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PHARMACOLOGICAL GROWTH INHIBITION OF  
PAPILLOMAVIRUS TYPE 16 E6E7-TRANSFORMED  
KERATINOCYTES BY THE CDK-INHIBITOR CYC202

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Abstract

In the present study we have analysed the effect of the pharmacological CDK-inhibitor CYC202 on HPV16 E6- and E7-transformed human keratinocytes. The proliferation rates of E6E7 keratinocytes treated with CYC202 were determined by measurement the <sup>3</sup>H-thymidine incorporation in the newly synthesised DNA molecules. The effect of CYC202 on some mitogen-activated protein kinases (MAPK) implicated in the regulation of cell proliferation, differentiation and apoptosis was also studied by Western blotting. CYC202 effectively inhibits the proliferation of E6E7 keratinocytes in a dose-dependent manner. Treatment with CYC202 strongly increases the activity of p38 MAP kinase and inhibits ERK1/2 at the highest concentration used. The phosphorylation of HSP27 and c-Myc are also changed in correlation with the upstream kinases from the MAPK family.

**Key words:** keratinocytes, HPV16 E6 and E7 genes, CYC202, MAP kinases

**Introduction.** Human papillomaviruses (HPV) have been identified as causative agents of more than 95% of the cervical carcinomas and are also linked to some oral and laryngeal cancers [1]. HPV16 early genes E6 and E7 efficiently immortalize normal human keratinocytes and are involved in the maintenance of the host's cell proliferation [2]. HPV16 E6 and E7 gene products stimulate the cell cycle transition G<sub>1</sub>-S in the presence of various G<sub>1</sub> arrest signals, through interaction with key components of cellular growth-regulatory pathways, including functional inhibition of the cellular cell cycle inhibitor p21<sup>Cip1</sup> [3,4].

The pharmacological agent CYC202 is a specific inhibitor of the cyclin-dependent kinases, particularly of CDK2 and CDK7 [5]. Most of the studies with CYC202 are directed to delineate its antitumour activities [6, 7] and CYC202 has entered clinical trials for cancer treatment [8]. In addition, CYC202 is a potent inhibitor for the cellular growth of normal fibroblasts [9] and normal epidermal keratinocytes [10].

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In the present study we have analysed the effect of the pharmacological CDK-inhibitor CYC202 on the proliferation of papilloma-transformed E6E7 human keratinocytes. CYC202 effectively inhibits the incorporation of  $^3\text{H}$ -thymidine in the newly synthesised DNA molecules in a dose-dependent manner. Treatment with CYC202 strongly increases the activity of p38 MAP kinase and the phosphorylation of its substrate HSP27. The decreased phosphorylation of ERK1/2 MAPK by the highest concentration of CYC202 used for treatment, and the corresponding decrease in the phosphorylation of one of their substrates – the transcriptional regulator c-Myc, indicates that CYC202 inhibits the activities of ERK1/2 MAP kinases.

**Materials and methods.** REAGENTS. (*R*)-2-[[9-(1-methylethyl)-6-[(phenyl methyl) amino]-9H-purin-2-yl] amino]-1-butanol (CYC202) is from Cyclacel Ltd (Dundee, UK). The recombinant human epidermal growth factor (EGF) is from R&D Systems (Minneapolis, USA).

**CELL CULTURE.** The cell line E6E7, derived from normal human keratinocytes through stable transfection with HPV16 E6 and E7 genes, was kindly provided by Dr. Mark Pittelkow (Mayo Clinic, Rochester, USA). The monolayer E6E7 cells were grown in Epilife keratinocyte medium (Cascade Biologics) supplemented with growth factors, hormones and antibiotics. At approximately 40% of culture confluence, the cells were grown under autocrine conditions by excluding all growth factors from the culture medium.

**DETERMINATION OF DNA SYNTHESIS.** E6E7 cells were plated in 24-well plates at  $5 \times 10^3$  cells/cm<sup>2</sup> and treated for 24 h with CYC202. For the last two hours of incubation 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]-thymidine (ICN) was added to each well. The radioactive medium was then removed and the cells were washed with ice-cold PBS and precipitated in 10% trichloroacetic acid (TCA). The cells were lysed in 0.3N NaOH and 0.1% sodium dodecyl sulphate (SDS). The radioactivity of each sample was counted in a Beckman Liquid Scintillation Counter. The protein content was determined with Bio-Rad DC protein assay (Bio-Rad Laboratories, France) and the rate of DNA synthesis was calculated per  $\mu\text{g}$  of protein.

**WESTERN BLOTTING.** After treatment with CYC202, the cells were lysed in sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2 M DTT). The protein lysates were cleared by centrifugation at 13000 rpm at 4 °C for 15 min. Protein concentration was quantified using Bio-Rad DC protein assay (Bio-Rad Laboratories, France) and equal amounts of protein were used for SDS-PAGE. Immunodetection was performed with rabbit anti-phospho-HSP27 (Ser82) IgG (Upstate, USA), rabbit anti-phospho c-Myc (Thr58/Ser62) IgG (Santa Cruz Biotech.), mouse monoclonal IgG anti-phospho ERK1/2 (Upstate, USA) and rabbit polyclonal anti-phospho p38[pTpY<sup>180/182</sup>] (Cell signalling tech., USA). After membrane-stripping, total HSP27, ERK 1/2, p38 and  $\beta$ -actin were detected with goat anti-HSP27 (Santa Cruz Biotech., USA), rabbit anti-ERK1/2 (Upstate, USA), rabbit anti-p38 MAPK (Biosource, USA) and monoclonal anti- $\beta$ -actin (Sigma, USA) antibodies. Primary antibodies were revealed with horseradish peroxidase (HRP)-conjugated antibodies and Luminol reagent (Santa Cruz Biotech., USA).

**Results and discussion.** Many epithelial malignancies, including cervical cancer, are often associated with small DNA viruses of the papilloma family. Mainly two viral genes – E6 and E7, are implicated in cell transformation leading to continuous proliferation and immortalization of normal cells [2]. One of the cell proteins, whose activities are interrupted by expression of the viral oncogenes, is the CDK-inhibitor p21<sup>Cip1</sup>. The abrogation of p21<sup>Cip1</sup>-mediated inhibition of CDK2 activity leads to indefinite cellular proliferation [4]. In this case, CYC202 might be a useful pharmacological inhibitor of

cell growth of papilloma-infected cells and perhaps could be applied for therapy of papilloma-induced malignancies. Here we report the action of CDK-inhibitor CYC202 on HPV16 E6- and E7-transformed keratinocytes.

The first step for the analysis of the effects of CYC202 on culture of E6E7-transformed keratinocytes was to determine the concentration inhibiting E6E7 cell's growth without affecting the cell viability. Therefore, the weakly toxic concentrations of the inhibitor were determined by measurement of the cell viability with trypan blue exclusion test (data not shown) and these concentrations were further used in the other experiments. The effect of CYC202 on cell proliferation was analysed by measuring the level of [<sup>3</sup>H]-thymidine incorporation in the newly synthesised DNA. The proliferation of E6E7 keratinocytes was inhibited by treatment with CYC202 for 24 h at all tested concentrations (Fig. 1). The concentration, at which the proliferation was inhibited to 50% (IC<sub>50</sub>), was determined by biostatistical GraphPad Prism 4 software (GraphPad Software, USA) around 4 μM CYC202 (3.5913 ± 0.575).

The effect of CYC202 on E6E7 proliferation was further analysed by testing the activity of some signalling molecules implicated in the regulation of this process. The mitogen-activated protein kinases (MAPK) have been shown to respond to a broad variety of external stresses and drugs including to inhibition with CYC202 [10-12]. In addition, the role of MAPKs in maintenance of the homeostasis between keratinocyte's proliferation and differentiation is well documented [13, 14].

The phosphorylation of ERK1/2 MAPK remained unchanged after treatment with CYC202 up to 10 μM (Fig. 2). Small decrease was observed at 20 μM CYC202. Mitogenic stimulation with EGF for 15 min increases the level of phosphorylated ERK1/2. However, EGF could not obviate the inhibitory effect of the highest concentration of CYC202 on the activation of ERK1/2, as at these conditions we observed inhibition

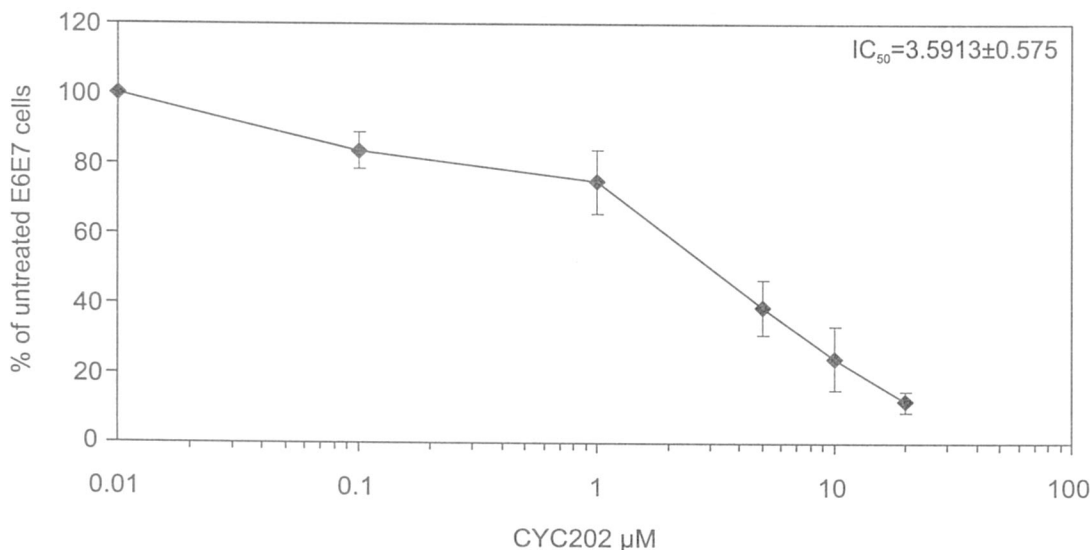


Fig. 1. CYC202 inhibits E6E7 proliferation in a dose-dependent manner. E6E7 keratinocytes were treated for 24 h with different concentrations of CYC202 (0.1–20 μM). The measurement of [<sup>3</sup>H]-thymidine incorporation into DNA was determined as it is described in Materials and methods. Data are presented as percent of untreated cells (incubated with DMSO only, 100%) ± SD averaged from five different experiments. The concentration of CYC202 which inhibited the cell proliferation to 50% (IC<sub>50</sub>) was calculated with GraphPad software

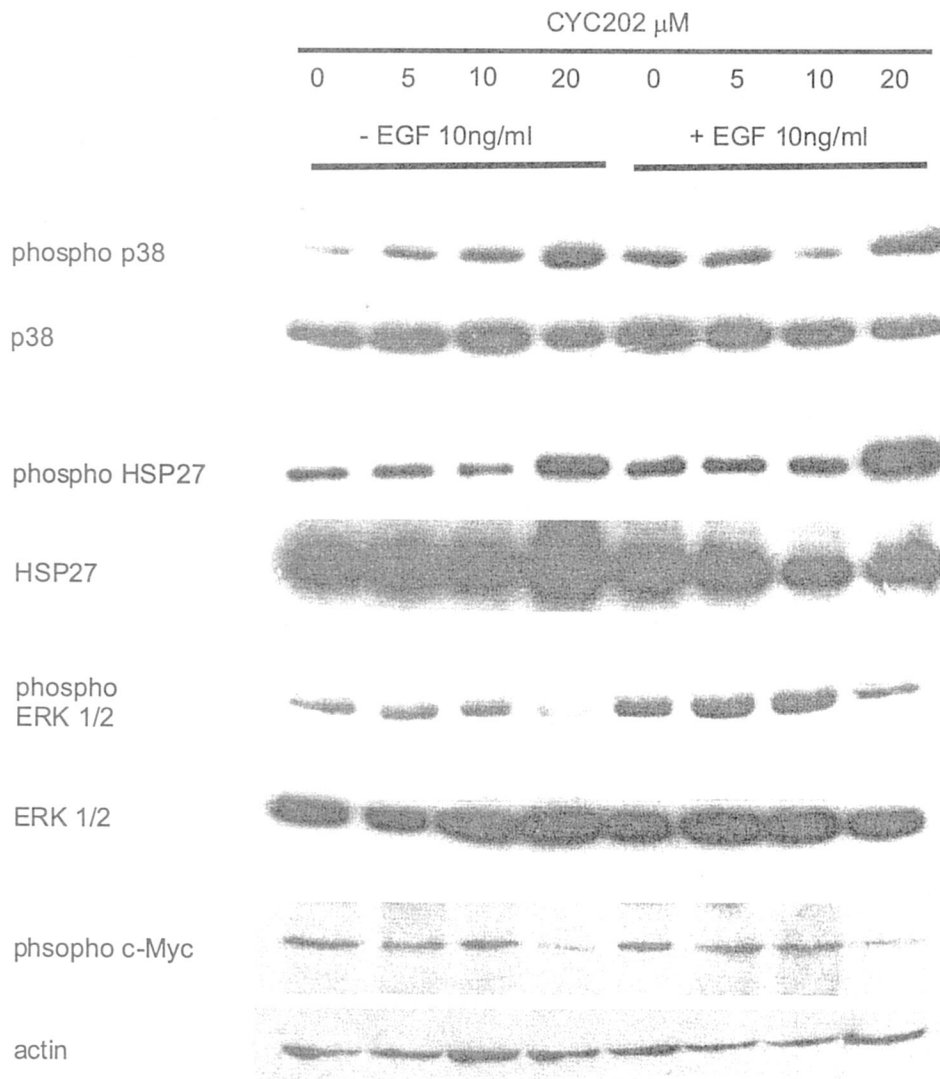


Fig. 2. Analysis of the phosphorylation of MAP kinases, c-Myc and HSP27 in E6E7 keratinocytes incubated with CYC202. E6E7 cells incubated with 0, 5, 10 or 20  $\mu$ M CYC202 for 24 h and (or without) recombinant EGF 10 ng/ml were lysed and equal amounts of proteins were used for Western blotting analysis of the basal and phosphorylated state of ERK 1/2, p38 MAPK, HSP27, phosphorylated c-Myc and  $\beta$ -actin (control for the protein loading)

again.

The transcriptional regulator c-Myc is necessary for keratinocyte proliferation and is one of the downstream effectors of ERK1/2 signalling pathway [15]. Therefore, the phosphorylation of c-Myc at Thr58/Ser-62 sites, targeted by active ERK1/2 MAPK, was inhibited by treatment with 20  $\mu$ M CYC202 in correlation with the phosphorylation of ERK1/2 (Fig. 2).

The level of the active (phosphorylated) p38 MAPK was dose-dependently elevated by CYC202 (Fig. 2). The most significant increase – about 7-fold than the control

cultures, was observed at 20  $\mu$ M CYC202. The effect of EGF on phosphorylation of p38 MAPK was comparable with this of the inhibitor. The combination of EGF with the highest dose of CYC202 results in analogical phosphorylation of p38 kinase, as the level observed with this concentration of CYC202 alone.

The activity of p38 MAP kinase was further tested by phosphorylation of one of its substrates, the small heat shock protein HSP27 [16, 18]. The phosphorylation of HSP27 was strongly increased by 20  $\mu$ M CYC202. Stimulation with EGF alone had a minimal effect on phosphorylation of HSP27, but when it was preceded by treatment with 20  $\mu$ M CYC202 the increase was significant (Fig. 2).

The reverse correlation between the activities of the two subfamilies MAP kinases: p38 MAPK and ERK1/2 is not surprising, as we have observed earlier analogical effect of CYC202 on normal keratinocytes [10]. Also, recent studies report the formation of a complex between ERK1/2 and p38 MAPK, where the activities of these kinases are reciprocally regulated [17].

In conclusion, in this study we demonstrate that HPV16 E6E7-transformed keratinocytes are sensitive to the action of CDK-inhibitor CYC202. The inhibition of the cellular proliferation was associated with changes in the activities of some MAP kinases participating in the regulation of this process. CYC202 inhibits the activity of ERK1/2 MAP kinases, but strongly activates the stress kinase p38 MAPK. These data provide information about the potency of the CDK-inhibitor CYC202 and its antiproliferative effects on HPV16 E6E7-transformed human keratinocytes.

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