et al.*, 2008) that blockade of the IGFIR or VEGFR2 signaling pathways inhibits autocrine growth and viability of DU145 cells *in vitro*. Our results indicate that both investigated typhostins (AG1024 and SU1498) at concentration  $\geq 10 \mu\text{M}$  are able to inhibit completely DU145 autocrine cell growth. These results suggest that the two growth factor receptors (IGFIR, VEGFR2) induce a common signaling route, or that activation of one of the investigated receptors influences the mitogenic pathway of the other. Further experiments have revealed that both the investigated inhibitors increase the percentage of DU145 cells in  $G_0/G_1$  phase of

cell cycle, indicating that AG1024 and SU1498 arrest cell growth in G<sub>0</sub>/G<sub>1</sub> phase, similarly as do known inhibitors of EGFR (Bonaccorsi *et al.*, 2004; Bellezza *et al.*, 2006).

A comparison of the IC<sub>50</sub> values for the anti-proliferative and enzyme inhibitory activities (12:1) of AG1024 suggests that the true ligand for IGFIR in the case of DU145 cells is IGFII rather than IGF1, and supports the hypothesis of IGFII/IGFIR autocrine loop in this type of cancer prostate cells (Figueroa *et al.*, 1995). We confirm that DU145 cells do not produce insulin-like growth factor I, and IGF1 cannot be an autocrine growth factor for these cells. In contrast, DU145 cells produce a great amount of VEGF (much more than TGF $\alpha$ ) and this protein may be the real autocrine growth factor of the investigated cells. Incubation of DU145 cells with AG1024 and SU1498 indicates a weak (about 25% of the control) and opposing effect of these tyrophostins on production of TGF $\alpha$ , which excludes the possibility that IGFs and VEGF stopped the growth of these cells by inhibition of synthesis or secretion of TGF $\alpha$ .

The mitogenic pathways stimulated by growth factors engage several proteins, including Akt (PKB), ERK(1/2), Src and STAT, the activity of which is regulated by phosphorylation/dephosphorylation. The obtained data indicate that all three tyrophostins inhibit intracellular signaling pathways, triggered by EGFR, IGFIR and VEGFR2 receptor, in a similar manner. A comparison of the effect of the investigated tyrophostins points to a similarity in TGF $\alpha$ , IGFII and VEGF-stimulated pathway signaling.

Unexpectedly, all the investigated inhibitors used at their respective IC<sub>50</sub> concentrations decreased phosphorylation of EGFR to the same extent. These results strongly suggest that activation of EGFR is the first (main) step in initiation of mitogen signaling in DU145 cells, regardless of the kind of ligands (TGF $\alpha$ , IGFs or VEGF) and their specific receptors.

The presented results show that autocrine growth of DU145 may be regulated by at least two additional autocrine loops: IGFII/IGFIR and VEGF/VEGFR2, apart from the well-documented TGF $\alpha$ /EGFR loop. Such multifactor regulation of the autocrine growth of the metastatic cancer cell line DU145 may explain the failure of single specific tyrosine kinase inhibitors in the therapy (monotherapy) of prostate cancer (Blackledge, 2003; Pu *et al.*, 2006). A role for the autocrine growth loop involving tyrosine kinases and their receptors in tumor progression has been suggested for a variety of cancers. Identifying biologically and physiologically important autocrine growth events may have important clinical consequences, also for the introduction of new strategies in anticancer therapies.

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