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Amidine analogue of chlorambucil is a stronger inhibitor of protein and DNA synthesis in breast cancer MCF-7 cells than is the parent drug

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Abstract

A novel amidine analogue of chlorambucil-*N*-(2-(4-(4-bis(2-chloroethyl)aminophenyl)butyryl)aminoethyl)-5-(4-amidinophenyl)-2-furancarboxamide hydrochloride (AB_1) and the parent drug were compared for their effects on collagen and DNA biosynthesis in breast cancer MCF-7 cells. IC_{50} values for chlorambucil and AB_1 for collagen biosynthesis were found to be about 33 and 13 μM, respectively. The greater potency of AB_1 to suppress collagen synthesis was found to be accompanied by a stronger compared with chlorambucil inhibition of prolidase activity and expression. The phenomenon was related to inhibition of β_1-integrin and IGF-I receptor-mediated signaling caused by this compound. The expression of β_1-integrin receptor, as well as Src, son of sevenless protein (SOS) and phosphorylated mitogen activated protein (MAP) kinases (MAPK), extracellular-signal-regulated kinase 1 (ERK_1) and kinase 2 (ERK_2) but not focal adhesion kinase $pp125^{FAK}$ (FAK), Shc, and Grb-2 was significantly decreased in cells incubated for 24 h with 10 μM AB_1 compared to the control, whereas in the same conditions chlorambucil did not evoke any changes in expression of all these signaling proteins, as shown by Western immunoblot analysis. Furthermore, AB_1 induced a stronger down-regulation of the expression of IGF-I receptor and evoked a higher antiproliferative effect. During 12 and 24 h of incubation AB_1 decreased DNA biosynthesis by about 33 % and 51 % of the control, whereas chlorambucil decreased it by about 19 % and 35 %, respectively. These data suggest that the amidine analogue of chlorambucil is a stronger inhibitor of protein and DNA synthesis in MCF-7 cells than is the parent drug.

Keywords: Chlorambucil; Collagen biosynthesis; Prolidase; β_1-integrins; IGF-I receptor; MCF-7

1. Introduction

Chlorambucil is a nitrogen mustard and is used in the pharmacotherapy of chronic lymphatic leukemia, lymphomas, and advanced ovarian and breast carcinomas (Armitage, 1993). Similar to other alkylating agents, the dose-limiting factor of chlorambucil is hematologic suppression (McLean et al., 1980). Localizing the cytotoxic action of chlorambucil to a target region would have the major advantage of diminishing the drug dose and, consequently, undesirable side effects in normal tissues. Targeting nitrogen mustards to DNA by attachment of a DNA minor groove binding carrier, such as distamycin or netropsin, reduces the loss of active drug due to reaction with other cell components and makes it possible to direct alkylation in both a sequence-specific and region-specific manner (Denny, 2001). In our previous study, we described the synthesis and cytotoxic activity of chlorambucil derivatives appropriately modified with linkers that allow attachment to specific DNA sequences (Bielawska et al., 2003). The compound AB_1 (Fig. 1) is the most cytotoxic amidine analogue of chlorambucil among a series of derivatives we have synthesized to date.

One of the characteristic features of breast cancer cells is a deregulation of their interaction with extracellular matrix proteins (Boudreau and Bissel, 1998). 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, mi-

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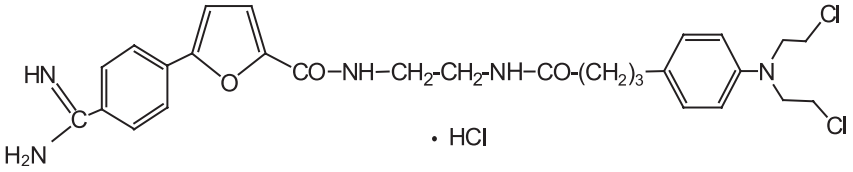

Fig. 1. The chemical structure of the amidine analogue of chlorambucil (AB_1).

gration, proliferation, progression and survival (Albeda and Buck, 1990). Therefore changes in the quantity, structure and distribution of collagens caused by anticancer agents may affect MCF-7 cell metabolism and function (Ioachim et al., 2002).

Collagen biosynthesis and prolidase activity are coordinately regulated in MCF-7 cells (Wołczyński et al., 2001). Prolidase [EC 3.4.13.9] catalyzes the hydrolysis of imidodipeptides (Myara et al., 1984), releasing proline, which is used for collagen resynthesis (Jackson et al., 1975; Yaron and Naider, 1993) and cell growth (Emmerson and Phang, 1993). Prolidase activity in MCF-7 cells is regulated by the signal mediated by activated β_1-integrin receptors (Pałka and Phang, 1994, 1998). Stimulated β_1-integrin receptors induce autophosphorylation of non-receptor focal adhesion kinase $pp125^{FAK}$ (FAK) (Hanks et al., 1992), 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), Ras and Raf proteins (Juliano, 1996) and subsequently, two mitogen activated protein (MAP) kinases (MAPK), extracellular-signal-regulated kinase 1 (ERK_1) and kinase 2 (ERK_2) (Seger and Krebs, 1995). 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 (Labat-Robert and Robert, 2000).

Stimulation of collagen biosynthesis and cell growth requires participation of the insulin-like growth factor I (IGF-I) (Goldstein et al., 1989). IGF-I, acting predominantly through the IGF-I receptor (Le Roith et al., 1995), has been demonstrated to stimulate the proliferation, promote the survival and enhance the metastatic potential of breast cancer cells (Gross and Yee, 2003) and to prevent them from undergoing apoptosis (Baserga et al., 1997). 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 (Butler et al., 1998).

In this study, the effects of the amidine analogue of chlorambucil (AB_1) on collagen and DNA biosynthesis, β_1-integrin receptor, IGF-I receptor, FAK, Src, Shc, Grb-2, SOS and phosphorylated MAP-kinases (ERK_1 and ERK_2) expression in MCF-7 cells were compared to those of chlorambucil.

2. Materials and methods

2.1. Materials

Anti-FAK antibody, anti-Human IGF-I receptor antibody, anti-Goat immunoglobulin antibody, anti-mouse immunoglobulin antibody, aprotinin, bacterial collagenase, chlorambucil, 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, monoclonal anti-v-Src antibody, phenylmethylsulfonyl fluoride and Protein A-Sepharose were provided by Sigma, 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, 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). Monoclonal anti-SOS, anti-Grb-2 and anti-Shc antibodies were obtained from Becton Dickinson, USA. Monoclonal anti-β_1-integrin antibody was obtained from ICN Biomedicals, 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, USA. [^{3}H] thymidine (6.7 Ci/mmol) was purchased from ICN Biomedicals and Scintillation Coctail "Ultima Gold XR" from Packard, USA.

2.2. Chemistry

The synthesis of *N*-(2-(4-(4-bis (2-chloroethyl) aminophenyl) butyryl) aminoethyl)-5-(4-amidinophenyl)-2-furancarboxamide hydrochloride (AB_1) was reported previously (Bielawska et al., 2003).

2.3. Tissue culture

All studies were performed with breast cancer MCF-7 cells cultured in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium-free phosphate-buffered saline, counted in hemocytometers, and plated at 5×10^5 cells per well in six-well plates (Nunc, Wiesbaden, Germany) in 2 ml of growth medium. Cells reached 80% of confluence at day 3 and in most cases such cells were used for the assays.

2.4. DNA production

After 12, 24, 48 and 72 h of treatment of cells with growth medium supplemented with 10 μM of chlorambucil or AB_1, 0.5 μCi [^{3}H] thymidine (6.7 Ci/mmol) was added to each well and the cells were incubated in 37 °C with 5% CO_2 for 4 h. After that time the cell surface was rinsed three times with 1 ml 0.05 mol/l Tris–HCl and two times with 5% trichloracetic acid. Then, the cells were dissolved in 1 ml 0.1 mol/l NaOH containing 1% SDS. Cell lysate was added to 4 ml of scintillation liquid and the radioactivity of the samples was measured.

2.5. Collagen production

Incorporation of radioactive precursor into proteins was measured after labeling of the cells in growth medium with varying concentrations of chlorambucil or AB_1 for 24 h with 5-[^{3}H] proline (5 μCi/ml, 28 Ci/mmol) as described previously (Oyamada et al., 1990). Incorporation of tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky (Peterkofsky et al., 1991). Results are shown as combined values for cell plus medium fractions.

2.6. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara (Myara et al., 1982). Protein concentration was measured by the method of Lowry (Lowry et al., 1951). Enzyme activity is reported as nanomoles of released proline during 1 min/mg of supernatant protein.

2.7. Immunoprecipitation

Subconfluent cells in 6-well plates were rinsed with phosphate-buffered saline, scraped out of the wells and centrifuged at $1000 \times g$ for 3 min. Then the cells (from 6 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 $10{,}000 \times g$ 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.

2.8. SDS-PAGE

Slab SDS/PAGE was used according to the method of Laemmli (1970).

2.9. Western immunoblot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 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, Src, 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, Src, 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 (5×5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

3. Results

Collagen biosynthesis and prolidase activity were measured in breast cancer cells treated for 24 h with different

concentrations of the studied compounds. As can be seen in Fig. 2 both chlorambucil and AB_1 decreased collagen biosynthesis in a dose-dependent manner; however, AB_1 was found to be a more potent inhibitor of the process. IC_{50} values of chlorambucil and AB_1 for collagen biosynthesis were about 33 and 13 μM, respectively. In both experiments IC_{50} values were calculated on the basis of the drug concentration in the medium of MCF-7 cells. Both chlorambucil and AB_1 induced a significant, dose-dependent decrease in prolidase activity, which plays an important role in the regulation of collagen biosynthesis. However, the potency of AB_1 to inhibit the enzyme activity was higher than that of chlorambucil (Fig. 3A). Furthermore, in both cases the inhibition of prolidase activity was accompanied by parallel changes in enzyme expression, as shown by Western immunoblot analysis (Fig. 3B).

Since it has been suggested that collagen biosynthesis and prolidase activity are activated by β_1-integrin-dependent signaling (Ivaska et al., 1999; Pałka and Phang, 1994), an expression of some proteins activated by this receptor was measured by Western immunoblot analysis. As can be seen in Fig. 4, a 24-h treatment of MCF-7 cells with 10 μM chlorambucil did not evoke changes in the expression of either β_1-integrin receptor or FAK, Src, Shc, Grb-2, SOS and phosphorylated MAP-kinases (ERK_1 and ERK_2) in comparison to that of the control cells. In contrast, a 24-h treatment with the same concentration of AB_1 led to a distinct decrease in the expression of β_1-integrin receptor, Src, SOS and phosphorylated MAP-kinases in comparison to that of the control cells. However, the expression of FAK, Shc and Grb-2 was the same as in the control cells or the cells incubated in the presence of chlorambucil (Fig. 4).

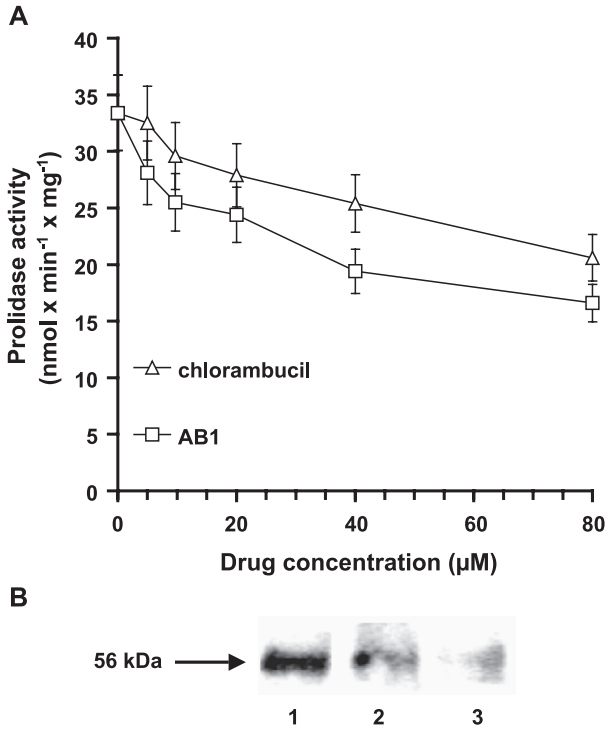

Fig. 3. (A) Prolidase activity in breast cancer MCF-7 cells treated for 24 h with different concentrations of chlorambucil or the amidine analogue of chlorambucil (AB_1). Mean values from three independent experiments done in duplicate are presented. (B) Western immunoblot analysis for prolidase in control breast cancer MCF-7 cells (1) and cells cultured for 24 h in the presence of 10 ìM chlorambucil (2) or the amidine analogue of chlorambucil (3). Samples used for electrophoresis consisted of 20 μg of protein from pooled cell extracts ($n = 6$). The arrows indicate the molecular mass of standards.

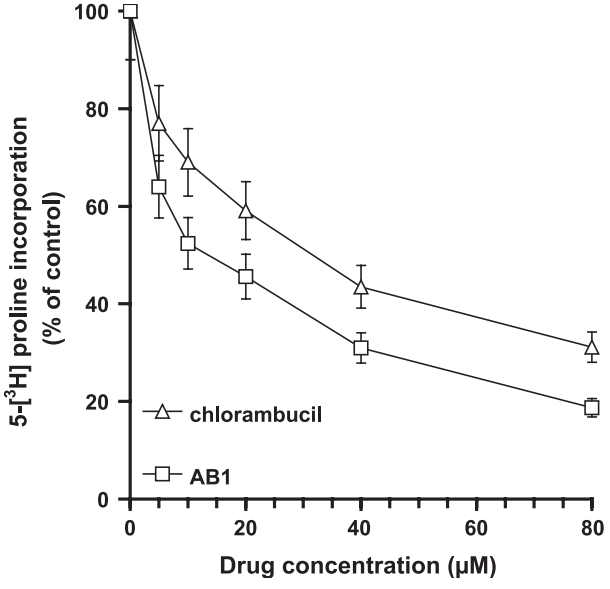

Fig. 2. 5-[^{3}H] proline incorporation into proteins susceptible to the action of bacterial collagenase in breast cancer MCF-7 cells cultured for 24 h in the presence of different concentrations of chlorambucil and amidine analogue of chlorambucil (AB_1). Mean values from three independent experiments done in duplicate are presented.

Considering the role of IGF-I receptor in cellular transformation, mitogenesis, collagen biosynthesis and inhibition of apoptosis, we decided to evaluate the effect of the compounds on the expression of this receptor. As shown in Fig. 5, both chlorambucil and AB_1 caused a slight decrease in IGF-I receptor expression; however, the down-regulation of the α subunit was more pronounced with AB_1.

The inhibitory effect of both drugs on IGF-I receptor expression indicates that this phenomenon may attenuate the proliferative activity of IGF-I. Therefore, [^{3}H] thymidine incorporation assay was performed as described in Materials and methods. As can be seen in Fig. 6, during the time course of the experiment DNA biosynthesis continuously decreased in the cells treated with either chlorambucil or AB_1. The antiproliferative activity of AB_1 appeared to be significantly higher than that of chlorambucil during 12 and 24 h of incubation. AB_1 decreased DNA biosynthesis in the cells by about 33% and 51% of control, whereas chlorambucil decreased it by about 19% and 35%, respectively. However, when the cells were

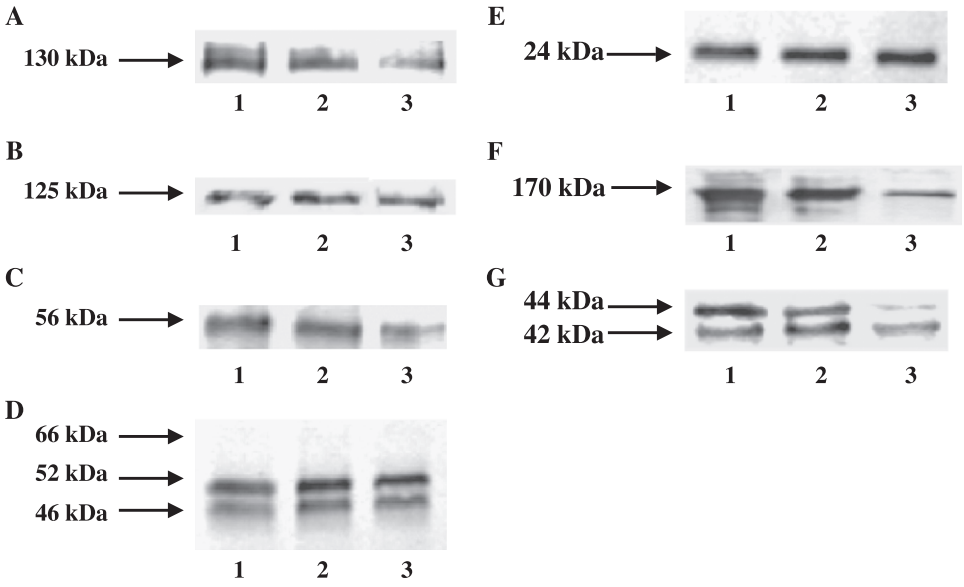

Fig. 4. Western immunoblot analysis for β_1-integrin receptor (A), FAK (B), Src (C), Shc (D), Grb-2 (E), SOS (F) and phosphorylated MAP-kinases-ERK_1, ERK_2 (G) in control breast cancer MCF-7 cells (1) and cells cultured for 24 h in the presence of 10 μM chlorambucil (2) or the amidine analogue of chlorambucil (3). Samples used for electrophoresis consisted of 20 μg of protein from pooled cell extracts ($n = 6$). The arrows indicate the molecular mass of standards.

incubated with the drugs for 48 and 72 h, a similar decrease in DNA synthesis was noticed.

4. Discussion

Alkylating agents are a major class of anticancer drugs for the treatment of various cancers. In our previous papers, we reported the synthesis and structure–activity studies of amidine analogues of chlorambucil derivatives, which appear to be a new class of cytotoxic minor groove binders and topoisomerase II inhibitors (Bielawska et al., 2003). The results of the present study suggest that this class of agents affect breast cancer cell metabolism and function more extensively than the parent drug, chlorambucil. First of all, AB_1 appeared to be more potent inhibitor of collagen biosynthesis and prolidase activity than chlorambucil. Inhibition of collagen biosynthesis may result in a decrease in the protein content of the extracellular matrix, which on one hand could promote neoplastic cells motility and invasion (Jonjic et al., 1993; Perumpanani and Byrne, 1999), but on the other could suppress cell growth or induce apoptotic cell death, if accompanied by an impaired collagen type I-integrin interaction (Boudreau et al., 1995; Juliano and Haskill, 1993). Furthermore, it has been demonstrated that blocking excessive β_1-integrin-mediated signals in breast cancer cells may induce morphological and biochemical reversion to a normal state (Hansen and Bissell, 2000; Weaver et al., 1997). We found that AB_1 caused a significant decrease in β_1-integrin subunit expression in the

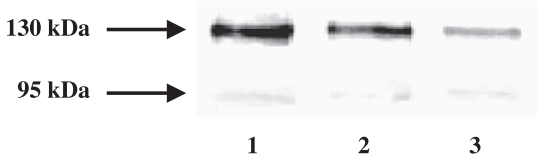

Fig. 5. Western immunoblot analysis for IGF-I receptor in control breast cancer MCF-7 cells (1) and cells cultured for 24 h in the presence of 10 μM chlorambucil (2) or the amidine analogue of chlorambucil (3). Samples used for electrophoresis consisted of 20 μg of protein from pooled cell extracts ($n = 6$). The arrows indicate the molecular mass of standards.

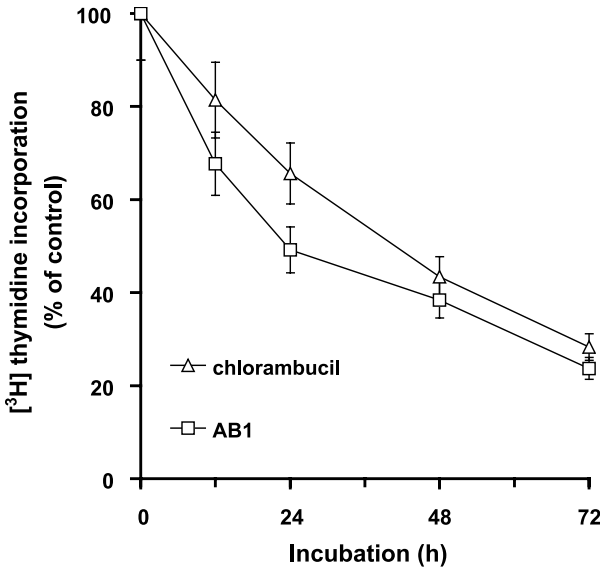

Fig. 6. Time course experiment for DNA biosynthesis (measured by [^{3}H] thymidine incorporation assay) in breast cancer MCF-7 cells incubated in the presence of 10 μM chlorambucil or the amidine analogue of chlorambucil (AB_1). Mean values from three independent experiments done in duplicate are presented.

studied cells, while the cells treated with the same concentration of chlorambucil showed no difference in receptor expression in comparison to the control. Interestingly, the down-regulation of β_1-integrins caused by AB_1 was not accompanied by any changes in FAK expression, even though the expression of Src, SOS and phosphorylated MAP-kinases (ERK_1 and ERK_2) significantly decreased. These data seem to confirm our previous findings showing that prolidase activity and collagen biosynthesis respond to a signal mediated by MAP-kinases, independently of FAK expression in MCF-7 cells (Surażyński and Pałka, 2001). The lack of a difference in the expression of FAK, Shc and Grb-2 between control cells and AB_1 or chlorambucil-treated ones may be due to activation of these proteins through other signal transduction pathways.

Nevertheless, the down-regulation of phosphorylated MAP-kinases (ERK_1 and ERK_2) expression evoked by AB_1 would be of benefit, considering the crucial role of these kinases in the promotion of cancer cell proliferation, differentiation and survival, as well as motility and invasion. Hyperexpression of MAP-kinases has been detected in various forms of breast cancer (Santen et al., 2002; Sivaraman et al., 1997) and, conversely, blocking the p44/42 MAPK signaling pathway by using MAPK kinase inhibitors, for instance 2-(2-amino-3-methoxyphenyl)-4*H*-benzopyran-4-one (PD 98059) has been found to have a strong antiproliferative effect on MCF-7 cells, which makes this pathway a potential target in the treatment of selected breast malignances (Hermanto et al., 2000).

IGF-I is known to play an important role in cell transformation, mitogenesis and inhibition of apoptosis (Baserga et al., 1997), and its involvement in carcinogenesis and cancer promotion is well established. It is also the most potent stimulator of collagen biosynthesis in tissues (Goldstein et al., 1989). The inhibition of IGF-I receptor signaling, through either receptor blockade (Maloney et al., 2003) or down-regulation of its expression (Xie et al., 1999), reduces cancer cell proliferation, promotes apoptosis and is thus a desirable goal in the suppression of tumor growth. Treatment of MCF-7 cells with chlorambucil or AB_1 decreased the expression of both the α and β subunits of IGF-I receptor and contributed to the inhibition of DNA biosynthesis, which was confirmed by the results of [^{3}H] thymidine incorporation assay. However, AB_1 was found to be a more potent inhibitor of these processes than chlorambucil. The down-regulation of IGF-I receptor α-subunit and antiproliferative activity during 12 and 24 h of incubation was much more pronounced with AB_1. Furthermore, the decrease in IGF-I receptor expression caused by this compound, as mentioned above, was accompanied by a decrease in the expression of SOS and phosphorylated MAP-kinases (ERK_1 and ERK_2), which suggests its strongest disruptive effect is on the IGF-I receptor-associated signaling pathway.

Taken together, the results of our study indicate that the amidine analogue of chlorambucil (AB_1) possesses a greater capacity to dysregulate MCF-7 cell metabolism than the parent drug, chlorambucil. The data presented here show that AB_1 evoked a stronger inhibition of prolidase activity and collagen biosynthesis, than did chlorambucil. Moreover, this novel anticancer agent induced a significant decrease in the expression of β_1-integrin receptor, Src, SOS and phosphorylated MAP-kinases (ERK_1 and ERK_2), whereas chlorambucil at the same concentration did not. Finally, AB_1 had a greater antiproliferative activity and induced a stronger down-regulation of the expression of IGF-I receptor. These data suggest that the amidine analogue of chlorambucil is a stronger inhibitor of protein and DNA synthesis in MCF-7 cells than is the parent drug.

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