Downregulation of clusterin mediates sensitivity to protein kinase inhibitors in breast cancer cells

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The efficacy of protein kinase inhibitors (PKIs) has been shown in clinical assays for cancer, but as isolated agents, they only have a modest effect. One of the most important characteristics of mitogen-activated PKIs is their ability to decrease the apoptotic threshold of cancer cells, sensitizing them to the action of other antiapoptotic agents. The secretory clusterin protein is an inhibitor of apoptosis with a cytoprotective function. We describe the use of clusterin-specific antisense oligonucleotides and siRNA to sensitize breast carcinoma cells to several PKIs. MCF-7 and MDA-MB-231 cells were treated with antisense oligonucleotide or siRNA to clusterin and the following PKIs: H-89, chelerythrine and genistein. The three inhibitors used in this study upregulated clusterin expression and treatments that included antisense oligonucleotide or siRNA to clusterin reduced the number of viable cells more effectively than did treatment with the drugs alone. Therefore, treatment with such combinations may benefit patients with breast cancer. *Anti-Cancer Drugs* 26:85–89

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Introduction

One of the primary goals in cancer research is to improve the effectiveness of cancer regimens. Numerous intracellular signalling proteins have been proposed as promising targets for blocking the malignancy of breast cancer cells, and protein kinases represent one such therapeutic target. Mitogen-activated protein kinase (MAPK) is very important in the regulation of proliferation and exerts a protective effect against apoptosis. The development of pharmacological inhibitors of specific clinical use for MAPK has opened up the possibility of utilizing the blockade of this pathway as a therapy to impede tumour growth. One of the most important characteristics of MAPK inhibitors is their ability to decrease the apoptotic threshold of cancer cells, sensitizing them to the action of other antiapoptotic agents [1].

The protein clusterin (CLU) plays an important role in various pathophysiological processes, such as tissue remodelling, lipid transport, complement regulation, reproduction and apoptosis [2]. It has also been associated with tumorigenesis and progression. This protein has been involved in two contrary functions, survival and apoptosis, which are carried out by two different forms of clusterin [secretory clusterin (sCLU) and nuclear clusterin (nCLU)]. Most authors are in agreement with the suggestion that tumour cell survival is related to the overexpression of sCLU and the loss of nCLU [3]. Only the cytoplasmic/sCLU form and not the nCLU is expressed in aggressive late stage tumours, in accordance with its antiapoptotic function [4].

The overexpression of exogenous CLU induces resistance to chemotherapy and radiation therapy [5,6]. In contrast, chemosensitivity is enhanced by anti-CLU treatment of various cell lines [7–10], which suggests that CLU expression is a survival factor in cancer cells, and for this reason, it is a target in clinical trials [11–13]. Given the known properties of the anticancer actions of protein kinase inhibitors (PKIs), we hypothesize that treatment with these agents and the simultaneous targeting of CLU may be a fruitful approach for the treatment of breast cancer patients. In this study, we examine the effects of various PKIs [genistein (Gen) H-89 and cheleritrine (Che)] and their combination with CLU antisense oligonucleotides or CLU interference RNA on cytotoxicity in two breast cancer cell lines.

Materials and methods

Cell lines

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and were maintained in RPMI 1640 (Sigma Chemical Co., St Louis, Missouri, USA) supplemented with 10% FBS.

Anticancer drugs

Gen, H-89 and Che were obtained from the Sigma Chemical Co. Stock solutions were prepared with PBS to the required concentrations before each experiment. The assays were performed at 8, 24 and 48 h. The following

concentrations were used: 50 μmol/l of Gen, 50 nmol/l of H-89 and 1.3 μmol/l of Che.

Western blot analysis

Cell extracts of the line MDA-MB-231 were subjected to electrophoresis under denaturing conditions in a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. They were then incubated for 90 min with the antibody to CLU (Merck Millipore, Billerica, Massachusetts, USA). The reactions were visualized by incubation with peroxidase-conjugated secondary antibody. β-Actin served as the internal control for equal loading.

RT-PCR

Isolation of total RNA was performed using the guanidinium isothiocyanate method. Reverse transcription into cDNA was performed using a commercial kit (Roche Molecular Biochemical, Mannheim, Germany) in accordance with the manufacturer's instructions. PCR was performed using the following primers to detect CLU and β-actin signals, respectively. CLU: 5′-GGCGACGA TGACCGGACTGT3′ (forward), and 5′-GGGACCGT CACAGTGATGGG-3′ (reverse); β-actin: 5′-GGCAT CGTGATGGACTCCG-3′ (forward) and 5′-GCTGGA AGGTGGACAGCGA-3′ (reverse).

Real-time RT-PCR was carried out using the SYBR Green PCR Master Mix. Using reverse-transcribed cDNA, the CLU PCR was performed in accordance with the following protocol: 5 min denaturation at 95°C, then 30 cycles of a 30 s denaturation step at 95°C, 1 min annealing at 64°C, 30 s elongation at 72°C and a final elongation step for 5 min at 72°C, and for the β-actin: 5 min denaturation at 94°C, then 25 cycles of a 30 s denaturation step at 94°C, 1 min annealing at 58°C, 30 s elongation at 72°C and a final elongation step for 7 min at 7 °C.

Gene quantification was based on the $-2\Delta\Delta C_t$ method [14]. Briefly, the mean C_t values for the CLU gene were subtracted from those for the control β-actin gene. The differences in the mean C_t values between genes (C_t) enabled us to calculate the relative levels of CLU.

Treatment of cells with oligonucleotides and siRNA

The cell lines were treated using a complementary sequence to CLU (ODN-Clu: 5′-GCACAGCAGGAGA ATCTTCAT-3′), and a two-base CLU mismatch ODN-control (5′-GCACAGCAGGAGGATATTCAT-3′), both provided by Roche Molecular Biochemical. This antisense oligonucleotide to CLU is produced for use in humans (OGX-011; OncoGeneX Technologies, Vancouver, British Columbia, Canada). To introduce ODN into the cell, we used lipofectin (Life Technologies, Glasgow, UK), previously mixed in a concentration of 1.5 μg/ml with a serum-free medium (Dulbecco's modified Eagle's) for 20 min at room temperature. Four hours after beginning incubation, the medium containing ODN and lipofectin was replaced with standard medium for 1 day. A concentration of 100 nmol/l of ODN was found to produce a maximal reduction in CLU mRNA levels [9].

Dose–response curves for PKIs were derived to determine the appropriate dosages. Thus, breast tumour cells were incubated for 1 day with antisense oligonucleotides at a concentration of 100 nmol/l and then incubated with PKIs for 1 day at the following concentration: 50 μmol/l of Gen, 50 nmol/l of H-89 and 1.3 μmol/l of Che.

siRNA transfection was performed using a method similar to that for ODN using 200 nmol/l of siRNA and control siRNA (Santa Cruz Biotechnology, Santa Cruz, California, USA) with FuGene 6 as the transfection reagent (Roche Molecular Biochemical). In this case, however, the breast tumour cells were incubated for 2 days with siRNA and then incubated with PKIs for 1 day at the same concentration as was used with antisense oligonucleotides. All experiments were conducted three times.

Determination of cell viability

The cells were distributed at 50 000 cells/well in 12-well plates, and treated with oligonucleotides or siRNA and the PKI drugs; floating and adherent cells were recovered. The number of viable cells was determined by trypan blue exclusion analysis.

Statistical analysis

Results are presented as means ± SE. Means were compared by one-way analysis of the variance. All *P*-values were two-sided and values less than 0.05 were considered statistically significant. All statistical calculations were carried out using SPSS.15 software (SPSS Inc., Chicago, Illinois, USA).

Results

Expression of CLU protein after treatment with PKIs

The expression of CLU under different PKIs was studied by western blot analysis in the breast cancer cell lines. These cells were treated with H-89, Che and Gen at different times (0, 8, 24 and 48 h). The cells expressed maximal levels of sCLU by western blot as early as 8 h after induction and these high levels were maintained even at 2 days after treatment (Fig. 1).

Expression of CLU mRNA after treatment with PKIs

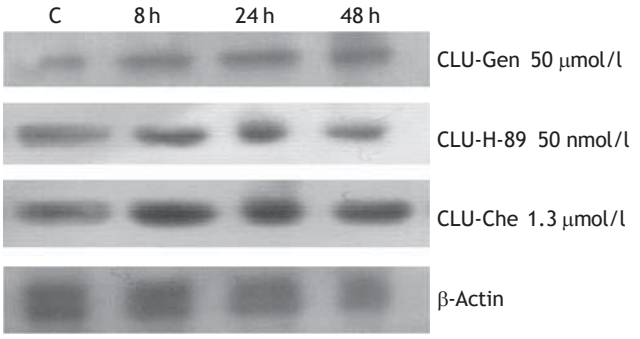
Real-time RT-PCR analysis was also used to determine the effects of PKIs on CLU mRNA expression in breast cancer cell lines. We compared the control time (time 0) versus stimulation at 8, 24 and 48 h with Gen (50 μmol/l), H-89 (50 nmol/l) and Che (1.3 μmol/l) in the lines MDA-MB-231 and MCF-7. We observed a statistically significant increase after treatment in all cases (Fig. 2).

PKIs increase the percentage of cellular mortality in breast cancer cell lines

The percentage of cellular mortality under different PKIs was studied in both breast cancer cell lines. As shown in

Table 1, the percentage of mortality at 24 h in MDA-MB-231 and MCF-7 breast cancer cell lines after treatment with different PKIs showed a significant increase in cellular mortality compared with the control, although in the case of Che, the difference was not statistically significant.

Fig. 1



Western blot analysis of clusterin after treatment by protein kinase inhibitors Gen, H-89 and Che and at different times: 0, 8, 24 or 48 h in the breast cancer cell line MDA-MB-231. After the incubation period, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies. Che, cheleritrine; Gen, genistein.

Antisense oligodeoxynucleotide and siRNA to CLU increase the cytotoxicity of PKIs

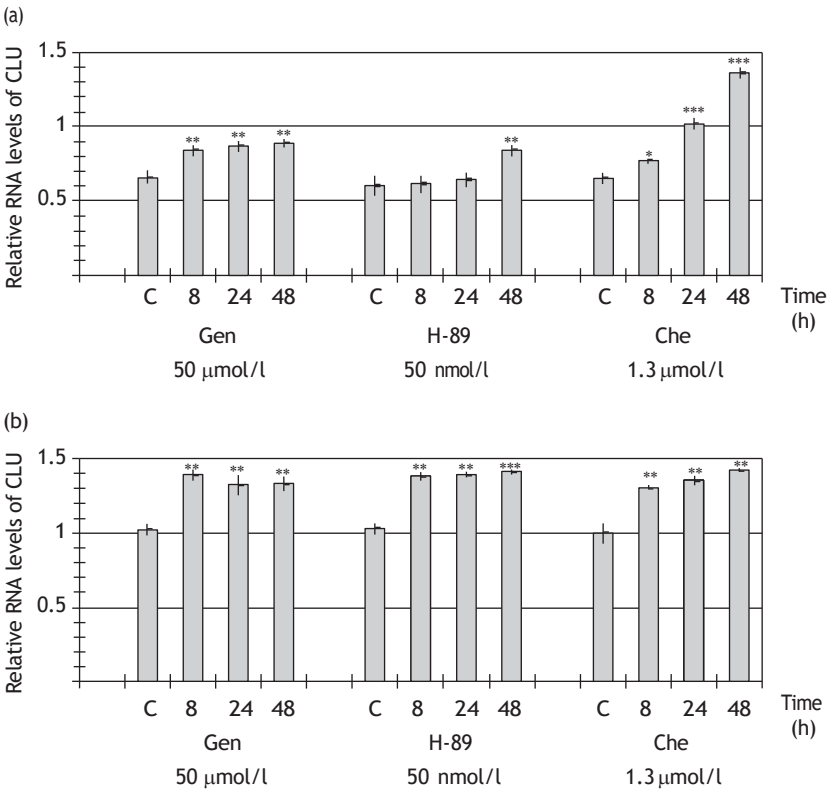
Above, we discussed the effect of treatment with antisense CLU ODN on protein and RNA CLU expression in breast cancer cell lines [9]. Now, we examine the additive effect of the different PKIs with CLU ODN or siRNA on the cell lines MCF-7 and MDA-MB-231.

Table 1 Percentage of mortality by PKIs H-89, Che and Gen

Cell line	Treatment	Control (%)	PKI (%)	*P*
MDA	Gen	12.8 ± 3.2	35.5 ± 4.5	$P \leq 0.001$
MCF-7	Gen	17 ± 1.1	34.5 ± 3.6	$P < 0.01$
MDA	H-89	12.8 ± 3.2	33 ± 2	$P < 0.001$
MCF-7	H-89	18.5 ± 3.3	49.4 ± 4	$P < 0.001$
MDA	Che	13.8 ± 3	17.7 ± 3	NS
MCF-7	Che	18.5 ± 5	22.1 ± 1.7	NS

Each data point represents the mean percentages of at least three independent experiments ± SEM. Cell viability was determined using the trypan blue exclusion test.
Che, cheleritrine; Gen, genistein; PKI, protein kinase inhibitor.

Fig. 2



Evaluation by real-time quantitative PCR of CLU after treatment by protein kinase inhibitors Gen, H-89 and Che, and for different times: control (C), 8, 24 or 48 h in the breast cancer cell lines (a) MDA-MB-231 and (b) MCF-7. Every experiment was conducted three times. Results are reported in arbitrary units as the mean of at least three independent experiments ± SEM. Comparison with respect to C: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Che, cheleritrine; CLU, clusterin; Gen, genistein.

The following tables show the results as a percentage of mortality at 24 h for both breast cancer cell lines with the different treatment options. The combination of ODN-Clu with the PKIs significantly increased the cytotoxicity in both cell lines (Table 2). Only in the MCF-7 cell line did Che and Gen fail to reach statistical significance.

Similarly, the combination of siRNA with the PKIs significantly increased the cytotoxicity in both cell lines (Table 3).

Therefore, the combination of antisense CLU ODN or siRNA with the PKIs produced a significant additive cytotoxic effect in both breast cancer cell lines.

Discussion

Protein kinases, which are present in the regulation of proliferation, differentiation and cell survival [15], are currently being used in the treatment of various cancers. Thus, Gen, Che and H-89 have been shown to reduce cell survival and proliferation [16–18]. These three inhibitors were chosen in view of their different sites of action in the metabolic pathway of protein kinases. Thus, Che is an inhibitor of protein kinase C, H-89 is an inhibitor of protein kinase A and Gen is an inhibitor of tyroxine kinase. Gen is dose dependent, and thus at high concentrations, it inhibits growth, whereas at low concentrations it stimulates the proliferation of ERα-positive breast cancer cells [19]. In the present case, a high concentration was used. Nevertheless, it should be taken into account that this effect could have been produced by early action of ERβ receptors [20] and therefore we do not know whether this fact might influence the expression levels of CLU.

Table 2 Effects of combined treatment with antisense ODN-Clu and PKIs on cytotoxicity of the MDA-MB-231 and MCF-7 breast cancer cell lines

Cell line	ODN-Clu (%)	Treatment	ODN-control + PKI (%)	ODN-Clu + PKI (%)	*P**
MDA	12.1 ± 11.8	Gen	27 ± 1	49.7 ± 4.3	$P \leq 0.01$
		H-89	20.5 ± 2.1	41 ± 1.8	$P < 0.001$
		Che	30.7 ± 1.7	47.3 ± 7.5	$P < 0.05$
MCF-7	19.7 ± 11.9	Gen	27 ± 1.8	29 ± 2	NS
		H-89	29.2 ± 1.9	36 ± 1.4	$P < 0.05$
		Che	26 ± 1.5	28.5 ± 1	NS

Each data point represents the mean percentages of at least three independent experiments ± SEM. Cell viability was determined using the trypan blue exclusion test.
Che, cheleritrine; Gen, genistein; ODN-Clu, clusterin oligonucleotide; PKI, protein kinase inhibitor.
**P* value is the comparison of the two previous columns.

Table 3 Effects of combined treatment with siRNA Clu and PKIs on the cytotoxicity of the MDA-MB-231 and MCF-7 cell lines

Cell line	siRNA (%)	Treatment	PKI (%)	PKI + siRNA (%)	*P**
MDA	25.2 ± 3.4	H-89	33 ± 2	44.2 ± 2.6	$P < 0.01$
MCF-7	53.5 ± 1.5	H-89	49.4 ± 4	57.2 ± 1.04	$P < 0.05$
MDA	26.7 ± 3.9	Che	17.7 ± 3	40 ± 2.6	$P < 0.001$
MCF-7	18.5 ± 2.8	Che	22.1 ± 1.7	32.7 ± 1	$P < 0.001$
MDA	38 ± 7	Gen	35.5 ± 4.5	56.2 ± 5	$P < 0.01$
MCF-7	45.2 ± 3.3	Gen	34.5 ± 3.6	51.2 ± 2.17	$P < 0.01$

Each data point represents the mean percentages of at least three independent experiments ± SEM. Cell viability was determined using the trypan blue exclusion test.
Che, cheleritrine; Clu, clusterin; Gen, genistein; PKI, protein kinase inhibitor.
**P* value is the comparison of the two previous columns.

The effects of PKIs, both *in vitro* and *in vivo*, are associated with an increase in cellular toxicity and an inhibition of cell growth [17,21].

Our results are in line with those reported in these earlier studies and thus we conclude that PKIs induce a statistically significant increase in cellular mortality in breast cancer cell lines.

However, given the disappointing results for PKIs as single agents in advanced cases of the disease, their combination with other agents is of interest. The present study shows for the first time that in breast cancer cell lines, CLU levels increase significantly after treatment with the PKIs H-89, chelerythrine and Gen.

We also found that in both breast cancer cell lines, combinations of anti-CLU treatment with PKI performed significantly better than the respective single-agent treatments alone.

We evaluated the effect of PKI treatment on the level of CLU expression in these breast tumour cell lines because this has been shown to be highly upregulated in various tissues undergoing apoptotic cell death [22,23]. Our results show that CLU expression in MCF-7 and MDA-MB-231 cells increases after PKI treatment, suggesting that CLU upregulation is likely to be an adaptive response that mediates resistance.

We also investigated the capacity of anti-CLU treatment to sensitize breast carcinoma cells to PKI, and searched for drug combinations that produce additive cytotoxicity. We found that antisense CLU oligonucleotides or siRNA efficiently inhibit CLU expression in MCF-7 and MDA-MB-231 cell lines, as reported previously [10], and that this activity is associated with a decrease in cell viability. A sequence control oligonucleotide and control siRNA did not increase the effect of PKI, which suggests that the sensitization of cells to apoptosis was because of the specific downregulation of CLU. These findings confirm the cytoprotective function of CLU in breast carcinoma cells, and suggest that there is a role for anti-CLU therapy in the treatment of breast carcinomas that mainly express CLU protein [10,24–26]. In fact, previous reports have shown that the combined use of antisense oligonucleotides with cytotoxic chemotherapy produces more potent antineoplastic effects [7,10,27,28].

Studies are currently being carried out with OGX-011 in humans. This drug is related to CLU, has a clinically

proven biological activity, its most effective dose is known and its side effects are tolerable [11,29].

Conclusion

Our study firmly establishes a role for CLU as a cell survival gene, and this role is heightened after PKI therapy, to inhibit tumour cell death. Treatment with PKIs and the simultaneous inhibition of CLU may represent a promising therapy for breast cancer patients. Further research is needed to obtain this result '*in vivo*'.

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Conflicts of interest

There are no conflicts of interest.

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