Research Article

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Targeting epidermal growth factor receptor pathway with irreversible tyrosine kinase inhibitor

Epidermal Büyüme Faktör yolağının dönüşümsüz tirozin kinaz inhibitörüyle hedeflenmesi

https://doi.org/10.1515/tjb-2017-0276
Received October 17, 2017; accepted May 8, 2018

Abstract

Background: Malignant mesothelioma (MM) is an endemic disease around central Anatolia region in Turkey, where people are exposed to erionite- and asbestos-contaminated soil. Aberrant EGFR signalling has implicated in several cancers including MMs. Tyrosine kinase inhibitors are new treatment options harbouring deregulated signalling network components. In this study, we aimed to investigate anti-proliferative effect of CL-387,785 in MM cells.

Materials and methods: Alteration of cell proliferation was evaluated with using MTS assay. Profile of EGFR, ERK, AKT, JNK and p38 proteins and ELK-1, JUN, STAT1, STAT3 and STAT5 genes were analysed by western blot and RT-PCR, respectively.

Results: Viability of MM cells was inhibited in dose- and time-dependent manner. CL-387,785 affected MM cells earlier and at higher extent compared to the mesothelial cells. CL-387,785 treatments suppressed EGF-induced phosphorylation of EGFR, ERK, AKT, STAT3 and STAT5 but not SAPK/JNK and p38 in SPC212 cells. RT-PCR analysis showed that expression of p21 increased, while Cyclin D and c-jun expressions decreased in SPC212 cells. However, ELK-1, STAT3 and STAT5, expressions did not change.

Conclusion: Our results propose that CL-387,785 could be an efficacious agent in the treatment of MMs with uncontrolled EGFR signalling.

Keywords: Malignant mesothelioma; CL-387,785; EGFR; MAPK; AKT; STATs.

Öz

Amaç: Malignant Mezotelyoma Türkiye'de insanların erionite ve asbestli topraklara maruz kalması neticesinde İç Anadolu Bölgesi civarında sıklıkla görülen endemik bir hastalıktır. Bozulmuş EGFR sinyalleri Malignant Mezotelyoma da dahil birçok kanserde görülmektedir. Tirozin kinaz inhibitörleri düzeni bozulan sinyal sistemleri için yeni bir tedavi seçeneği sunmaktadır. Bu çalışmada CL-387,785'in Malignant Mezotelyoma (MM) hücrelerindeki anti-proliferatif etkisinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: CL-387,785'nin hücre canlılığına etkisi, MTS assay ile analiz edilmiştir. EGFR, MAPK/ERK, SAPK/JNK, p38MAPK, PI3K/AKT ve STAT yolaklarının protein analizleri Western Blot; ELK-1, JUN, STAT1, STAT3 ve STAT5 gen analizleri RT-PCR yöntemleriyle belirlenmiştir.

Bulgular: CL-387,785 MM hücreler üzerinde konsantrasyona ve zamana bağımlı olarak anti-proliferatif etki göstermiştir. Kanser ve kontrol hücrelerine etkinliği karşılaştırıldığında, CL-387,785 SPC212 hücrelerini MeT-5a hücrelerine göre daha erken sürede ve daha düşük dozda baskılamıştır. CL-387,785, SPC212 hücrelerinde EGFR ile başlatılan EGFR, ERK, AKT, STAT3 ve STAT5 fosforilasyonlarını düşürürken; JNK ve p38 fosforilasyonları üzerinde etkin olmamıştır. Gen analizlerinde CL-387,785 uygulanan hücrelerde, CylinD ile c-jun mRNA seviyeleri azalırken, p21 seviyesinde artış gözlenmiştir. ELK-1, STAT3 ve STAT5 ekspresyonlarında ise anlamlı bir değişim belirlenmemiştir.

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Sonuç: Sonuçlarımız CL-387,785'in kontrolsüz EGFR sinyalleri taşıyan Malignant Mezotelyoma tedavisinde kullanılmaya aday bir ajan olabileceğini düşündürmektedir.

Anahtar Kelimeler: Malignant mezotelyoma; CL-387,785; EGFR; MAPK; AKT; STATs.

Introduction

Malignant mesothelioma (MM) is a lethal cancer arising from long time exposure to asbestos [1]. Domestic use of asbestos contaminated soil and local exposure of erionite have increased the rate of MMs especially in central Anatolia region of Turkey [2]. To our best knowledge, despite intense chemotherapeutic regimens and radiotherapy, no curative agent has proven to treat MM patients so far.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase, over-expressed at least 50% of the epithelial tumours including breast, lung, head, neck and bladder [3–7]. It is well known that uncontrolled EGFR signalling leads to increased survival, proliferation, invasion and angiogenesis but decreased apoptosis, subsequently contributing to development of malignant phenotype [8]. The overexpression of EGFR was reported in MMs [9]. Lately, neutralising antibodies or small molecule tyrosine kinase inhibitors have employed in EGFR-targeted cancer therapies [10]. However, the main problem seems to be the rapid clearance of existent molecules from plasma and/or resistance to treatment. N-(4-((3-bromophenyl) amino)-6-quinazolinyl)-2-butynamide or, CL-387,785 (CL) is an irreversible EGFR kinase inhibitor having advantage of accumulating in the cell after clearance of drug from the plasma and tumour [11]. Threonine 790 (T790) is an important residue in adenosine triphosphate (ATP) binding pocket of EGFR and acts as a gatekeeper. The mutation in that residue substitutes the threonine to methionine (T790M) and causes a steric hindrance. This interference results in the resistance to tyrosine kinase inhibitors (TKI) [12]. It is known that T790M mutation on EGFR leads to drug resistance in non-small cell lung cancers [13]. Irreversible EGFR tyrosine kinase inhibitors are ATP mimetics and they can bind covalently to the EGFR which inhibits the EGFR kinase activity even in the presence of T790M [14]. Irreversible TKI CL covalently binds to erythroblastic leukemia viral oncogene homolog 1 (ErbB-1) with the cysteine 773 (C773) region therefore T790M effect cannot be observed [15]. In the present work, the effect of irreversible EGFR inhibitor on EGFR dependent signalling has evaluated in mesothelial and MM cells for the first time. Our result indicates that CL has a potential as an anti-proliferative agent against to MM cells with aberrant EGF signalling.

Materials and methods

Culture of cell lines and CL

Mesothelioma (MeT-5A) (ATCC, Manassas, VA, USA) and MM (SPC212) (The Institute of Histology and General Embryology, University of Fribourg, Switzerland) cells were cultured with Medium 199 (Sigma Aldrich, Darmstadt, Germany) and RPMI 1640 (Sigma Aldrich, Darmstadt, Germany) with 10% (v/v) FBS (Hyclone, Logan, UT, USA) and 1% pen/strep (Hyclone, Logan, UT, USA), respectively. Both cell lines were maintained in a 5% CO2 incubator at 37°C under humidified conditions. CL was purchased from (Calbiochem, Darmstadt, Germany) dissolved in DMSO and diluted 1000 fold with serum free media.

Viability assay

MTS assay was used to measure viability of the MeT-5A and SPC212 cells. Briefly, 5×10^3 cells seeded in 96-well plates overnight and, then incubated with different concentrations of CL (1, 5, 10, 20, 30 μM) for 0–96 h. Measurements of assay were performed with microplate reader at A490 nm absorbance wavelength value.

Protein analysis

1×10^6 cells were seeded in Petri dishes after a 24-h serum starvation cells treated with different concentrations of CL (5, 10, 50, 100, 500, 1000 nm). Before each lysis, cells were incubated with 100 ng/mL EGF for 15–20 min. Samples were collected and washed with cold PBS. After discarding PBS, lysis buffer (with 1% protease-phosphatase inhibitors) was added to each sample and apply 1 mL syringe for cell burst. Following centrifuge, supernatant was removed into fresh tube. Samples were separated for BCA analysis (Pierce BCA Protein Assay Kit, Thermo-Fisher Scientific, Waltham, MA, USA), and used immediately or stored at –20°C for further usage. Protein lysate were mixed with 4× sample loading dye and 15-30 μg protein loaded to the 10–12% SDS-PAGE gel. Samples were transferred to PVDF membranes, then were subsequently probed with specific antibodies [p-EGFR, EGFR p-AKT, AKT, p-JNK, JNK, p-STAT3, STAT3, p-STAT5, STAT5, β-actin, anti-rabbit (Cell Signalling Technology, Danvers, MA, USA), p-ERK, ERK1, anti-mouse (Santa Cruz Biotechnology, Dallas, TX, USA)]. Finally, membrane was developed using an ECL kit (Pierce ECL Plus Western Blotting Substrate, Thermo-Fisher Scientific, Waltham, MA, USA). Image quantification software

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(Biorad Image lab 6.0, Hercules, CA, USA) was used to determine protein expression levels, relative to β-actin. Each experiment was repeated at least 3 times.

Total RNA isolation and cDNA synthesis

After a 24-h serum starvation SPC212 cells were exposed to 100 nM concentration of CL and after 3 h cells (1×10^6) scrapped with 1× PBS (Before lysis, cells were incubated with 100 ng/mL EGF for 15–20 min). Total RNA was isolated with RNeasy Mini Kit (Qiagen 74104, Hilden, Germany). Elution made with 40 µL RNAse free water, concentration and purity measurements did with spectrophotometer. cDNA synthesis was started with 100 ng RNA and preceded with Transcription High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). The final 10–20 µL cDNA samples were stored at –20 °C.

RT-PCR

PCR mix was prepared using Light Cycler 480 Probe Master Kit (Roche, Basel, Switzerland). Alterations of cellular jun

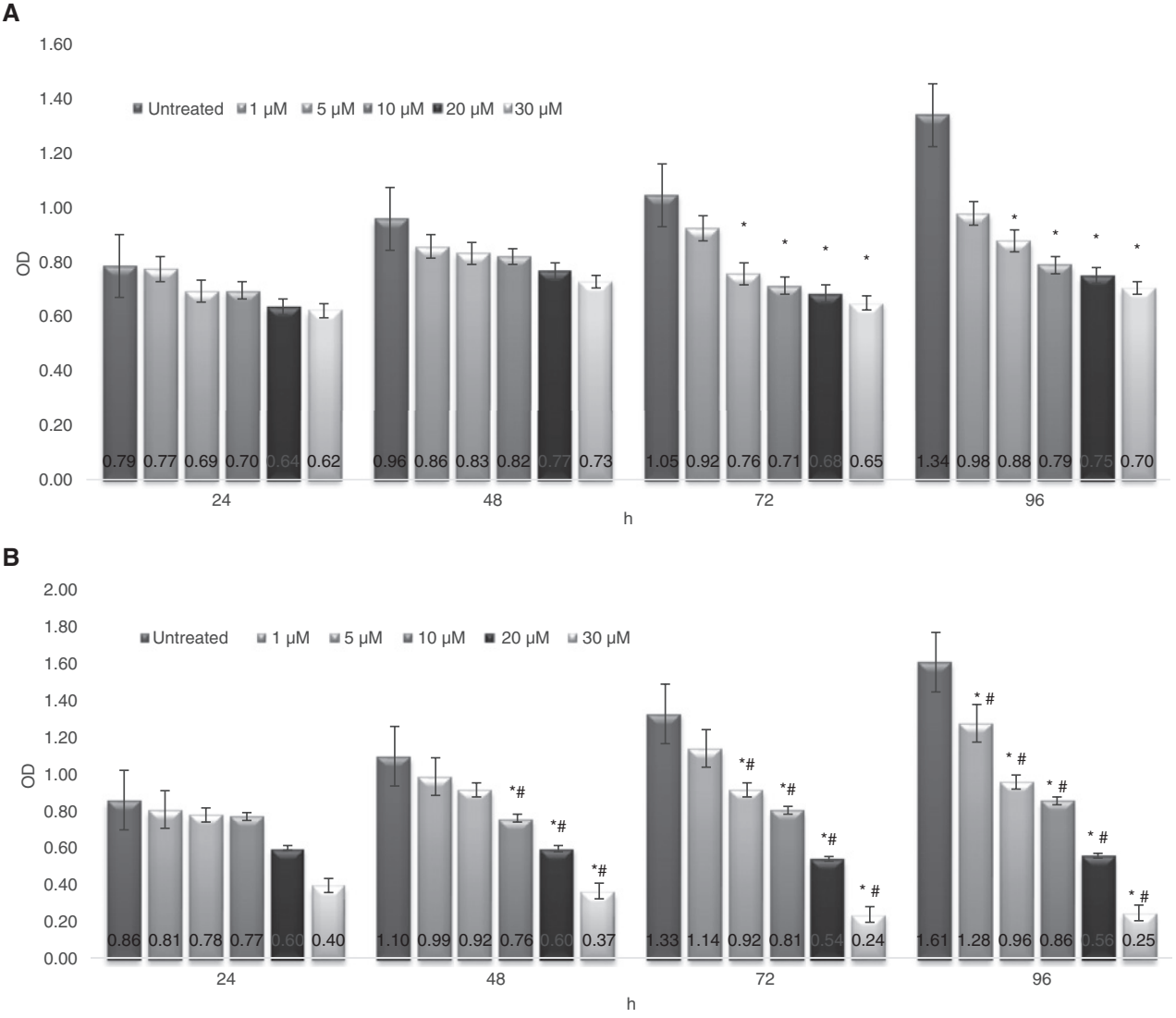


Figure 1: Effect of CL on MeT-5A (A) and SPC212 (B) cells viability.
Cells were induced by CL-387,785 CL at several concentrations for 96 h. Viability of both cells was decreased by dose- and time-dependent manner. However, CL affected SPC212 cells earlier and at higher degree compared to MeT-5A cells. Experiment repeated three times independently and averages of result calculated by SPSS with One way ANOVA and Bonferroni tests. P-value was calculated as *$p < 0.005$ and #$p < 0.001$.

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(c-jun), Elk-1, STAT3, STAT5, cyclin D and p21 genes were investigated. β-actin (ACTB) and hypoxanthine phosphoribosyl transferase 1 (HPRT1) (Roche, Basel, Switzerland) were used as reference genes. Normalisations were made by using values of control groups. Results were analysed by using ΔΔCT method.

Statistical analysis

Results were expressed as mean ± S.D. of three independent experiments containing duplicated samples. Data has been statistically evaluated by one-way ANOVA and Bonferroni tests. The levels of significances have chosen as $*p < 0.005$ and $\#p < 0.001$ between control groups and multiple doses of CL.

Results

CL-387,785 reduces viability of mesothelioma and MM cells

To examine the effect of CL on cell viability of two cell lines, we exposed control 'MeT-5A' and malignant 'SPC212' cells to various concentrations of CL in 96 h. Cell viability reduced at concentration and dose-dependent manner in both cell lines. However, CL was more potent in decreasing MM cell viability when compared to MeT-5A (Figure 1).

CL inhibits EGFR/MAPK signalling in EGF treated SPC212 cells

To study the effect of CL on EGFR/MAPK signalling, tumour cells were used as a model. Cells were serum-deprived to reduce the background level of several growth factors and EGF is used to stimulate EGFR signalling pathway after serum starvation. As shown in Figure 2, EGFR and ERK1/2 activations were suppressed upon CL treatment in dose-dependent pattern when compared to the control cells in 3 h. Inhibition of EGFR and ERK phosphorylation observed in concentrations at greater than 10 nM and 100 nM, respectively.

CL-387,785 shows no effect on stress activated MAPK pathways

JNK and p38 MAPK pathways are often associated with stress and apoptosis, however, depending on cell line the controversial evidence exist, such as induction of cell survival and inhibition of apoptosis. For that reason, the alteration of JNK and p38MAPK pathways were also, investigated in CL treated SPC212 cells. JNK1/2 and p38MAPK activations were determined in 3 h. No change observed

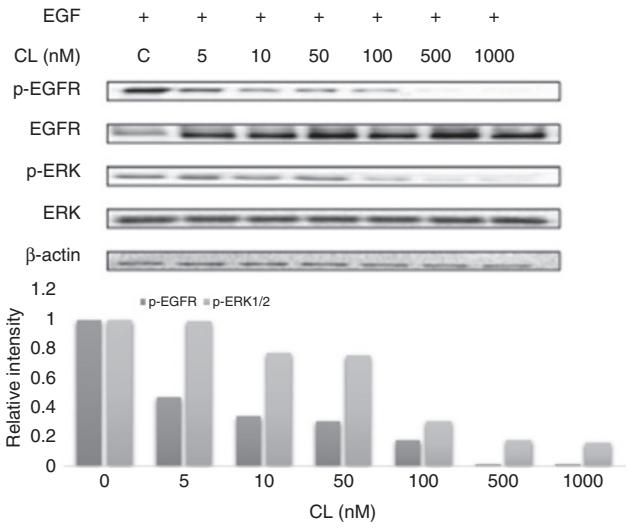


Figure 2: Concentration-dependent effects of CL on EGFR and ERK1/2 signalling.
Serum starved cells treated with CL (0–100 nM) and samples were stimulated for 15–20 min with 100 nM EGF before lysed. Whole-cell lysates were immunoblotted for p-EGFR, total EGFR, p-ERK1/2, total ERK1/2 and β-actin as a loading control. EGFR/ERK signalling was reduced by increased doses of CL in SPC212 cells. Total and activated proteins were analysed in 3 h. One representative figure is shown between experiments, which were repeated 3 times with similar results.

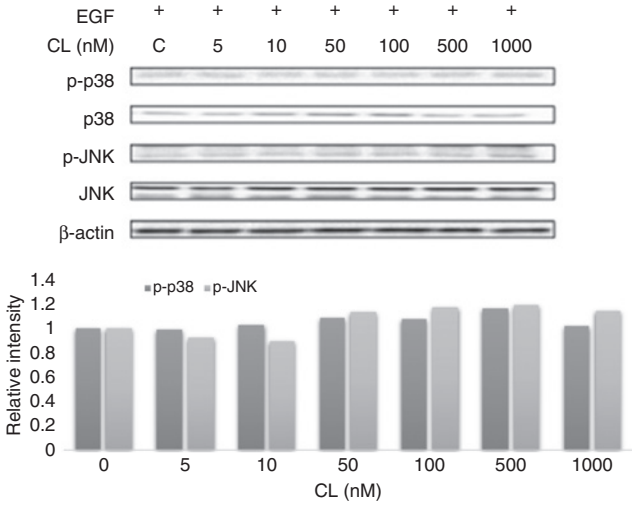


Figure 3: Concentration-dependent effects of CL on JNK1/2 and p38 signalling.
Total and activated JNK and p38 stress proteins were analysed in 3 h. β-actin was used as a loading control. No change was observed in stress proteins analysed in SPC212 cells.

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in protein phosphorylation and expression levels of stress proteins (Figure 3).

CL-387,785 treatments inhibit EGFR/PI3K/AKT signals

To investigate the effect of CL on cell survival pathway, activation and expression of AKT protein were investigated in EGF induced SPC212 cells. Results showed that starting from 100 nM with increasing increments of 100–1000 nM of CL reduced AKT phosphorylation but not the expression levels (Figure 4).

CL restrains phosphorylation of STAT3 and STAT5 proteins but not expressions

To examine two foremost STAT proteins, cells treated with increasing concentrations of CL in 3 h. Phosphorylation of STAT3 and STAT5 were decreased after CL treatments, however, EGF-induced STAT expressions were not influenced, when indicated CL doses were applied to SPC212 cells (Figure 5).

CL-387,785 changes expression of genes involved in proliferation

To investigate the effect of CL on EGF-induced gene expressions, c-jun, ELK-1, STAT3, STAT5, Cyclin D, p21 mRNA

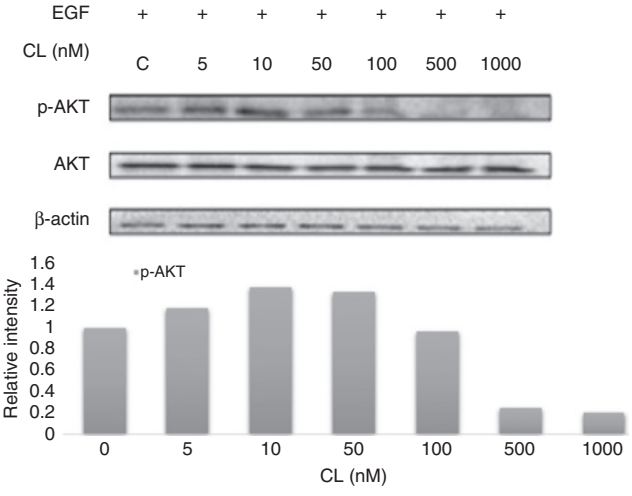


Figure 4: Concentration dependent effects of CL on AKT. Activation of AKT protein was suppressed dose-dependently by CL in EGF-stimulated SPC212 cells. Samples were immunoblotted for p-AKT, total AKT, and β-actin (loading control) antibodies.

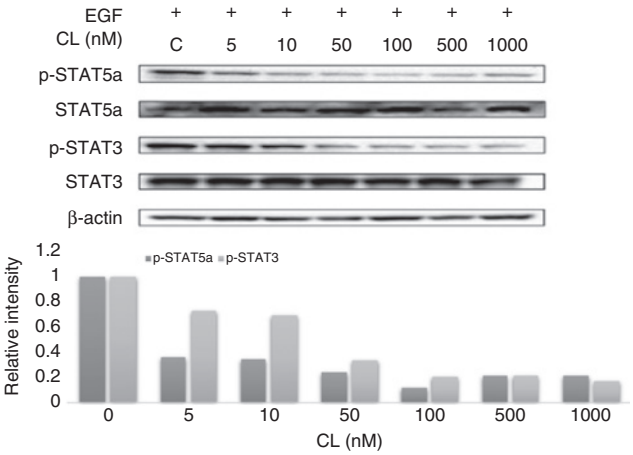


Figure 5: Concentration dependent effects of CL on STAT proteins. Samples were treated with different doses of CL and induced by EGF. Blots were exposed to p-STAT3, p-STAT5 and STAT3 and STAT5 antibodies. The activation of STAT3 and STAT5 were suppressed but not to the total protein levels in 3 h.

levels were analysed in SPC212 cells. Cells were exposed to 100 nM concentration of CL and real-time assay were performed for designated time intervals. Relative amount of mRNA changes was measured by using ΔΔCt method. Results showed that while p21 mRNA level was upregulated 3 fold, the mRNA level of cyclin D and c-jun were reduced 50% respective to control cells. However, no significant changes observed at the expression of Elk-1, STAT3 and STAT5 in 3 h (Figure 6).

Discussion

Using small molecule kinase inhibitors have become a new strategy for the targeted therapies lately. However, the major problem of these treatment is development of resistance to inhibitors due to mutations on the targeted regions. Today, it is well known that EGFR mutations render them drug resistant [16]. As a result, the efficacy of drug diminishes in time, specific mutations or combination of mutations changes the dynamic interactions between EGFR and its inhibitors. To avoid these consequences, irreversible inhibitors have been developed to be used alone or in combination. Afatinib, gefitinib and erlotinib are promising EGFR inhibitors for the NSCLC treatment in recent years [17–19]. However, studies show that patients who carry T790M mutation developed resistance in the 50% of the clinical cases [20, 21]. EGFR overexpression reported in epithelial cancers as well as MMs [3–7, 9]. In this study, anti-proliferative effect of irreversible receptor tyrosine kinase inhibitor CL was investigated

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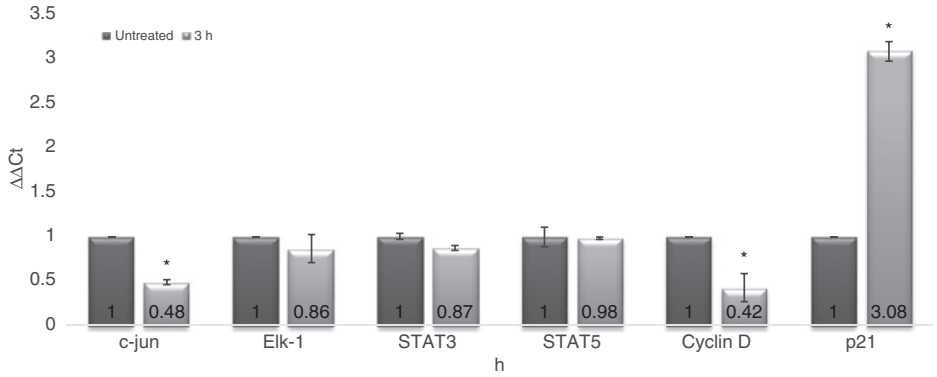


Figure 6: Effect of CL on transcription of EGF-induced genes.
CL treated cells induced with EGF, then c-jun, Elk-1, STAT3, STAT5, cyclin D, p21 mRNA levels were analysed for 3 h. ACTB and HPRT genes were used as reference genes. Cyclin D and c-jun mRNA levels were down regulated and p21 mRNA level was upregulated in SPC212 cells. However, Elk-1, STAT3 and STAT5 expressions were not influenced when 100 nM of CL were applied to the cells.

in mesothelial and MM cells for the first time. Our results showed that viability of both cells reduced at dose- and time dependent manner after CL treatments in 96 h. The effect of CL on cancer cell viability was more drastic than control cells ($p < 0.01$). Besides, cancer cells were affected earlier. This might indicate that CL is selectively toxic to cancer cells. However, this argument needs to clarify with other non-tumorigenic cell lines. It is documented that Ras/MAPK, PI3K/AKT and JAK/STAT signalling pathways are activated by EGFR [22–25]. Considering the uncontrolled induction of cell growth and survival, Ras/MAPK, PI3K/AKT and STAT pathways contribute to oncogenesis [22–24, 26–28]. Inhibition of these pathways results in the reduction of tumour viability and proliferation. Therefore, the effects of CL on EGFR, ERK, AKT, STAT3 and STAT5 proteins were evaluated in SPC212 cells. CL significantly inhibited EGFR, ERK, AKT, STAT3 and STAT5 phosphorylation at concentrations greater than 100 nM, however, CL made no alteration in their expressions within 3 h. According to results presented here, inhibition of EGFR phosphorylation occurs in lower concentration while ERK phosphorylation occurs in higher concentration suggesting EGFR exploits diverse pathways instead rather than in addition to MAPK, AKT and STAT. Our previous study also confirmed this suggestion for ERK and AKT phosphorylation within 6 h, 12 h and 24 h [29]. Relevantly, we have analysed expression of a few genes activated in response to EGFR signals. In normal route, expression of Cyclin D1 increased in G1 in order to activate its CDK partner and related target proteins to ensure progress through the S phase. In our experiments, expression of cyclin D gene was downregulated and p21 gene was upregulated with 100 nM CL applications in 3 h in SPC212 cells. These results support the scheme that CL capable of hampering EGF stimulated MAPK and AKT pathway activation in MM cells. Moreover, it is known that MAPK/ERK1/2 and PI3K/AKT downstream signals regulate transcription factor ELK-1 that induces proliferation and survival signals [30, 31]. In our cells, ELK-1 expression was down regulated not significantly but slightly in response to CL within 3 h. Elk-1 regulates proto-oncogene c-fos so this repression can be useful for the uncontrolled proliferation of cancer cells. c-jun is one of the components of activated protein 1 complex besides c-fos. Some studies show that p38 MAPK, ERK1/2 and ERK5 needed for the UV- or EGF-induced c-jun expression. It has been shown that constitutively active ERK increases the activity and stability of c-jun expression in melanoma cells [32]. Another study indicates that expression of c-jun mRNA level shows a 5.5 fold increase in 30 min after EGF treatment in fibroblast cell. In addition, suppressed MAPK pathway may suggest that ERK1/2 is needed for the c-jun expression [33]. As shown in Figure 6, exposing 100 nM CL to SPC212 cells resulted in 50% reduction in, c-jun mRNA levels ($p < 0.05$) in 3 h. In our cells, application of CL showed no effect on JNK and p38 protein activations and expressions at least in 3 h which shows that stress-activated pathway is not affected by CL. STATs, as latent transcription factors, regulate transcription of several genes including Bcl-2 [34], cyclin D [35] and p21 [36]. However, expression levels of STAT proteins were not changed in CL treated SPC212 cells (Figure 6). This data suggests that transcription of cyclin D and p21 may be independent of STAT transcription in SPC212 cells. However, this argument needs to be elucidated by future work. Similar to our results inhibition of EGFR signalling by CL were reported in mouse embryonic, lung isogenic cells and NSCLC cell lines [37–40]. This is the first report showing anti-proliferative effect of CL on mesothelioma

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and MM cells. CL decreased cell viability, suppressed EGF-dependent proliferation and survival but it had no effect on the stress-activated pathways. For these reasons CL might be a good candidate for the MM treatments with aberrant EGFR signalling.

Acknowledgement: This work was supported by Gebze Technical University Grant (formerly Gebze Institute of Technology Research Project 2012-BAP-A-03).

Conflict of interest statement: The authors report no declarations of interest.

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