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Novel amidine analogue of melphalan as a specific multifunctional inhibitor of growth and metabolism of human breast cancer cells

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Abbreviations:
AB4, methyl 3-(4-(bis(2-chloroethyl) amino)phenyl)-2-(2-(4-[(N-isopropyl) amidino)]phenyl)furan-5-carboxamido) propanoate hydrochloride (amidine analogue of melphalan)
DCI, N,N′-carbonyldiimidazole
MTT, 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide
DMEM, Dulbecco's minimal essential medium

ABSTRACT

A novel amidine analogue of melphalan (AB4) was compared to its parent drug, melphalan in respect to cytotoxicity, DNA and collagen biosynthesis in MDA-MB-231 and MCF-7 human breast cancer cells. It was found that AB4 was more active inhibitor of DNA and collagen synthesis as well more cytotoxic agent than melphalan. The topoisomerase I/II inhibition assay indicated that AB4 is a potent catalytic inhibitor of topoisomerase II. Data from the ethidium displacement assay showed that AB4 intercalated into the minor-groove at AT sequences of DNA. The greater potency of AB4 to suppress collagen synthesis was found to be accompanied by a stronger inhibition of prolidase activity and expression compared to melphalan. The phenomenon was related to the inhibition of β_1-integrin and IGF-I receptor mediated signaling caused by AB4. The expression of β_1-integrin receptor, as well as Sos-1 and phosphorylated MAPK, ERK_1 and ERK_2 but not FAK, Shc, and Grb-2 was significantly decreased in cells incubated for 24 h with 20 μM AB4 compared to the control, not treated cells, whereas in the same conditions melphalan did not evoke any changes in expression of all these signaling proteins, as shown by Western immunoblot analysis. These results indicate the amidine analogue of melphalan, AB4 represent multifunctional inhibitor of breast cancer cells growth and metabolism.

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ERK_1 and ERK_2, extracellular-signal-regulated kinase 1 and kinase 2
FAK, non-receptor focal adhesion kinase $pp125^{FAK}$
FBS, fetal bovine serum
GRB2, growth-factor receptor-bound protein 2
IGF-I, insulin-like growth factor I
MAPK, mitogen activated protein kinases
PAGE, polyacrylamide gel electrophoresis
SDS, sodium dodecylsulfate
Sos-1, son of sevenless protein 1

1. Introduction

The nitrogen mustards are among the earliest, effective antitumor drugs used in human cancer chemotherapy [1,2]. Mechlorethamine and melphalan are two of the most currently used anticancer agents, and these cytotoxic drugs are believed to exert pharmacologic activity by inducing interstrand cross-links in the major groove of DNA that represents the toxicity of all alkylation events [1,2]. Numerous nitrogen mustard have been modified during the last 30 years in order to increase their cytotoxicity and target affinity [3–8]. Among them are amidine analogues of alkylating drugs. It was found that the amidine moiety of the drugs may direct the alkylation both sequence specifically and regiospecifically [3–8]. The well-known representative of this class of drugs that was clinically tested is tallimustine, a benzoic acid nitrogen mustard, derivative of distamycin. Recently our studies on the targeting of chlorambucil to DNA by the use of 5-[4-(*N*-alkylamidino)phenyl]furans have shown that this strategy can greatly enhance both the in vitro cytotoxicity and the in vivo antitumor activity of the mustard moiety, when compared with untargeted mustards of similar reactivity [5–7,9,10].

One of the characteristic features of breast cancer cells is a deregulation of their interaction with extracellular matrix proteins [11]. Collagen is the most abundant component of extracellular matrix and is responsible for the maintenance of the architecture and the integrity of connective tissue. It is known that the interaction between integrin receptors and extracellular matrix proteins, e.g. collagen, can regulate neoplastic cell attachment, migration, proliferation, progression and survival [12]. Therefore changes in the quantity, structure and distribution of collagens caused by anticancer agents may affect human breast cancer cells metabolism and function [13].

Collagen biosynthesis and prolidase activity are coordinately regulated in MCF-7 and MDA-MB-231 cells [14]. Prolidase [EC 3.4.13.9] catalyzes the hydrolysis of imidodipeptides [15], releasing proline, which is used for collagen resynthesis [16] and cell growth [17]. Prolidase activity is regulated by the signal mediated by activated β_1-integrin receptors [18]. Stimulated β_1-integrin receptors induce autophosphorylation of non-receptor focal adhesion kinase $pp125^{FAK}$ (FAK) [19], which is then capable of interacting with adaptor-proteins, such as Grb2, through Src and Shc proteins. This interaction allows to activate further cascade of signaling pathway through son of sevenless protein (Sos-1), Ras and Raf proteins [20] and subsequently, two mitogen activated protein (MAP) kinases (MAPK), extracellular-signal-regulated kinase 1 (ERK_1) and kinase 2 (ERK_2) [21]. The result of this phenomenon is induction of transcription factors and stimulation of the expression of genes for integrins, metalloproteinases, proteases and many other proteins involved in the regulation of cell growth and differentiation [22].

Collagen biosynthesis and cell growth is stimulated by insulin-like growth factor I (IGF-I) [23]. IGF-I, acting predominantly through the IGF-I receptor [24], has been demonstrated to stimulate proliferation, promote survival, enhance metastatic potential of breast cancer cells [25] and prevent apoptosis [26]. The MAP-kinase (ERK_1 and ERK_2) pathway induced by activated IGF-I receptor is considered to play a central role in carcinogenesis and tumor progression. IGF-I receptor signaling involves the same proteins and kinases as the β_1-integrin transduction pathway, except for the participation of FAK kinase and Src protein [27].

Prolidase plays an important role in stimulation of a collagen production. Collagen through interaction with integrin receptors activate signaling cascades that regulate cell growth and is involved in cancer metastasis [11–13]. The inhibition of prolidase may therefore inhibit variety of integrin-dependent cellular responses. Previous results of our studies proved higher potency of amidine analogues of alkylating drugs to affect breast cancer cells metabolism than the parent drugs [9,10]. The mechanisms of the activity was related to inhibition of DNA and collagen biosynthesis, inhibition of β_1-integrin signaling cascade, down regulation of IGF receptor expression and inhibition of prolidase activity [9,10]. Alkylating compounds also induced cell death and expression of mediators participating in the recruitment of inflammatory cells. These activities may represent an additional beneficial actions of the anticancer compounds.

The amidine analogue of melphalan (AB4) has been synthesized (Fig. 1) and its cytotoxicity has been tested in both MCF-7 and MDA-MB-231 breast cancer cells. The mechanism of action of AB4 was studied employing the

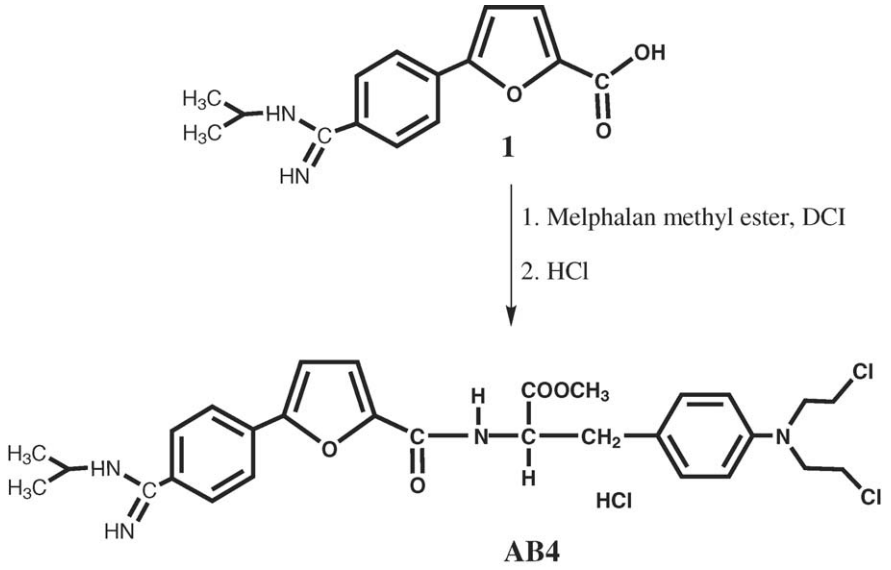

Fig. 1 – Synthesis of amidine analogue of melphalan (AB4).

topoisomerase I/II inhibition assay and ethidium displacement assay using calf thymus DNA, T4 coliphage DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$ [5–7]. In this study, the effects of the amidine analogue of melphalan AB4 on collagen and DNA biosynthesis, β_1-integrin receptor, IGF-I receptor, FAK, Shc, Grb-2, Sos-1 and phosphorylated MAP-kinases (ERK_1 and ERK_2) expression in human breast cancer cells were compared to those evoked by melphalan.

2. Materials and methods

2.1. Chemistry

Melting points were determined on Büchi 535 melting-point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on Brucker AC 200F apparatus using tetramethylsilane as an internal standard. Chemical shifts are expressed in δ value (ppm). Multiplicity of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quarter (q), and multiplet (m). Elemental analysis of C, H, and N was performed on a Perkin–Elmer 240 analyser and satisfactory results within ±0.4% of calculated values were obtained. Melphalan, dimethylformamide, acetone, triethylamine, *N*,*N*′-carbonyldiimidazole and LiOH were purchased from Sigma Chemical Co (USA).

2.1.1. *Methyl 3-(4-(bis(2-chloroethyl)amino)phenyl)-2-(2-(4-[(N-isopropyl)amidino)]phenyl)furan-5-carboxamido)propanoate hydrochloride (AB4)*

To a solution of 5-(4-(*N*-isopropyl)phenyl)-2-furancarboxylic acid hydrochloride **1** (0.96 g, 3 mmol) [28] in 5 ml of anhydrous DMF in an ice bath was added *N*,*N*′-carbonyldiimidazole (0.49 g, 3 mmol). The solution was stirred for 30 min and a solution of melphalan methyl ester hydrochloride (1.1 g, 3 mmol) and triethylamine (0.42 ml, 3 mmol) in 5 ml of anhydrous DMF was added. It was stirred at 0 °C for 4 h, then the temperature was allowed to rise to the ambient and stirring was continued for 12 h. The volume of the reaction mixture was reduced to 2 ml by evaporation and the remaining mixture was diluted with acetone. The resulting solid was filtered, washed with acetone and dried under vacuum at room temperature. The crude product was basified with 1 M LiOH to pH above 9. A gummy solid was filtred, washed with water, and dried under vacuum. The free base was converted into the salt by taking up in 3 ml of methanol, treated with 2 ml of 2 M HCl and stirred for 30 min. Acetone was added and the final product was filtered, washed with acetone and recrystallized from absolute ethanol to give 1.14 g of AB4. Yield 63%; mp 269–270 °C; ^{1}H NMR (DMSO-d_6) δ 9.62 (br, 1H), 9.45 (br, 1H), 9.07 (br, 1H), 8.01 (d, 2H), 7.90 (d, 2H), 7.38 (d, 1H), 7.36 (d, 1H), 4.05 (m, 1H), 1.32 (t, 3H), 1.27 (d, 6H), 3.67 (s, 3H), 4.14 (m, 1H), 2.89 (d, 2H), 6.54 (d, 2H), 7.00 (d, 2H), 3.47–3.67 (m, 8H), 7.25 (br, 1H); ^{13}C NMR (DMSO-d_6) δ 158.6, 154.5, 145.1, 133.1, 129.3, 129.0, 124.2, 119.8, 110.3, 45.1, 21.2, 156.3, 51.9, 171.6, 55.9, 37.7, 112.0, 125.7, 130.7, 144.9, 53.4, 40.4. Anal. calcd. for $C_{31}H_{40}N_5O_3Cl_2 \cdot HCl \cdot H_2O$ (655.5): C, 56.75; H, 6.56; N, 10.68; found: C, 56.74; H, 6.58; N, 10.72.

2.2. Pharmacology

2.2.1. Materials

Ethidium bromide, netropsin, distamycin, calf thymus DNA, T4 coliphage DNA, homopolymers poly(dA-dT)·poly(dA-dT), and poly(dG-dC)·poly(dG-dC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (USA). Topoisomerase II was purchased from Amersham Pharmacia Biotech (USA). Topoisomerase I was purchased from Topogen (USA). Stock cultures of breast cancer MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection, Rockville, MD (USA). Dulbecco's minimal essential medium (DMEM) and foetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^{3}H]-thymidine (6.7 Ci/mmol) was

the product of NEN (USA). Anti-FAK antibody, anti-Human IGF-I receptor antibody, anti-Goat immunoglobulin antibody, anti–Mouse immunoglobulin antibody, aprotinin, bacterial collagenase, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT), leupeptin, L-glycyl-proline, L-proline, monoclonal anti-posphorylated MAPK (ERK_1 and ERK_2) antibody, Nonidet P-40, phenylmethylsulfonyl fluoride and Protein A-Sepharose were provided by Sigma Chemical Co. (USA), as were most other chemicals and buffers used. The Dulbecco's minimal essential medium (DMEM) and fetal bovine serum used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). Nitrocellulose membrane (0.2 μm), sodium dodecylsulfate (SDS), polyacrylamide, molecular weight standards and Coomassie Brilliant Blue R-250 were received from Bio-Rad Laboratories (USA). 5-[^{3}H]-proline (28 Ci/mmol) was purchased from Amersham (UK). Mouse monoclonal anti-Sos-1 (clone 25), anti-Grb-2 (clone 81) and anti-Shc (clone 30) antibodies were obtained from Becton Dickinson Co. (USA). A Goat polyclonal anti-β_1-integrin antibody (N-20) and a rabbit polyclonal anti-α_2-integrin antibody (H-293) were obtained from Santa Cruz Biotechnology (USA). Polyclonal (rabbit) anti-human prolidase antibody was a gift from Dr. James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA). Anti-Rabbit immunoglobulin was obtained from Promega Corp. (USA). [^{3}H]thymidine (6,7 Ci/mmol) was purchased from ICN Biomedicals, USA and Scintillation Coctail "Ultima Gold XR" from Packard (USA).

2.2.2. Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate buffered saline, counted in hemocytometers and plated at 5×10^5 cells per well of six-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluency at day 3 and in most cases such cells were used for the assays.

2.2.3. DNA synthesis assay

MCF-7 and MDA-MB-231 cells were seeded in six-well plates and were incubated with varying concentrations of AB4 or melphalan and 0.5 μCi of [^{3}H]-thymidine for 24 h at 37 °C. The cells were then harvested by trypsinization, washed with cold phosphate-buffered saline and centrifuged for 10 min at $1500 \times g$ several times (4–5) until the dpm in the washes were similar to the reagent control. Radioactivity was determined by liquid scintillation counting. [^{3}H]-thymidine uptake was expressed as dpm/well.

2.2.4. Cell viability assay

The assay was performed according to the method of Carmichael using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [29]. Confluent cells, cultured for 24 h with various concentrations of studied compounds in six-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37 °C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of breast cancer cells cultured in the presence of studied compounds was calculated as a per cent of control cells. The experiments were performed in triplicates. After treatment of the cells with drug, the ratio of survived to dead cells in tested and control (untreated) cells was calculated for each drug concentration. Cell number was plotted versus drug concentration, and IC_{50} values were calculated from dose–response curves as the concentration of drugs that reduce the number of viable cells to 50% of control using an Origin 7.5 software.

2.2.5. Relaxation assay of topoisomerase I and II

PBR322 plasmid DNA (0.083 μg) was incubated with 1 unit of human topoisomerase I (reaction buffer: 10 mM Tris–HCl (pH 7.8), 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 spermidine, 5% glycerol) or human topoisomerase II (reaction buffer: 30 mM Tris–HCl (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 3 mM ATP, 15 mM mercaptoethanol) in the presence of varying concentrations of the test compound. The mixture was incubated at 37 °C for 1 h and the reaction was terminated by addition of 2 μl of 10% SDS and 2 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to electrophoresis through a 0.8% agarose gel containing 0.5 mg/ml ethidium bromide in TBE buffer (90 mM Tris–borate and 2 mM EDTA). The gels were stained with ethidium bromide and photographed under UV light. For the quantitative determination of topoisomerase concentration activity, photographic negatives were scanned and the area representing supercoiled DNA, migrating as a single band at the bottom of the gel was measured using UVI-KS4000i gel documentation and analysis system (SyngenBiotech, USA). The concentrations of the inhibitor that prevented 50% of the supercoiled DNA from being converted into relaxed DNA (IC_{50} values) were determined by averaging the data from at least three experiments.

2.2.6. Ethidium displacement assay

Fluorescence was measured using a Hitachi spectrophotometer F-2500 FL (Tokyo, Japan) at room temperature. The DNA-ethidium complex was excited at 546 nm and the fluorescence was measured at 595 nm. To 2 ml of ethidium bromide (5.0×10^{-6} M) in 10 mM Tris–HCl (pH 7.4), 75 mM NaCl buffer solution, containing 25 μl of DNA solution ($A_{260} = 2$) was added, and the maximum fluorescence was measured. Aliquots of a 10 mM stock test compound solution were then added to the DNA–ethidium solution, and the fluorescence was measured after each addition until a 50% reduction of fluorescence had occurred. Theoretical curves were fit to the fluorescence intensity data points with nonlinear least-squares computer routines. The apparent binding constant was calculated from $K_{EtBr}[EtBr] = K_{app}[drug]$, where [drug] = the concentration of the test compound at a 50% reduction of fluorescence and K_{EtBr} is known [30].

2.2.7. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of the cells in growth medium with varying concentrations of melphalan or AB4 for 24 h with 5-[^{3}H] proline (5 μCi/ml, 28 Ci/mmol) as described previously [31].

Incorporation of tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky [32]. Results are shown as combined values for cell plus medium fractions.

2.2.8. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara [33]. Protein concentration was measured by the method of Lowry [34]. Enzyme activity is reported as nanomoles of released proline during 1 min/mg of supernatant protein.

2.2.9. Immunoprecipitation

Subconfluent cells in six-well plates were rinsed with phosphate-buffered saline, scraped out of the wells and centrifuged at 1000 × *g* for 3 min. Then the cells (from six wells) were solubilized with lysis buffer containing 10 mM Tris–HCl, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, at 4 °C for 10 min. The insoluble material was removed by centrifugation at 10,000 × *g* for 5 min at 4 °C. Supernatant containing 100 μg of protein was added to 100 μg of Protein A-Sepharose that had been linked to primary antibody in the following manner. Protein A-Sepharose was washed three times with lysis buffer and 100 μl of suspension containing about 100 μg of beads was incubated for 1 h at 4 °C with 20 μl of primary antibody. Then, the conjugate was incubated for 1 h at 4 °C with shaking. Immunoprecipitate was washed four times with lysis buffer. Proteins were released from the beads by boiling in SDS sample buffer and loaded onto a 10% SDS-polyacrylamide gel. The immunoprecipitates were analyzed by Western immunoblot.

2.2.10. SDS-PAGE

Slab SDS/PAGE was used according to the method of Laemmli [35].

2.2.11. Western immunoblot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris–HCl, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2-μm pore-sized nitrocellulose at 100 mA for 1 h by using a LKB 2117 Multiphor II electrophoresis unit. The nitrocellulose was incubated with polyclonal antibody against human prolidase at concentration 1:3000, monoclonal antibody against IGF-I receptor at concentration 1:500, monoclonal antibody against β_1-integrin subunit, FAK, and Shc at concentration 1:1000, monoclonal antibody against Grb-2 and phosphorylated MAPK at concentration 1:5000 or monoclonal antibody against Sos-protein at concentration 1:250 in 5% dried milk in Tris buffered saline with Tween 20 (TBS-T) (20 mmol/l Tris–HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 h. In order to analyze prolidase and FAK, second antibody-alkaline phosphatase conjugated anti-Rabbit immunoglobulin (whole molecule) was added at concentration 1:5000; in order to analyze the β_1-integrin subunit, Shc, Grb-2, Sos-protein and phosphorylated MAP-kinases second antibody-alkaline phosphatase conjugated anti-Mouse immunoglobulin (whole molecule) was added at concentration 1:7500; and in order to analyze IGF-I receptor second antibody-alkaline phosphatase conjugated anti-Goat immunoglobulin (whole molecule) was added at concentration 1:5000. All antbodies were diluted in TBS-T and incubated for 60 min under gentle shaking. Then nitrocellulose was washed with TBS-T (5 min × 5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

2.2.12. Statistical analysis

In all experiments, the mean values for three assays ±standard deviations (S.D.) were calculated. The results were submitted to statistical analysis using the Student's *t*-test. Differences were considered as a significant at $p < 0.05$. Mean values, the standard deviations and the number of measurements in the group (*n*) are presented in the figures. These statistical analyses were carried out using Origin 7.5 software (OriginLab, USA).

3. Results

The synthetic route of AB4 is outlined in Fig. 1. In our previous work the 5-[4-(*N*-isopropylamidino)phenyl]-2-furancarboxylic **1** was synthesized [28] by using convenient routes in good yields. The amidinoacid **1** was conjugated with a melphalan methyl ester in the presence of *N*,*N*′-carbonyldiimidazole (DCI) as condensing agent in DMF at 0 °C to give the compound AB4 in good yield (Fig. 1). Melphalan methyl ester was synthesized

Table 1 – Viability of MCF-7 and MDA-MB-231 cells treated for 24 h with different concentrations of AB4 and melphalan

Concentration (μM)	Viability of cells (% of control)[a]			
	AB4		Melphalan	
	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
0	100	100	100	100
10	77 ± 2	65 ± 2	91 ± 2	92 ± 2
25	68 ± 2	57 ± 2	80 ± 2	82 ± 2
50	59 ± 2	47 ± 2	74 ± 2	74 ± 2
75	44 ± 2	35 ± 2	65 ± 2	66 ± 2
100	37 ± 2	25 ± 2	58 ± 2	57 ± 2
125	30 ± 2	22 ± 2	50 ± 2	52 ± 2
150	23 ± 2	18 ± 2	45 ± 2	47 ± 2

[a] Mean values ± S.D. from three independent experiments done in duplicate are presented.

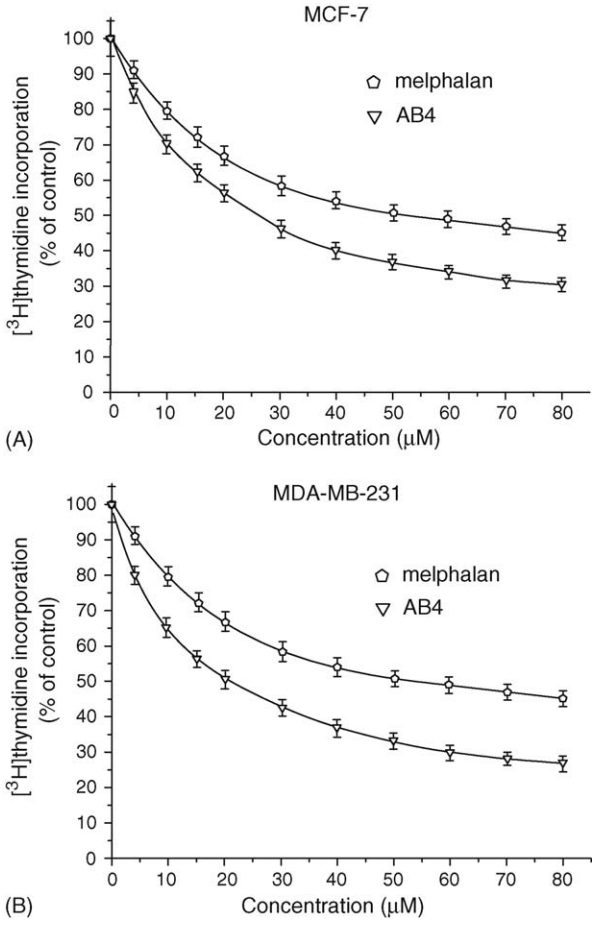

Fig. 2 – Antiproliferative effects of melphalan and AB4 in cultured breast cancer MCF-7 (A) and MDA-MB-231 (B) cells as measured by inhibition of [^{3}H]thymidine incorporation into DNA. Mean values ± S.D. of three independent experiments (n = 4) done in duplicates are presented.

according to the method reported by Rachele [36] in which melphalan was reacted with 2,2-dimethoxypropane in the presence of conc. HCl. AB4 was isolated as the hydrochloride salt by dissolving the free base in acetone and adding a few drops of hydrochloric acid. The chemical structure of AB4 was proved by NMR and elemental analysis.

In order to compare cytotoxicity of AB4 and the parent drug, cell viability of breast cancer cells was measured by the method of Carmichael et al. [29] using tetrazolinum salt (Table 1). We have found that amidine analogue of melphalan, AB4 decreased the number of viable cells in both estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells. Although the cytotoxicity was concentration-dependent in both cell lines, it was more pronounced at shorter times in MDA-MB-231 than in MCF-7. AB4 in both MDA-MB-231 and MCF-7 was proved to be more potent than melphalan, with IC_{50} values of 45 ± 2 and 62 ± 2 μM, respectively, compared to 130 ± 2 and 125 ± 2 μM for melphalan.

The data were corroborated by cell proliferation assay (Fig. 2). The profiles of DNA synthesis were found to be similar in MCF-7 and MDA-MB-231 (Fig. 2). The concentrations of AB4 required to inhibit [^{3}H]-thymidine incorporation into DNA by 50% (IC_{50}) in MDA-MB-231 was found to be 23 ± 2 μM, suggesting higher cytotoxic potency compared to melphalan ($IC_{50} = 77 \pm 2$ μM). The concentrations of AB4 and melphalan required for 50% inhibition of [^{3}H]-thymidine incorporation into DNA in breast cancer MCF-7 (IC_{50}) was found to be 30 ± 2 and 75 ± 2 μM, respectively.

To test whether cytotoxic and antiproliferative properties of AB4 were related to DNA-binding and topoisomerase I/II inhibition, the drug was evaluated in a cell-free system. The binding affinities of AB4, melphalan, netropsin and distamycin to calf thymus DNA, T4 coliphage DNA, and synthetic polymers poly(dA-dT)$_2$ and poly(dG-dC)$_2$ were compared by using the ethidium displacement assay [30,37]. Melphalan appeared totally inactive in the EtBr/DNA displacement assay at concentrations up to 200 μM, contrasting with the effects of AB4 or netropsin and distamycin A. Table 2 summarizes the results for those ligands that affected the fluorescence due to the intercalated ethidium at pH 7.4. The affinity constant of AB4 was found at about 2×10^5 which indicates a moderate interaction with the calf thymus DNA. The high value of binding constants of AB4 for T4 coliphage DNA provide evidence for its minor-groove selectivity, because the major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residues [38]. The homopolymer DNA-binding data shown in Table 2 characterizes the affinity of AB4 for a more limited set of DNA-binding sites and can indicate of base-sequence specificity for DNA-binding molecules. AB4 was found to interact with a GC base pair, though the binding affinity were weak compared with that for an AT base pair (Table 2). The binding constant of AB4 to poly(dC-dG)$_2$ polymer is almost four times lower than the association constant for binding of AB4 to poly(dA-dT)$_2$. Since calf thymus DNA contains random sequences and therefore fewer AT sites than poly(dA-dT)$_2$, the selectivity of AB4 is further demonstrated by their much weaker binding to calf thymus DNA compared to poly(dA-dT)$_2$.

Table 2 – DNA binding and topoisomerase I/II inhibitory effect of netropsin, distamycin and AB4

Ligand	ct DNA[a] ($K_{app} \times 10^5$ M^{-1})	T4 DNA[a] ($K_{app} \times 10^5$ M^{-1})	poly(dA-dT)$_2$[a] ($K_{app} \times 10^5$ M^{-1})	poly(dG-dC)$_2$[a] ($K_{app} \times 10^5$ M^{-1})	Inhibition of topo[b] I (μM)	Inhibition of topo[b] II (μM)
Netropsin	8.7	8.3	875	2.5	20	5
Distamycin	7.5	6.4	340	2.0	30	5
AB4	2.0	1.8	3.9	1.1	125	25

[a] The error for netropsin, distamycin and AB4 is $\pm 0.2 \times 10^5$ M^{-1}.
[b] Topo, topoisomerase.

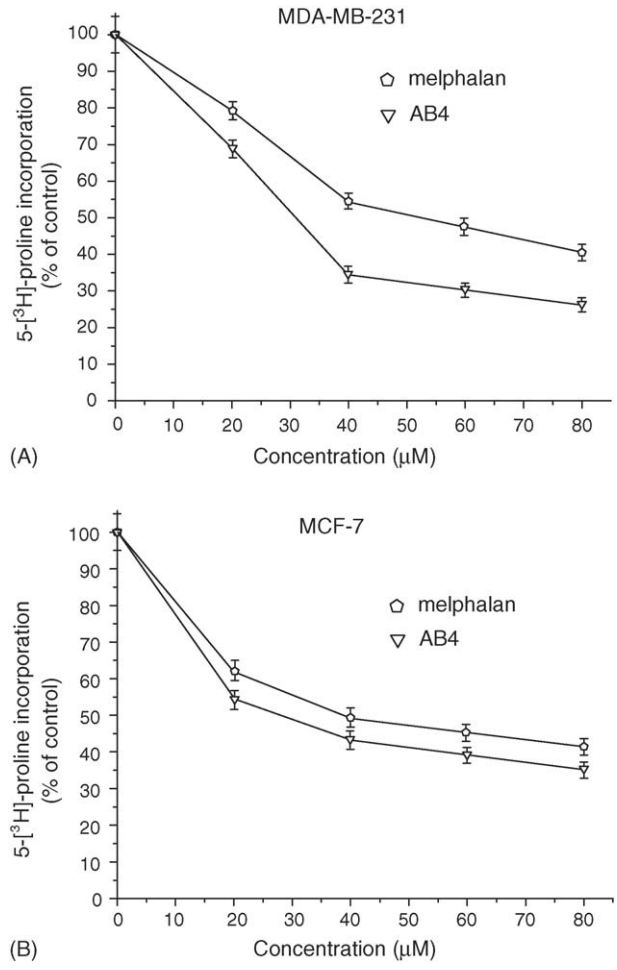

Fig. 3 – Collagen synthesis, measured by 5-[^{3}H]-proline incorporation into proteins susceptible to the action of bacterial collagenase, in breast cancer MDA-MB-231 (A) and MCF-7 (B) cells cultured for 24 h in the presence of different concentration of melphalan and AB4. Mean values from three independent experiments done in duplicates ±standard deviation (S.D.) are presented.

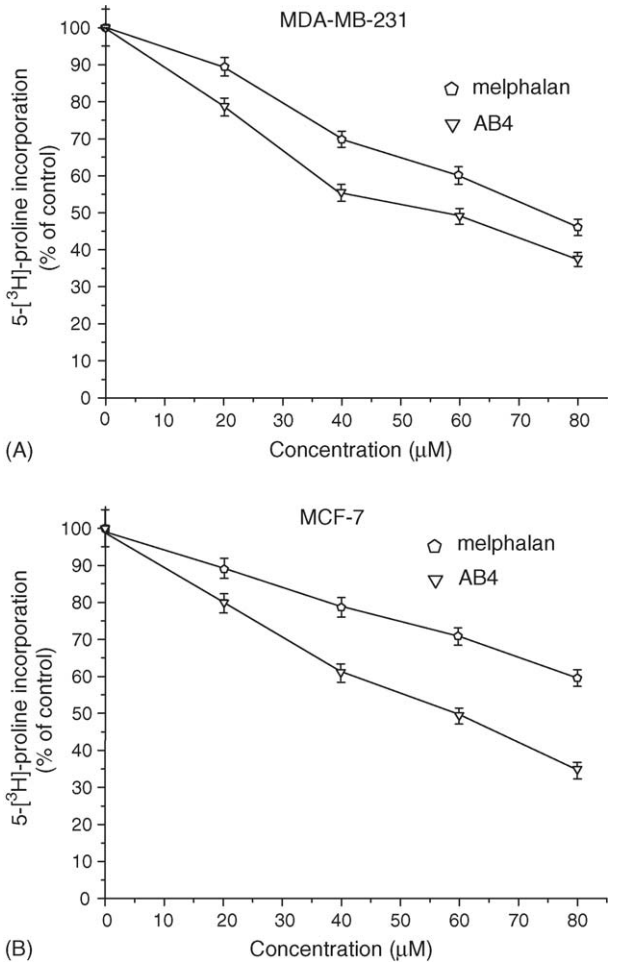

Fig. 4 – Total protein synthesis, measured by 5-[^{3}H]-proline incorporation into proteins, in breast cancer MDA-MB-231 (A) and MCF-7 (B) cells cultured for 24 h in the presence of different concentration of melphalan and AB4. Mean values from three independent experiments done in duplicates ±standard deviation (S.D.) are presented.

The ability of AB4 to inhibit topoisomerase I and II activity was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis. Melphalan as a control was ineffective in this assay. It was found that AB4 evokes topoisomerase II inhibitory activity with 50% inhibitory concentrations (IC_{50}) of 25 μM (Table 2). Moreover, AB4 inhibited the topoisomerase I—mediated relaxation of supercoiled DNA at a concentration of 125 μM. It may suggest that the topoisomerase II inhibiting activity contributes to the cytotoxicity of AB4.

Collagen biosynthesis was measured in MCF-7 and MDA-MB231 breast cancer cells treated with various concentrations of melphalan and AB4 for 24 h. As shown on Fig. 3 in both cell lines AB4 was found to be more effective inhibitor of collagen biosynthesis than melphalan. The inhibitory effect was dose dependent. IC_{50} for melphalan and AB4 (in MCF-7: 40 and 30 μM, in MDA-MB231: 57 and 32 μM, respectively) showed specific inhibitory effect of AB4 on collagen biosynthesis. Similar effect was observed regarding total protein synthesis except higher IC_{50} values (Fig. 4).

Important role in collagen biosynthesis plays prolidase. In MCF-7 cells treated with melphalan prolidase activity was not affected. However AB4 decreased the enzyme activity to 78% of control value (Fig. 5B). In MDA-MB-231 cells melphalan inhibited the enzyme activity to 64% of control value (Fig. 5A). AB4 was found to be more effective inhibitor of prolidase. It reduced the activity to 31% of control value (Fig. 5A). Decreased prolidase activity in both cell lines was related to the changes in the enzyme expression as shown by western immunoblot analysis (Fig. 6A and B).

Collagen biosynthesis and prolidase activity are regulated by signal generated by β_1-integrin receptor. The expression of the receptor in both cell lines was measured by western immunoblot. As shown on Fig. 7 treatment of the cells with 20 μM of AB4 evoked stronger inhibitory effect than 20 μM of melphalan on the protein expression in both MDA-MB231 and MCF-7 cell lines.

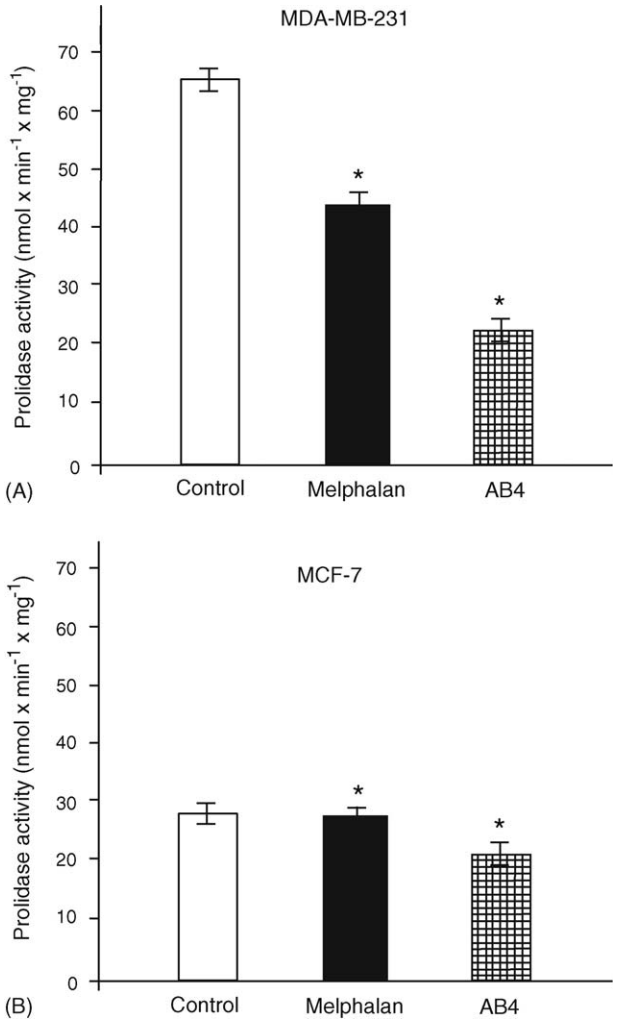

Fig. 5 – Prolidase activity in breast cancer MDA-MB-231 (A) and MCF-7 (B) cells treated for 24 h with 20 μM melphalan and 20 μM of AB4. Mean values from three independent experiments done in duplicates ±standard deviation (S.D.) are presented. *$P < 0.05$, significantly different when compared with control (Student's *t*-test).

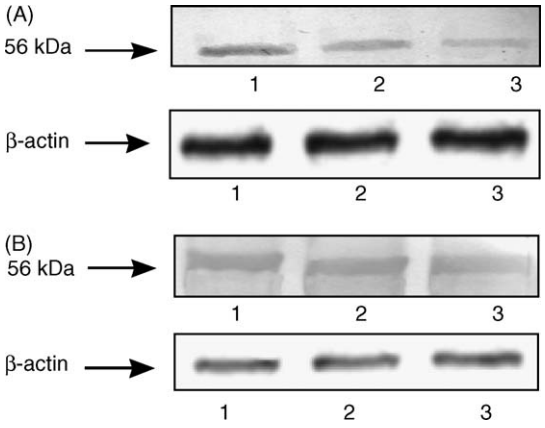

Fig. 6 – (A) Western immunoblot analysis for prolidase in control breast MDA-MB-231 cells (1) and the cells cultured for 24 h in the presence of 20 μM melphalan (2) or AB4 (3). (B) Western immunoblot analysis for prolidase in control breast MCF-7 cells (1) and the cells cultured for 24 h in the presence of 20 μM melphalan (2) or AB4 (3), (C) β-actin served as a loading control. This profile is representative of at least three different experiments. Samples used to experiments consisted of 30 μg of protein from pooled cell extracts ($n = 6$). The arrows indicate the molecular mass of standards.

Expression of proteins involved in signaling cascade activated by β_1-integrin receptor was also evaluated. As shown on Fig. 7 treatment of both cell lines with 20 μM melphalan for 24 h did not affected the expression of Shc, Grb-2, Sos-1 and phosphorylated MAP-kinases ERK1 and ERK2. Expression of FAK-kinase was decreased in MDA-MB231, as compared to control cells while in MCF-7 cells the expression of the protein was unchanged. In opposite to that the AB4 inhibited expression of Shc and MAP-kinases in both cell lines. Decreased expression of FAK-kinase was found only in MDA-MB-231 cells. Grb-2 and Sos-1 proteins expression was not affected in both cell lines (Fig. 7).

Collagen biosynthesis is also known to be regulated by IGF-I receptor. Activation of the receptor plays important role in cellular transformation, mitogenesis and inhibition of apoptosis. The expression of the receptor in both cancer cell lines treated with 20 μM melphalan and 20 μM of AB4 for 24 h was inhibited by both compounds. Down regulation of the receptor was more pronounced by AB4 (Fig. 7).

4. Discussion

The present study was undertaken to extend our recent findings related to antineoplastic activity of amidine analogues of alkylating compounds [5–7,9,10]. We have found that amidine analogue of melphalan is a more potent inhibitor of DNA biosynthesis than the parent drug, melphalan in MDA-MB-231 and MCF-7 breast cancer cell lines. However the inhibitory effect was more pronounced in MDA-MB-231 cells. The effect could be related to the ability of AB4 to bind to minor-groove in AT sequences of DNA, that results in increase of antiproliferative activity of AB4 in both cell lines as compared to melphalan. AB4 showed also higher cytotoxicity in both cell lines, than the parent drug.

The amidine analogue of melphalan AB4 as netropsin and distamycin forms hydrogen bonds along the minor groove of DNA through the terminal amidine moiety and amide group. The binding of AB4 in the minor groove of DNA may prevent binding of regulatory proteins or transcription factors to DNA promotors, as has been shown for other amidines [6,7,43]. It is well known that sites of topoisomerase mediated DNA cleavage are not randomly distributed in DNA fragments, hence this enzyme seems to recognize specific sequences in a given substrate. This topological enzyme binds at least in part to AT rich sequences in the minor groove [39–42]. The AT base pair specificity shown by amidine analogue of melphalan (AB4) (Table 2) could be the result of the preferential binding of highly electropositive

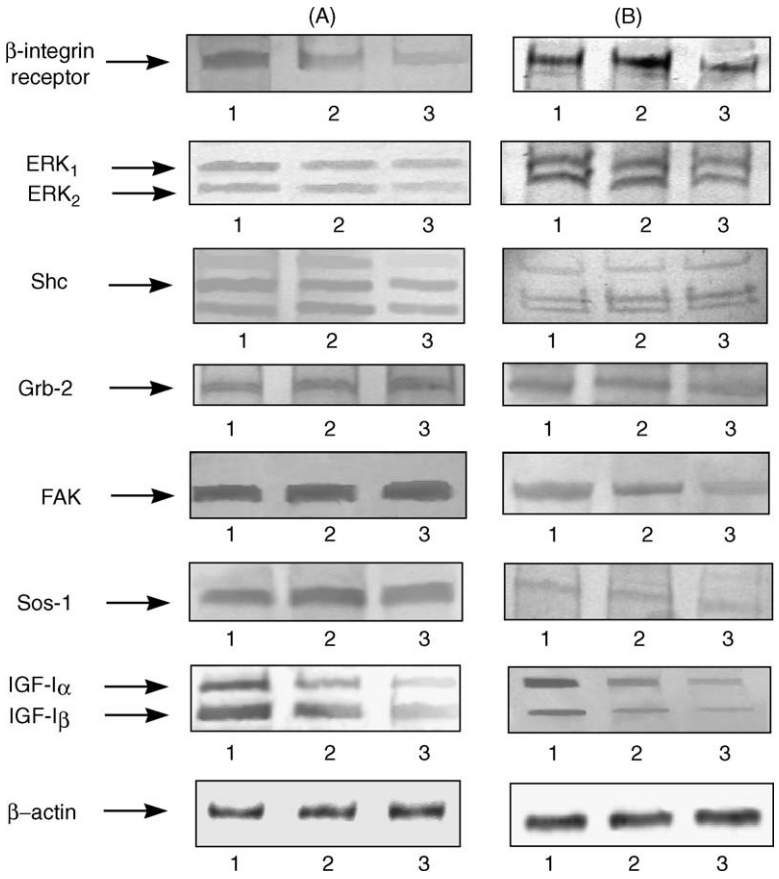

Fig. 7 – (A) Western immunoblot analysis for β_1-integrin receptor, phosphorylated MAP-kinases ERK_1 and ERK_2, Shc, Grb-2, FAK, Sos-1 and IGF-I receptor in control breast cancer MCF-7 cells (1) and the cells incubated for 24 h in the presence of 20 μM melphalan (2) or 20 μM AB4 (3). (B) Western immunoblot analysis for β_1-integrin receptor, phosphorylated MAP-kinases ERK_1 and ERK_2, Shc, Grb-2, FAK, Sos-1 and IGF-I receptor in control breast cancer MDA-MB-231 cells (1) and the cells incubated for 24 h in the presence of 20 μM melphalan (2) or 20 μM AB4 (3). β-actin served as a loading control. The results represents at least three different experiments. Samples used to the experiments consisted of 30 μg of protein from pooled cell extracts (n = 6).

AB4 to the most electronegative region of DNA, rich in AT bases in the minor groove [43]. It is likely that the ability of AB4 to inhibit the activity of topoisomerase II (Table 2) is due to blockade of the binding of the enzyme to DNA. It is possible that AB4 acts through inducing a conformational change in the DNA and hindering the formation of the cleavable complex.

The topoisomerase II-targeting drugs can be classified as either topoisomerase II poisons, which act by stabilizing enzyme-DNA cleavable complexes leading to DNA breaks, or topoisomerase II catalytic inhibitors, which act at stages in the catalytic cycle of the enzyme where both DNA strands remain intact and no DNA strand breaks occur [39–42]. Treatment with the classical topoisomerase II poison, e.g. etoposide, results in the production of linear DNA which demonstrates that these compounds stabilise DNA–topoisomerase II covalent complexes and hence stimulate a double-strand cleavage by the enzyme. Conversely, with AB4 no band corresponding to linear DNA was detected in the presence of enzyme (data not shown), implying that this compound do not act as topoisomerase poisons.

DNA binding and DNA synthesis assays suggest that the combined effects of alkylation and DNA minor groove binding might be responsible for the cytotoxic activity of AB4. The mechanism by which the alkylation induced by AB4 leads to cytotoxicity remains unclear. We hypothesize, on the basis of the present results, that simultaneous DNA-binding and inhibition of topo II activity leads to increased cytotoxicity of AB4 compared to the parent drug, melphalan. It is probable that deregulation of DNA replication and transcription by inhibition of topoisomerase II activity contribute significantly to the cytotoxicity of AB4 in addition to primary drug-DNA reaction products.

The results of the study suggest that AB4 may have another consequences for metabolism of breast cancer cells. We showed that AB4 is a stronger inhibitor of collagen biosynthesis than melphalan. Decreased amount of collagen in extracellular matrix is known to enhance motility and invasion of neoplastic cells [44], but it also contribute to inhibition of cell growth and induction of apoptosis [45].

The new compound AB4 shows also higher potency than melphan to inhibit prolidase activity in MDA-MB-231 than

melphalan. Prolidase activity in MCF-7 was only slightly affected by both studied compounds. This is probably due to very low activity of this enzyme in MCF-7 cells. Prolidase is a metaloproteinase which supplies free proline for collagen biosynthesis and cell growth. Lack of the enzyme impedes the efficient recycling of proline for collagen resynthesis [48] and cell growth [49]. The efficiency of recycling of proline for collagen biosynthesis was found to be about 90% [48]. Previously we found the link between collagen synthesis and prolidase activity in cultured skin fibroblasts treated with antiinflammatory drugs [50], during experimental aging of the cells [51], fibroblasts chemotaxis [52], and cell surface integrin receptor ligation [53]. This phenomenon could be another factor determining higher efficiency of AB4 to inhibit collagen biosynthesis and growth of MDA-MB-231 in comparison to the MCF-7 cells.

An important role in induction of these processes plays β_1-integrin receptor. Our findings show that incubation of MDA-MB-231 and MCF-7 cells in the presence of 20 μM of AB4 for 24 h caused significant decrease in expression of β_1-integrin subunits as compared to the control cells or cells incubated in the presence of 20 μM of melphalan. This function of AB4 may suppress growth of cancer cells, induce apoptosis [45,46] as well attenuate invasion of breast cancer cells [47]. We found that down regulation of the expression of β_1-integrin receptor by AB4 was accompanied by decrease in the expression of Shc and MAP-kinases in both cell lines. No differences however in the expression of those proteins were found in both cell lines treated with melphalan. AB4 also inhibited the expression of FAK in MDA-MB-231 cells. However, no differences in the expression of Grb-2 and Sos-1 between control cells and cells treated with AB4 or melphalan were found. It suggests that other signaling pathways, that involve Grb-2 and Sos-1 participation, may be resistant to the action of studied compounds.

Another important finding of AB4 activity is inhibition of phospho-ERK's activation. Up regulation of those kinases was found in various breast cancers [54]. Blocking of those kinases was found to have pro-apoptotic and antiproliferative effect on MDA-MB231, that indicates a new target in the treatment of breast malignancies [55,56].

Treatment of the cells with 20 μM of AB4 decreased significantly expression of IGF-I receptor in both cell lines comparing to the control cells or cells treated with 20 μM melphalan. IGF-I receptor is involved in cellular transformation, mitogenesis and inhibition of apoptosis [26]. Therefore inhibition of the receptor may represent approach to the inhibition of tumor growth. Blockade of the receptor [57] or down regulation of its expression [58] reduces cancer proliferation and induces apoptosis. Decreased expression of IGF-I receptor by AB4 could explain also decreased biosynthesis of collagen in the cells treated with AB4, since IGF-I is a most potent stimulator of collagen biosynthesis [59]. Although the mechanism of antitumour action of AB4 is not yet fully elucidated, it may be due to its ability to inhibit the binding of some transcription factors to their consensus sequences in DNA, thereby preventing transcription.

The presented data suggest that amidine analogue of melphalan (AB4) impairs more efficiently growth and metabolism of MDA-MB-231 and MCF-7 breast cancer cells than the parent drug.

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