Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 824–836
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effects of the cyclin-dependent kinase inhibitor CYC202 (*R*-roscovitine) on the physiology of cultured human keratinocytes

Ganka Atanasova [a,b], Ralph Jans [a], Nikolai Zhelev [c], Vanio Mitev [b], Yves Poumay [a,*]

[a] *Department Histology–Embryology, University of Namur, 61 Rue de Bruxelles, B-5000 Namur, Belgium*
[b] *Department of Chemistry and Biochemistry, Medical University of Sofia, 1431 Sofia, Bulgaria*
[c] *School of Contemporary Sciences, University of Abertay, Dundee, UK*

Received 11 May 2005; accepted 1 June 2005

Abstract

CYC202 (*R*-roscovitine) is a potent cyclin-dependent kinase inhibitor, investigated as a potential anti-cancer agent. The knowledge of the action of this pharmacological agent on normal human cells is still limited. In this study, we have explored the effects of the cyclin-dependent kinase inhibitor CYC202 on normal human epidermal keratinocytes. The loss of cell viability induced by this compound was strongly dependent on the rate of keratinocyte proliferation. At slightly cytotoxic doses, CYC202 inhibited the proliferation of subconfluent keratinocytes in a dose-dependent manner, and at higher concentrations induction of early apoptosis was observed, evidenced by caspase-3 activation. The signal transduction pathways in subconfluent keratinocytes were altered, as CYC202 increased the phosphorylation of p38 MAP kinase. The activation of this kinase was confirmed by the increased phosphorylation of p38 MAPK substrate, the small heat shock protein HSP27. Prolonged inhibition of highly proliferative cells with CYC202 for 48 and 72 h altered the expression of epidermal differentiation markers. The use of the selective p38 kinase inhibitor PD169316 demonstrated that involucrin mRNA was upregulated by CYC202 via p38 MAPK pathway. These effects were strongly dependent on cell density and were observed only in highly proliferative keratinocytes. We concluded that CYC202 although highly potent against cancer cells inhibits also the proliferation and induces early apoptotic events in autocrine culture of normal human keratinocytes, activates p38 MAP kinase pathway and alters the expression of the epidermal differentiation markers. These results suggest that despite this potency against tumour cells, CYC202 must be used attentively in the clinical practice.

Keywords: Roscovitine; Cell cycle inhibition; p38 Signaling; Gene expression; Keratinocytes; Involucrin

1. Introduction

Keratinocytes represent the main cell type in the epidermis, where they produce the cornified barrier at the surface of the skin. This production and maintenance of the barrier is made possible by complex regulation of keratinocyte proliferation and differentiation. In some pathological situations, the equilibrium between epidermal proliferation and differentiation is altered, as for instance, is the case in psoriasis, where benign hyperproliferation of keratinocytes is associated with incomplete differentiation [1,2] or as it is also the case with the development of malignant tumours.

Inhibitors of the cell cycle, more specifically inhibitors of cyclin-dependent protein kinases (CDKs) have been suggested as potential candidates to inhibit cell proliferation in different kind of hyperproliferative diseases, including keratinocyte proliferation in psoriasis [3]. CDKs are serine/threonine kinases that become activated by association with cyclin subunits. Cyclins are named after their characteristic appearance and disappearance during the cell cycle. CDK/cyclin complexes function to control cell cycle progression by phosphorylating crucial proteins involved in DNA replication and cell division, but recently, their involvement in other cellular processes like differentiation or apoptosis has further come to light [4,5].

Abbreviations: CDK, cyclin-dependent protein kinases; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; HSP27, heat shock protein 27

* Corresponding author. Tel.: +32 81 724257; fax: +32 81 724272.
E-mail address: yves.poumay@fundp.ac.be (Y. Poumay).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.06.005

Amongst inhibitors of CDKs, (*R*)-2-[[9-(1-methylethyl)-6-[(phenyl methyl) amino]-9*H*-purin-2-yl] amino]--1-butanol (CYC202), an *R*-enantiomer of roscovitine, binds to the ATP-binding site of CDKs and has shown highest activity in inhibiting CDK2/cyclin E and CDK7/cyclin H complexes [6,7]. Roscovitine has been shown to effectively block cell cycle progression of different cell types, including normal human fibroblasts [6,8] and many tumour-derived cell lines [6,9]. The different cell types exhibit variable sensitivity to roscovitine, but now, because of a high sensitivity of highly proliferative cells, this compound, CYC202, has entered clinical development for the treatment of cancer [4,10]. For therapeutic use of this compound, the knowledge of its effects on normal cells, in addition to its effects on malignant cells, is important to investigate. The results obtained in vitro are required to support the clinical trials of CYC202.

In order to evaluate whether CYC202 has effect on normal epidermal keratinocytes, we have undertaken the present study. Thus, the cytotoxicity of CYC202 towards keratinocytes has been evaluated, and we investigated whether CYC202 can inhibit the proliferation of growing keratinocytes. Since cell growth-arrested keratinocytes are normally committed to terminal differentiation, we studied also the effect of CYC202 on epidermal differentiation, and potential modulations of cell signalling pathways involved in the control of keratinocyte proliferation, differentiation, apoptosis and survival were also investigated. Considering the importance that the selective anti-tumour agents must spare normal human cells, the induction of apoptosis in proliferating normal keratinocytes by CYC202 was examined. A431 epidermoid cancer cells were also studied in order to compare the effect of CYC202 on tumour cells. Our data provide first information relevant to the effects of this CDK inhibitor in the control of epidermal keratinocyte physiology.

2. Materials and methods

2.1. Reagents

(*R*)-2-[[9-(1-Methylethyl)-6-[(phenyl methyl) amino]-9*H*-purin-2-yl] amino]-1-butanol is from Cyclacel Ltd. CYC202 is the *R*-enantiomer of roscovitine, a specific CDK inhibitor [7]. For use in culture medium, CYC202 was dissolved at 100 mM in DMSO and stored frozen at −20 °C. The inhibitor of p38 MAPK 4-(4-fluor ophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole (PD169316) was obtained from Calbiochem, dissolved at 150 mM in DMSO and stored frozen at −20 °C. The recombinant human epidermal growth factor (EGF) was purchased form R&D Systems, dissolved according to instructions (50 μg/ml stock solution) and stored at −20 °C.

2.2. Cell culture and treatment

The cell cultures were incubated at 37 °C and 5% CO_2 in a humidified incubator. Human keratinocytes were isolated by trypsin float technique from normal adult skin samples obtained at plastic surgery (Dr. B. Bienfait, Clinique St. Luc, Bouge-Namur). Primary cultures were initiated in KGM-2 keratinocyte medium (Biowhittaker). Proliferating keratinocytes were harvested by trypsinization of the subconfluent primary culture and were then plated into secondary cultures at 6×10^3 to 7×10^3 cells/cm^2 in epilife keratinocyte medium supplemented with growth factors, hormones and antibiotics (Cascade Biologics). This medium was used for all subcultures of keratinocytes. At approximately 40% of culture confluence, keratinocytes were grown under autocrine conditions by excluding all growth factors from the culture medium [11]. In those autocrine growth conditions, subconfluent cultures contain hyperproliferating undifferentiated keratinocytes and confluence of the culture is concomitant with appearance of differentiating keratinocytes as revealed by the expression of suprabasal epidermal differentiation markers [12].

For treatment of keratinocytes with CYC202, different dilutions of the stock CYC202 solution in DMSO were added to the autocrine culture medium in order to get 0.1–200 μM CYC202.

The human epidermoid carcinoma cell line A431 was cultured in Dulbecco's modified Eagle's medium (DMEM, Biowhittaker) supplemented with 10% fetal bovine serum. When A431 cells reached approximately 50% of culture confluence, CYC202 was added to this complete medium for 24 h of treatment.

Control cultures of both keratinocytes and A431 cells were incubated with DMSO only.

2.3. Determination of cell viability using the MTT assay

Normal human keratinocytes were plated at 6×10^3 to 7×10^3 cells/cm^2 and A431 cells at 2×10^3 to 4×10^3 cells/cm^2 in 24-well plates. After incubation for 24 h with different concentrations of CYC202 in a dose range between 0.1 and 200 μM, the medium was removed and substituted with 500 μl/well of MTT solution (0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) in culture medium) and the cells were incubated for 2 h in the dark at 37 °C in a humidified incubator. MTT solution was then removed, and the cells were rinsed with phosphate-buffered saline (PBS). Formazan crystals in each dish were dissolved within 250 μl of isopropanol during 30 min incubation on a rocker platform. Depending on the intensity of the dye concentration, 100 μl from each sample was diluted with isopropanol and distributed in a 96-well plate. The absorbance was spectrophotometrically measured at a wavelength of 570 nm. The

measurement was converted into a percentage of cell viability, using control cultures to determine 100% of cell viability. Each measurement was performed on quadruplicate culture wells and the experiment was repeated five times. The IC_{50} was determined (curve fitting by GraphPad software) in order to compare the sensitivity of different cell cultures [6].

2.4. Determination of DNA synthesis

Keratinocytes and A431 tumour cells were plated as above in 12-well plates and treated for 24 h with varying concentrations of CYC202. For the last 2 h of incubation, 1 μCi [^{3}H] thymidine (ICN) was added to each well. The radioactive medium was then removed and the cells were washed three times with ice-cold PBS and precipitated in 10% trichloroacetic acid (TCA) for 10 min at 4 °C. The cells were lysed in 1 ml of 0.3N NaOH and 0.1% sodium dodecyl sulphate (SDS). The radioactivity in 500 μl of each sample was counted in a Beckman Liquid Scintillation Counter after addition of Aqualuma. Triplicates of cultures for each concentration of CYC202 were analysed and the experiment was performed several times. The cellular protein content was determined by the method of Lowry et al. [13], and the rate of DNA synthesis was calculated per microgram of protein content of the culture.

Alternatively, subconfluent keratinocyte cultures were plated on coverslips in a 12-well plate and treated with varying concentrations of CYC202 for 24 h. For the last 2 h of the incubation, 10 μM solution of 5′-bromo-2′-deoxyuridine (BrdUrd, Sigma) was added to each coverslip. After formaldehyde fixation, the cellular DNA was denatured by incubation in 2N hydrochloric acid (30 min, 37 °C) and neutralized with 0.1 M sodium tetraborate (pH 8.5). After two washes with PBS/BSA, the coverslips were covered for 90 min in a humidified chamber with a mixture of an anti-BrdUrd monoclonal antibody (NovoCastra). After two washes in PBS/BSA, the coverslips were incubated in the same conditions with the secondary fluorescent antibody, anti-mouse FITC-conjugated IgG (Biosys). Finally, the coverslips were washed and mounted with Mowiol mounting medium (Molecular Probes) and observed using fluorescence microscopy.

2.5. Detection of clonogenicity of keratinocytes

Subconfluent keratinocytes were treated with 1–20 μM CYC202 for 24 h as above. The highest concentration for this experiment was chosen because it reduces cell viability by less than 50% in the MTT assay. After the treatment, the cells were trypsinized and then counted. A total number of 2000 cells were replated in 60 mm Petri dishes into fresh complete culture medium (containing all factors required for growth of keratinocytes). These cultures were incubated for 7 days. At the end of the culture period, the cells were fixed with 4% formaldehyde solution in PBS, and then stained with 0.2% solution of crystal violet for 5 min at room temperature. The staining solution was discarded and the dishes were washed three times with distilled water before taking photograph of the cell colonies.

2.6. Northern blot analysis

Poly (A) RNA was prepared from subconfluent or confluent cultures of keratinocytes using the method described by Schwab et al. [14]. The samples were resolved on 1.2% agarose–formaldehyde gel by electrophoresis for 3 h at 75 V and then transferred on Zeta-Probe GT membrane (Bio-Rad). Hybridizations were made overnight at 43 °C with randomly primed and [α-^{32}P] dCTP-labeled DNA probes for basal keratin 14, suprabasal keratin 10 and involucrin as already described [15]. In order to check equivalent loading and transfer of RNA, the blotted membranes were further hybridized with 36B4 cDNA probe [16,17]. The expression of poly (A) RNA was detected and measured with the Cyclone™ Storage Phosphor System (Packard BioScience Company) after proper exposure time of the membrane. Northern blot analysis for each culture condition was performed at least two times with different strains of keratinocytes.

2.7. Signal transduction analysis

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 in deionised water), detached by scraping and then cleared by centrifugation at 13,000 rpm at 4 °C for 15 min. Protein concentration was quantified using the Bio-Rad DC protein assay, and equal amounts of protein were used for analysis on 10 or 12% SDS-PAGE. Resolved proteins were transferred overnight at 4 °C to hybond-P, PVDF membrane (Amersham Biosciences). The membrane was then incubated with blocking buffer (5% non-fat dry milk in 0.1% Tween-20/PBS). The immunodetection of phosphorylated HSP27 and MAP kinases (Erk 1/2 and p38) was performed, respectively, with rabbit anti-phospho-HSP27 (Ser82) IgG (Upstate), mouse monoclonal IgG anti-phospho MAP kinase (ERK 1/2), clone 12D4 (Upstate) and rabbit polyclonal anti-phospho p38[pTpY$^{180/182}$] (Cell Signalling Technology). After membrane-stripping, total HSP27, ERK 1/2 and total p38 were detected, respectively, with the goat anti-HSP27 (Santa Cruz Biotechnology), rabbit anti-MAP kinase (ERK 1/2, Upstate) and the rabbit anti-p38 MAP kinase (Biosource). Primary antibodies were then revealed with anti-mouse, anti-goat and anti-rabbit horseradish peroxidase-conjugated antibodies (Dako Diagnostics). The detection was performed using the POD chemiluminescence blotting substrate (Roche Diagnostics). These

analyses were performed at least two times with different strains of keratinocytes.

2.8. Apoptosis detection by immunofluorescent staining of active caspase-3

The cells were grown on glass coverslips. After incubation with CYC202 or with 1 μM staurosporine for apoptosis induction, the cells were fixed with formaldehyde and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The incubation with the anti-ACTIVE® caspase-3 pAb (Promega) was performed overnight at 4 °C, followed by incubation with Cy®3-conjugated secondary antibody for 2 h in a humidified incubator in the dark. The coverslips then were mounted with Mowiol mounting medium (Molecular Probes) and observed under a fluorescence microscope.

3. Results

3.1. CYC202 exhibits higher toxicity in subconfluent cultures of keratinocytes than in confluent cultures

In order to assess the effects of CYC202 on normal human keratinocytes, we first determined the viability of both subconfluent and confluent keratinocytes cultured in autocrine conditions and treated for 24 h with a large range of concentrations (0.1–200 μM) (Fig. 1). In such culture conditions, subconfluent cultures contain undifferentiated highly proliferative cells, which mimic the growth of keratinocytes during wound healing. Conversely, confluent cultures contain mainly growth-arrested differentiating keratinocytes and their low proliferating capacity is probably closer to the keratinocyte proliferation in normal epidermis at equilibrium [12]. In both subconfluent and confluent cultures, CYC202 induces a dose-dependent loss in cell viability; however, the proliferating keratinocytes were more sensitive than growth-arrested keratinocytes at concentrations between 1 and 20 μM, with IC_{50} values at 17 μM for subconfluent cells and 35 μM for confluent cells. These values are in perfect agreement with data comparing the cytotoxicity of CYC202, which is highest against rapidly proliferating cells than against quiescent cells [6]. In order to compare normal keratinocytes with tumour cells, the viability of the rapidly growing A431 epidermoid cancer cells was determined in a range of CYC202 concentrations between 0.1 and 50 μM (Fig. 2B). The IC_{50} value was estimated around 10 μM for this cell type.

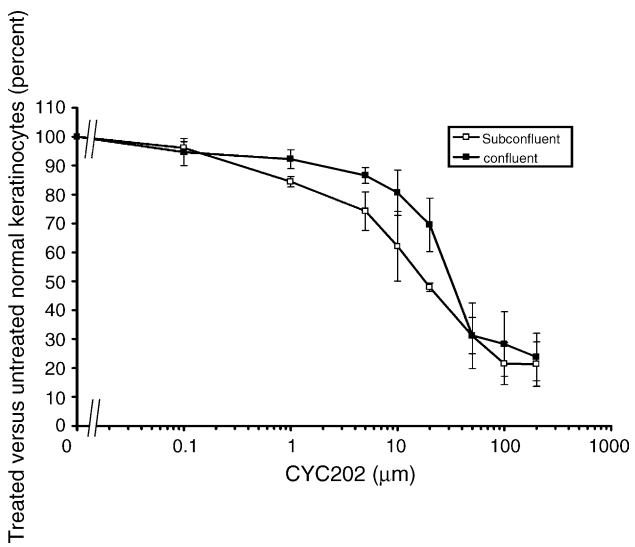


Fig. 1. Effect of CYC202 on the viability of subconfluent and confluent keratinocyte cultures. After 24 h of incubation of subconfluent or confluent keratinocyte cultures with different concentrations of CYC202 (0.1–200 μM), an MTT assay was performed as described in Section 2 in order to determine the cell viability in each condition. This value was compared to the value obtained in either subconfluent or confluent untreated keratinocyte cultures, where DMSO only had been added for the 24 h incubation. The viability of untreated cultures was chosen as 100%. Data are presented as percentage of keratinocyte viability ± S.D. averaged from five different experiments.

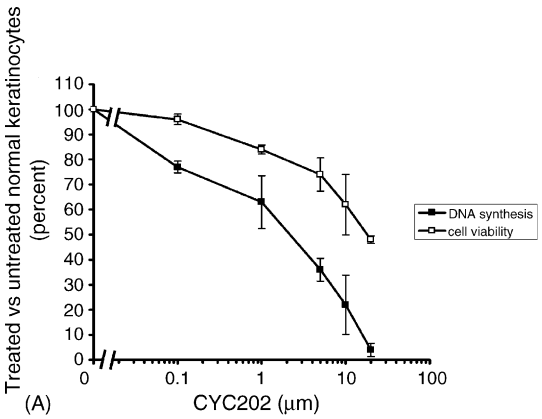
3.2. CYC202 inhibits cell proliferation in a dose-dependent manner at concentrations weakly cytotoxic

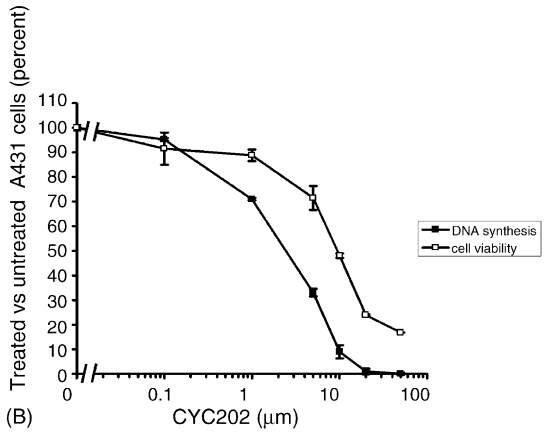
We next examined whether CYC202 inhibits the DNA synthesis in subconfluent growing keratinocytes (Fig. 2A) or in A431 cells (Fig. 2B). As shown in Fig. 2A, in normal epidermal keratinocytes CYC202 inhibited significantly the incorporation of [^{3}H] thymidine in DNA at all tested concentrations after 24 h of incubation, but a similar inhibition was found already after 4 h of treatment (data not shown). Compared to the loss in cell viability, a more important decrease in the DNA synthesis happens at lower concentrations, suggesting that this decrease does not result solely from toxicity (Fig. 2C). Similar data were found with A431 cells, except that 0.1 μM CYC202 was ineffective on DNA synthesis in this cell type (Fig. 2B). Altogether, data on proliferating keratinocytes and A431 cells indicate that CYC202 is a drug with growth-inhibiting activity on proliferating cell types, either normal or malignant.

Similar results were obtained, when we analysed the incorporation of the thymidine analogue 5′-bromo-2′-deoxyuridine in keratinocytes treated with CYC202. BrdUrd-labeled cells were observed in untreated subconfluent cultures or in cultures treated with CYC202 up to 5 μM. The absence of incorporation of BrdUrd in keratinocyte cultures treated with concentrations above 5 μM, is a strong indication that DNA synthesis, i.e. cell cycling, is inhibited by CYC202 in normal cells (Fig. 3).

3.3. The inhibition of cell growth by CYC202 in epidermal keratinocytes is reversible

In case, CYC202 could be interesting for therapeutic purposes, its inhibitory effect on the proliferation of normal cells should be reversible after withdrawal of the drug. In





(C)

	subconfluent keratinocytes	confluent keratinocytes	A431 cell line
IC50 (μM ± SD)	17.3 ± 7.2591	34.75 ± 1.6405	9.534 ± 6.265
EC50 (μM ± SD)	6.1 ± 1.9598	-	3.015 ± 2.0274

Fig. 2. Comparison between cell viability and [^{3}H] thymidine incorporation in subconfluent cultures of keratinocytes or in cultures of A431 cells incubated with CYC202. Subconfluent cultures of keratinocytes (A) or cultures of A431 cells (B) were treated for 24 h with different concentrations of CYC202 (0.1–20 μM (A) or 0.1–50 μM (B), respectively) before the determination of cell viability or the measurement of [^{3}H] thymidine incorporation. The viability was determined as in Fig. 1. The [^{3}H] thymidine incorporation into DNA was measured by adding 1 μCi [^{3}H] thymidine to the culture medium for the last 2 h of incubation with CYC202 and analysing the radioactivity incorporated into DNA per mg of cell protein. The viability and the [^{3}H] thymidine incorporation measured in untreated cells were chosen as 100%. Data are presented in percent of untreated cells ± S.D. averaged from five different experiments (A) or from triplicates (B). The concentrations which cause 50% reduction in cell viability IC_{50} and which inhibit cell proliferation by 50% EC_{50} were calculated by curve fitting with GraphPad software (C).

order to evaluate whether the inhibition of keratinocyte growth observed between 1 and 20 μM of CYC202 could be reversed, subconfluent keratinocytes were treated for 24 h at specific concentrations (1, 5, 10 and 20 μM); then, the cells were trypsinized in order to plate them at low density in a clonal assay [18]. Fig. 4 illustrates that low concentrations of CYC202 (1 and 5 μM) allow clonogenicity of keratinocytes similar to the one observed with cells incubated in control culture conditions (0 μM). However, the number of colonies was slightly decreased after an incubation with 10 μM CYC202, and less numerous smaller colonies were observed with cells treated with 20 μM CYC202, a concentration shown to allow less than 50% of viability by the MTT assay.

3.4. Long-term inhibition with CYC202 changes the expression of differentiation markers in normal human keratinocytes

Since the naturally occurring growth-arrest in epidermal keratinocytes is concomitant with some induction of terminal differentiation [12], we analysed whether the inhibition of cell proliferation by CYC202 in subconfluent cultures of keratinocytes could induce the expression of differentiation markers by those cells. Northern blot analysis was used to study the expression of keratins 10 and 14, together with the expression of involucrin in keratinocytes treated with concentrations of CYC202 (5, 10 and 20 μM) high enough in order to allow less than 50% of [^{3}H] thymidine incorporation. With this technique, we found (Fig. 5) that the expression of keratin 14, a marker for undifferentiated cell phenotype, was not affected by CYC202. Simultaneously, the suprabasal keratin 10, an early marker of epidermal differentiation, whereas slightly expressed in control cells, was not induced by CYC202. Similar observation was done with the expression of involucrin, a late marker of differentiation (Fig. 5). Interestingly, we extracted only very small amount of mRNA from keratinocyte cultures treated with 20 μM CYC202. This is not surprising, as it was already reported that roscovitine inhibits RNA synthesis in human skin fibroblasts and in colon carcinoma cells [19]. On the other hand, while studying the mRNA expression in confluent cultures of keratinocytes, we experienced no problem in collecting mRNA from cells treated with 20 μM CYC202 (Fig. 5). Very likely, the problem with the RNA extraction from subconfluent cultures treated with 20 μM CYC202 is due to the low viability of subconfluent keratinocytes at this concentration (Fig. 1) than due to inhibition of RNA synthesis by CYC202. In confluent cultures of keratinocytes, CYC202 had again no effect on the expression of keratin 14 or on the expression of involucrin, however, a concentration of 20 μM CYC202 resulted in a markedly inhibited expression of keratin 10 (Fig. 5).

Surprisingly, longer incubations (48 and 72 h) with CYC202 caused in subconfluent keratinocytes induction of involucrin mRNA (Fig. 6). This induction is strongest at a concentration of 20 μM CYC202, despite the lower viability observed at this concentration and the lower amount of RNA extracted. Both the expressions of keratin 10 mRNA and keratin 14 mRNA in those conditions were also slightly increased by 20 μM CYC202. However, the effect on involucrin mRNA expression was strongly dependent on cell density, as it was not observed, when the cells reached confluence. On completely confluent cultures, keratin 10 mRNA was inhibited by CYC202. The expres-

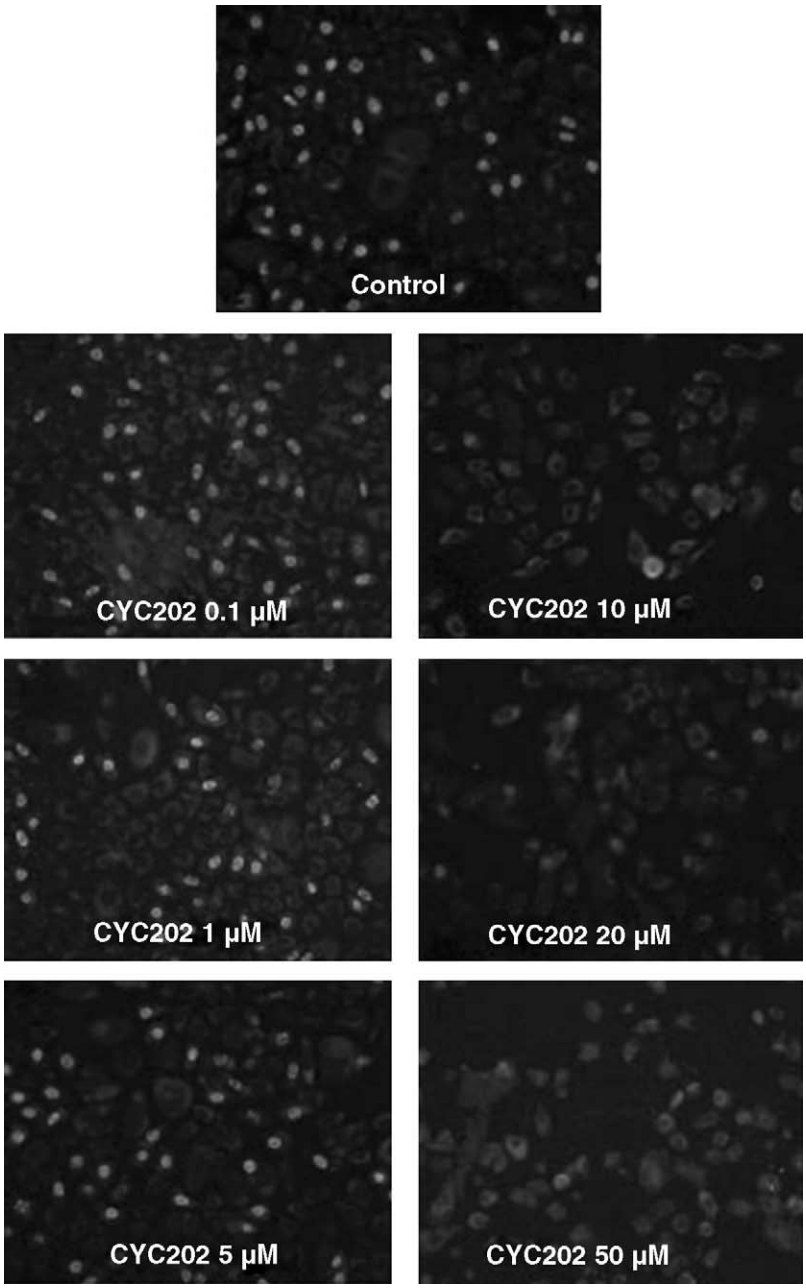


Fig. 3. Immunofluorescent staining for 5′-bromo-2′-deoxyuridine (BrdUrd) incorporated in newly synthesised DNA. Keratinocytes grown on coverslips were incubated with various concentrations of CYC202 for 24 h and with 10 μM 5′-bromo-2′-deoxyuridine for the last 2 h of the treatment. After cell fixation, the coverslips were incubated with an anti-BrdUrd monoclonal antibody and with secondary FITC-conjugated IgG. The coverslips were observed under a fluorescence microscopy.

sion of involucrin in these conditions was unchanged and that of keratin 14 mRNA was slightly increased only at a concentration of 20 μM CYC202 (Fig. 6).

3.5. P38 phosphorylation but not ERK 1/2 phosphorylation is increased by CYC202 in subconfluent keratinocytes

Various signalling cascades regulate the expression of differentiation markers in keratinocytes. So, we focused our attention on cell signalling in keratinocytes treated with CYC202.

Very recently, it has been reported that treatment of carcinoma cell lines with CYC202 increases ERK 1/2 (p42/p44) phosphorylation [20]. These MAP kinases are involved in the induction of keratinocyte proliferation [21–23]. Because our results show that CYC202 inhibits the proliferation of normal epidermal keratinocytes, we analysed the activation level of ERK 1/2. In subconfluent keratinocytes, the basal phosphorylation of ERK 1/2 remained unchanged after 24 h with 1, 5 or 10 μM CYC202, but it was decreased at 20 μM. Incubation with 10 ng/ml EGF, one of the natural inducers of the MAPK cascade [24], strongly augmented the phosphorylation of ERK 1/2. This induction

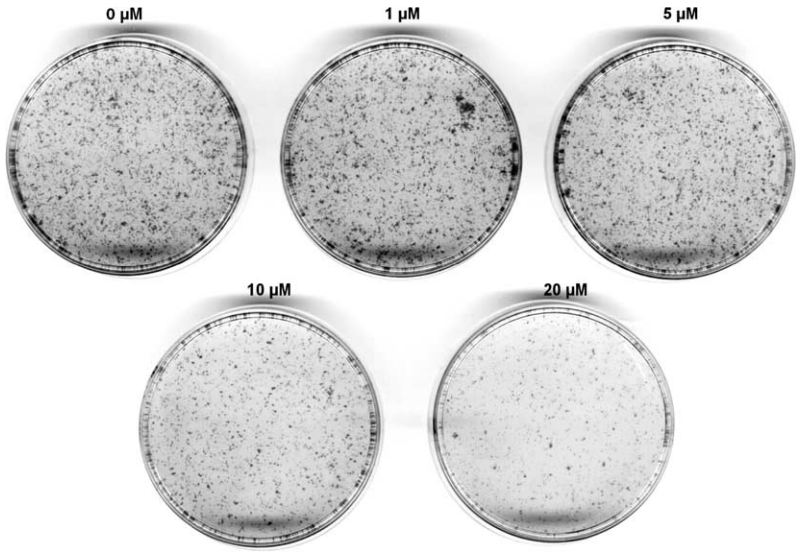


Fig. 4. Clonogenicity of keratinocytes after CYC202 treatment. Subconfluent cultures of growing keratinocytes were treated for 24 h with different concentrations of CYC202 (1–20 μM); then, the inhibitor was washed and the different cultures trypsinized. The cells were counted and 2000 cells from each treatment were reseeded in 60 mm diameter Petri dishes using fresh complete culture medium containing all growth factors necessary for keratinocyte proliferation. After 7 days, the cell colonies were fixed and stained with crystal violet. The bottom of stained dishes was photographed in a scanner. Keratinocytes cultures treated with DMSO only were used as control.

by EGF was unchanged by pre-treatment with CYC202 at any concentration tested (Fig. 7A).

In confluent cells, the basal phosphorylation of ERK 1/2 seems slightly stronger than in subconfluent keratinocytes and CYC202 does not affect its level. Again, an increased phosphorylation of these kinases can be observed after treatment for 30 min with 10 ng/ml EGF, either in control keratinocytes and in keratinocytes pre-incubated for 24 h with various concentrations of CYC202 (Fig. 7A).

Because of this lack of effect on ERK 1/2, we next examined the activity of another member of the MAP kinase family, the p38 MAP kinase, which is also known

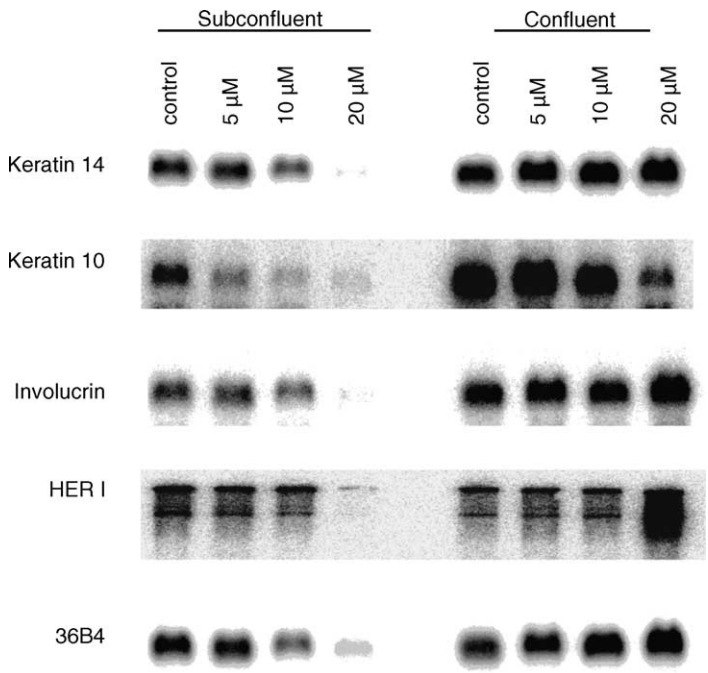


Fig. 5. Northern blot analysis of the expression of epidermal differentiation markers in keratinocytes incubated with CYC202. After 24 h of incubation of subconfluent or confluent keratinocyte cultures with concentrations of CYC202 (5, 10 and 20 μM) known to induce blockade of DNA synthesis in proliferating cells or after 24 h of incubation of cultures with DMSO only (control), poly (A) RNA were extracted and analysed by Northern blotting. Probes labeled with ^{32}P by random priming and used for hybridization of the membrane were cDNAs specific for keratin 14, keratin 10, involucrin and the house-keeping gene 36B4 (in order to check equivalent loading).

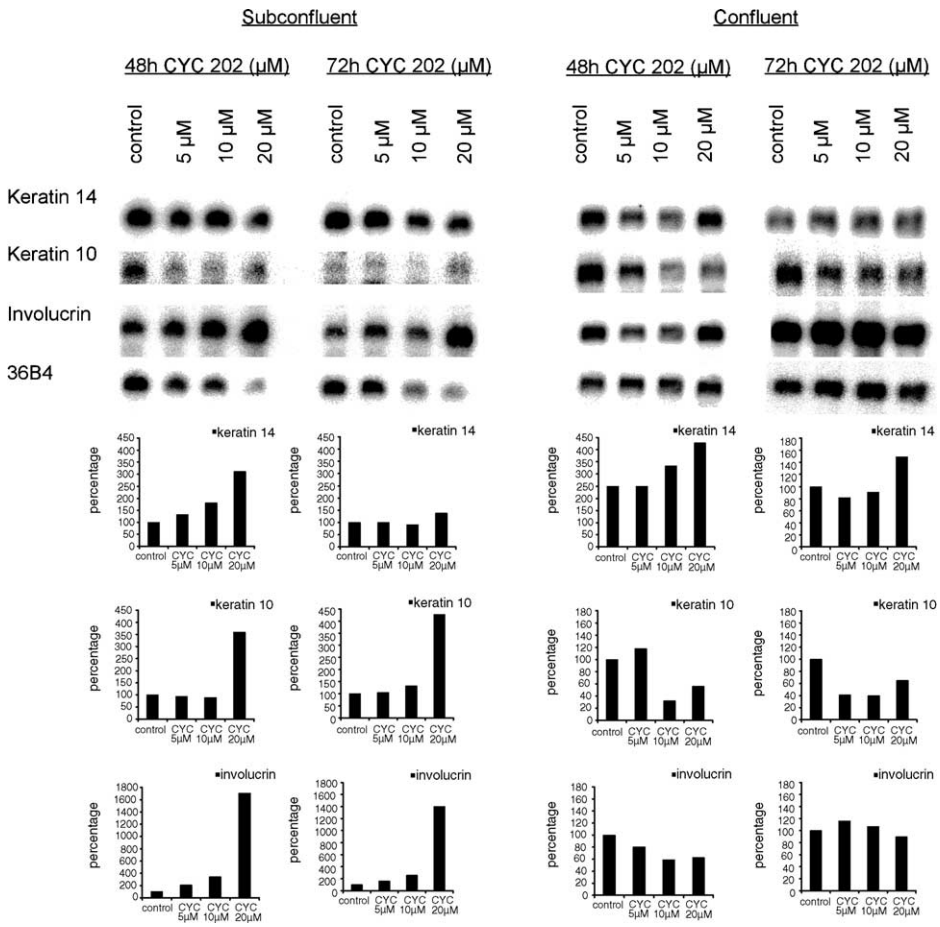


Fig. 6. Northern blot analysis of the expression of epidermal differentiation markers in keratinocytes incubated with CYC202 for 48 and 72 h. Poly (A) RNA were extracted from subconfluent and confluent keratinocytes after 48 and 72 h of incubation with CYC202 or DMSO (for the control probes), and analysed by Northern blotting. Specific c DNAs probes for keratin 14, keratin 10, involucrin and house-keeping gene 36B4 were labeled with ^{32}P by random priming and used for hybridization. Densitometry was performed to determine the relative expression of mRNA for each epidermal marker (the ratio of the marker expression compared to the expression of the house-keeping gene) and is presented as a percentage of the expression in the control probes, which is arbitrarily fixed at 100%.

as involved in the regulation of keratinocyte proliferation, as well as in the regulation of their differentiation, apoptosis and responses to stress [25]. The phosphorylation of the p38 MAP kinase was increased in subconfluent keratinocytes by a treatment with CYC202. However, this was not the case in confluent cultures. In subconfluent keratinocytes the level of p38 phosphorylation was highest at 20 μM CYC202. At this concentration of CYC202, the activated p38 correlated with decreased ERK 1/2 phosphorylation and with decreased cell viability (Figs. 1 and 7A). The basal phosphorylation of the p38 MAP kinase, like that of ERK 1/2, was also slightly higher in confluent cells and unaltered by CYC202, by treatment with EGF alone or by combination of both the inhibitor and the growth factor. Similar to ERK 1/2 phosphorylation, the activity of p38 also was increased by recombinant EGF 10 ng/ml added to untreated subconfluent keratinocytes or treated with CYC202 (Fig. 7B).

A time-course experiment was performed for p38 activation after incubation with 20 μM CYC202, a concentration, which is slightly higher then the IC_{50} in proliferating keratinocytes. The increase in p38 phosphorylation was time-dependent and was observed after 16 h of treatment with CYC202 and extended through the 24 h incubation period. The induced p38 phosphorylation remained elevated, when the inhibitor was withdrawn from culture medium for up to 24 h of incubation (Fig. 7C). In certain experiments, an earlier induction of p38 MAP kinase phosphorylation could be observed after 6 h of incubation with 20 μM CYC202 (data not shown).

3.6. The induction of involucrin expression after long-term treatment with CYC202 is a result of p38 MAP kinase activation

Several previous reports have already shown that the activation of p38 MAP kinase in normal human keratinocytes is leading to an increased expression of the late differentiation marker involucrin [27,28]. As already described above, the incubation of keratinocytes with CYC202 for 24 h increases the phosphorylation of p38 MAP kinase (Fig. 7B) and long-term incubations for 48 or 72 h resulted in an increased expression of the differentiation marker involucrin (Fig. 6). We tested whether a

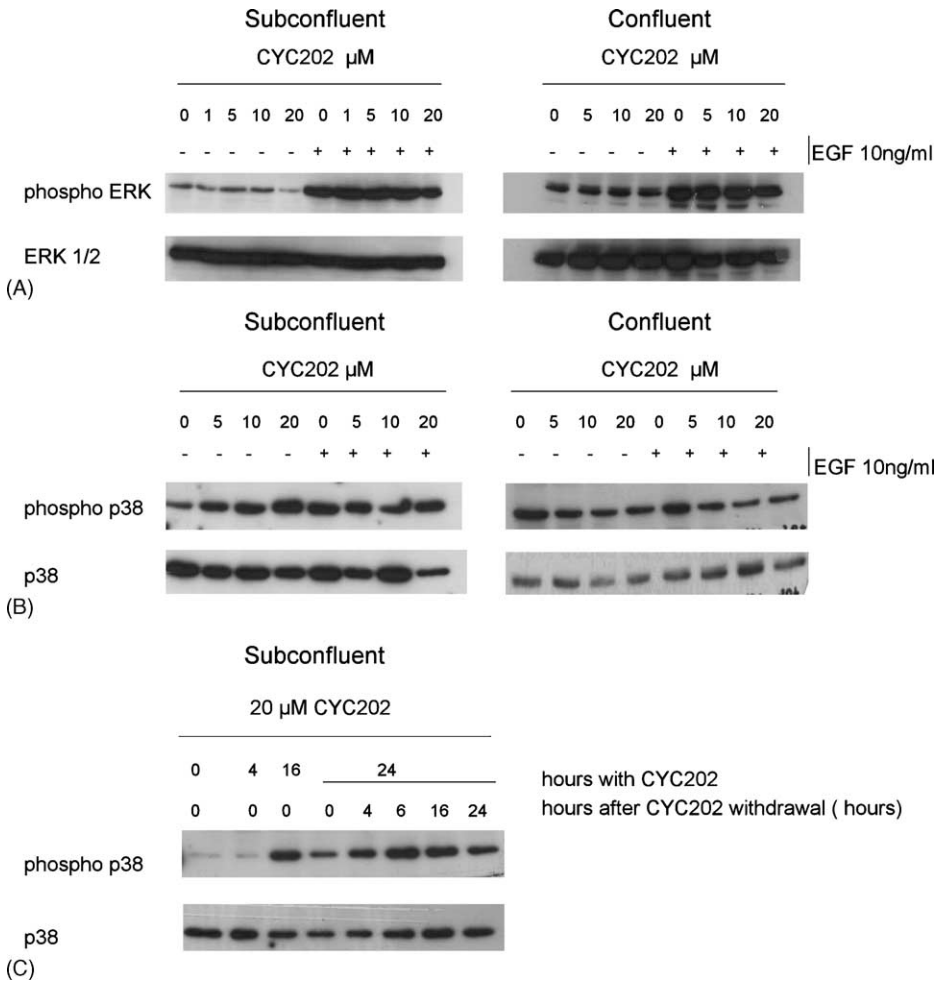


Fig. 7. Analysis of the phosphorylation of ERK 1/2 and p38 MAP kinase in keratinocytes incubated with CYC202. Cells incubated with 0, 5, 10 or 20 μM CYC202 for 24 h and (or without) recombinant EGF10 ng/ml were lysed, and equal amounts of proteins were used for analysis of the basal and phosphorylated state of ERK 1/2 (A) and of p38 (B). The kinetics of p38 phosphorylation was estimated after incubation of subconfluent keratinocytes with 20 μM CYC202 for different time intervals before lysis or withdrawal of the inhibitor after 24 h and a change into fresh medium for 0, 4, 16 or 24 h before lysis. Western blotting with an antibody to the total p38 was used for control of gel loading (C).

relationship exists between the effect of the CDK–inhibitor CYC202 on the expression of involucrin mRNA and p38 activation in normal epidermal keratinocytes. For this investigation, we performed incubation of the cultures with PD169316, a chemical inhibitor of p38 MAP kinases. Indeed, PD169316 effectively blocks the induction of involucrin expression by 20 μM CYC202, after a 48 h of treatment of subconfluent cells (Fig. 8), suggesting that involucrin expression after CYC202 treatment is induced by an activated p38 MAP kinase.

3.7. The p38 MAP kinase, activated by CYC202, phosphorylates the small heat shock protein 27 (HSP27)

It is well-documented that in keratinocytes and other cell types, the activation of p38 MAP kinase by various stimuli, results in phosphorylation of the small heat shock protein 27 [29–32]. We investigated whether CYC202, when activating p38 MAP kinase, also induces the phosphorylation of HSP27 in keratinocytes. An increased phosphorylation of HSP27 was induced by CYC202 only in subconfluent keratinocyte cultures, when p38 MAP kinase was activated. The phosphorylation of HSP27 was induced by all concentrations of CYC202 tested in this experiment. Furthermore, the phosphorylation of this protein was strongly inhibited by PD169316, a specific p38 MAP kinase inhibitor (Fig. 9). In confluent keratinocyte cultures, the basal level of HSP27 phosphorylation is constitutively higher then in subconfluent keratinocytes and remained unaltered by treatment with CYC202 (data not shown).

3.8. The cytotoxicity of CYC202 towards proliferating normal keratinocytes correlates with induction of apoptotic cell death

From a clinical point of view, in order to be used as a pharmacological agent, CYC202 should be as selective as possible in the suppression of the tumour growth and ideally, the induction of cell death should be restricted

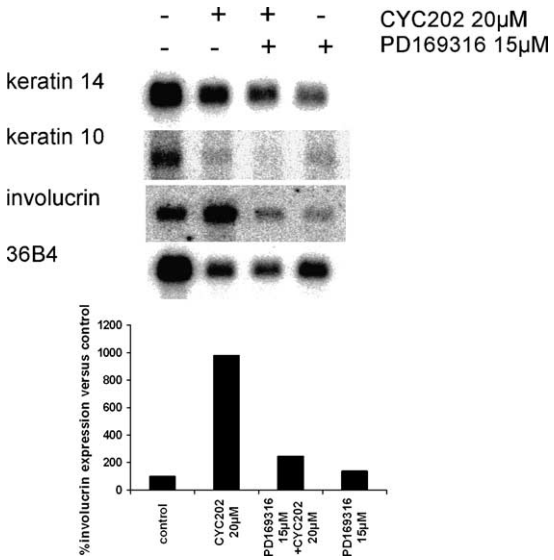


Fig. 8. Pre-treatment with p38 MAP kinase inhibitor PD169316 impairs the induction of involucrin expression by CYC202. The cells were incubated for 48 h with 20 μM CYC202 or pre-incubated for 1 h with 15 μM PD169316 before adding 20 μM CYC202 for 48 h. Control cells were incubated for 48 h with DMSO (control) or 15 μM PD169316. Poly (A) RNA was extracted and analysed by Northern blotting for the expression of epidermal markers keratin 14, keratin 10, involucrin and the house-keeping gene 36B4. The relative expression of involucrin comparing to the control cells (100%) was quantified by densitometry and presented as a graph.

to the tumour or hyperproliferating cells. It has been shown that CYC202/roscovitine induces apoptosis in tumour cells [9,35,36,39]. The activation of the p38 kinase is regularly associated with the pathway of the programmed cell death [21,25]. In order to explore if the cytotoxicity of CYC202 in keratinocytes was due to apoptotic cell death, we performed assay for the activity of caspase-3, the key executor of apoptosis. The proteolytic cleavage of procaspase-3 to its active form is indicative of early induction of apoptosis. Detection of the active form was assessed by an immunocytochemical assay. The active caspase-3 was not observed in cultures treated with CYC202 at concentrations between 0.1 and 5 μM, which correlates with the observed high cell viability after treatment of keratinocytes with these concentrations. Cleavage of caspase-3 was evident at concentrations of CYC202 above 10 μM

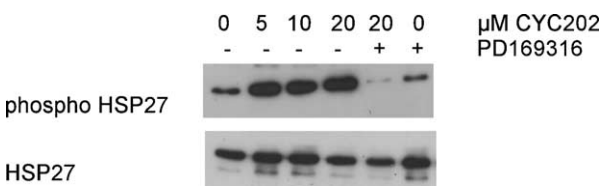


Fig. 9. Phosphorylation of HSP27 by activated p38 MAP kinase after incubation with CYC202. Subconfluent cultures of keratinocytes were incubated for 1 h with 0 or 15 μM of p38 MAPK inhibitor PD169316. CYC202 was added in concentrations of 0, 5, 10 or 20 μM and the cells were incubated for additional 24 h. After lysis, Western blotting was transferred, and equal amounts of proteins were used for detection of phosphorylated and total HSP27 using specific antibodies.

(Fig. 10), concentrations inhibiting the cell proliferation and decreasing the cellular viability of the cultured keratinocytes.

4. Discussion

Because of its high anti-tumoural potency, the CDK–inhibitor CYC202 (*R*-roscovitine) has received a lot of attention regarding its effects on cancer cells [6]. Recently, clinical trials have already been initiated with this inhibitor [4]. Several years ago, it was suggested that cell cycle inhibitors like roscovitine could be used also for inhibition of hyperproliferation in diseases like psoriasis [3]. Still there are no data about the effects of CYC202 on normal or psoriatic epidermal cells. In this study, we evaluate the effects of the cyclin-dependent kinase inhibitor CYC202 on normal keratinocyte proliferation and differentiation, as well as on signal transduction pathways involved in the regulation of these processes in this epidermal cell type.

As was expected after treatment with CYC202, the proliferation of normal epidermal keratinocytes was strongly inhibited. Because of the specific action of CYC202 on the cell cycle, mainly on CDK2–cyclin E complexes controlling G1/S transition, epidermoid A431 cancer cells were very sensitive to this compound, highly proliferating subconfluent keratinocytes exhibited also a high sensitivity, whereas mostly growth-arrested confluent keratinocytes exhibited lower sensitivity. This responsiveness of highly proliferative cells is in perfect agreement with previous observations with CYC202 [6]. It is of highest interest that CYC202 inhibits cell growth effectively at concentrations, which allow a viability of keratinocytes still higher than 50%. Of high interest also for its potential use on normal cell types is the reversibility of the growth-arrest obtained with CYC202. Indeed, after removal of the inhibitor used at concentrations below 10 μM, the viable keratinocytes overcome the growth-arrest and demonstrate ability to reinitiate proliferation necessary for tissue renewal and maintenance.

Usually, the commitment of keratinocytes towards epidermal differentiation is associated with growth-arrest and high cellular levels of the natural CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ [33]. Incubation with the chemical CDK–inhibitor roscovitine of epithelial cells in the organ of Corti was already found to induce cell differentiation [34]. Although we hypothesized that inhibition of the cell cycle with CYC202 could also induce differentiation in normal human epidermal keratinocytes, incubations for 24 h with this inhibitor did not perturb the physiology of cells undergoing terminal differentiation, as treatment of keratinocytes with CYC202 did not induce expression of the differentiation markers keratin 10 and involucrin. However, this observation was denied after long-term inhibition with CYC202. Indeed, treatment for 48 and 72 h with CYC202 strongly induced involucrin mRNA

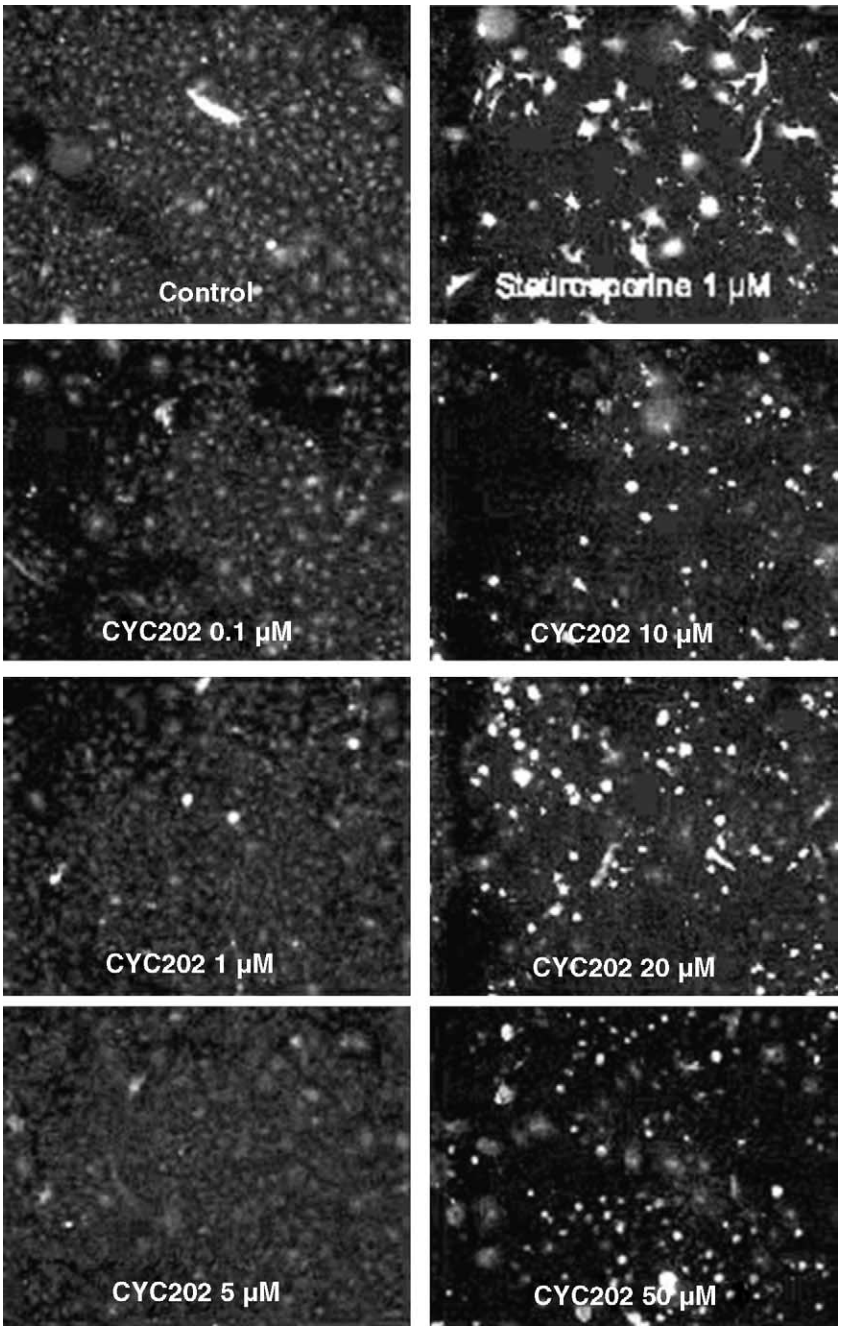


Fig. 10. Immunocytochemical staining for the active form of caspase-3. Keratinocytes grown on coverslips were incubated for 24 h with CYC202 (0.1–50 μM) and with the apoptosis inducer stauropsorine (1 μM). The incubation with the anti-active® caspase-3 antibody (Promega) was performed according the instructions of the manufacturer and the specific staining was observed under a fluorescence microscope.

expression. Interestingly, this effect was observed only after treatment of highly proliferating subconfluent keratinocytes, and not in keratinocytes growth-arrested by confluence of the culture. Because these confluent cells are already differentiating and express high amounts of involucrin, we suppose that CYC202 cannot increase further this expression. On the other hand, in proliferating keratinocytes, we hypothesize that the induction of involucrin expression could probably be part of a kind of stress response and probably serves as a protective mechanism for still viable cells. It is well-documented that amongst upstream activators of involucrin gene expression are the p38 stress MAP kinases [25,27,28]. The specific p38 MAP kinase pathway is involved in the cellular response to a variety of stress agents, including chemotherapeutic drugs, which induce mitotic arrest [26]. In addition, it was recently reported that CYC202 induces p38 phosphorylation in colon cancer cells [20]. In our hands also, CYC202 at concentration close to the IC_{50} (i.e. 20 μM) induces activation/phosphorylation of p38 MAP kinase in subconfluent keratinocyte cultures. Furthermore, investigation with the p38 MAPK inhibitor PD169316 clearly shows

that the induction of involucrin expression is a consequence of the activation of p38 MAPK.

A well-known target of p38 MAP kinase is the small heat shock protein 27. Activation of HSP27 by p38 MAP kinase in keratinocytes and other cells is observed in response to a variety of stresses, like UV, osmotic stress or heat shock [29–32]. Treatment with CYC202 also increases the phosphorylation of HSP27 in subconfluent keratinocytes and this phosphorylation of HSP27 is dependent on the activity of p38 kinase, since this process is strongly inhibited by PD169316. The activation of HSP27 is certainly part of a cellular protective mechanism. For instance, one of the functions of HSP27 is to stabilize actin cytoskeleton.

Another consequence of the activated p38 MAPK could be apoptosis. The function of several CDK inhibitors or antagonists as apoptotic agents in transformed cells has been documented [36]. Roscovitine and thus CYC202 can be an apoptotic inducer in tumour cells [9,37,39], although roscovitine has shown anti-apoptotic effects in cerebellar granule cells [37]. When activated, caspase-3 has a pivotal role in the apoptotic cell death, and is considered as a point of no return. Normal proliferating keratinocytes treated with CYC202 were committed to apoptosis, as evidenced by caspase-3 activation at concentrations of CYC202 corresponding to a strong decrease in cell viability. This suggests that the cytotoxicity of CYC202 is, in fact, a result of the programmed cell death induced by the inhibitor.

The phosphorylation of p38 kinase induced by CYC202 persists for at least 24 h after withdrawal of the inhibitor. This suggests that the activation of p38 is not a direct effect of the presence of CYC202, but is rather mediated by long-term changes in the signalling network of keratinocytes. Despite the fact that usually caspase activation is downstream of p38 MAPK, there are data indicating that caspase-3 can function upstream of p38 MAPK [40–42]. It is possible that the activation of caspase-3 induced by CYC202 is responsible for the phosphorylation and activation of p38 MAPK, but the exact mechanism remains to be identified.

Available data from in vitro kinase assays with CYC202 demonstrated that this inhibitor is also potent against the MAP kinase ERK 2, but IC_{50} toward this kinase was reported about 10 times greater than the value measured with CDK2/cyclin E [6]. These data can explain why the highest concentration of CYC202 used in our experiments (i.e. 20 μM) inhibits ERK 1/2 phosphorylation. Conversely, in a very recent report, a surprising activation of ERK 1/2 by CYC202 has been demonstrated in human colon cancer cells [20]. However, the concentrations of the inhibitor used in that study are among the concentrations found cytotoxic for normal human keratinocytes in our experiments. This is a good example that such an inhibitory compound may have opposite effects in normal versus cancer cells. These observations strongly suggest that the effects of CYC202 should ideally be tested in different experimental systems in order to be better understood.

The coincidence between ERK inhibition and p38 activation at concentration 20 μM of CYC202 is particularly interesting in keratinocytes. Indeed, an inverse correlation between the two MAPK pathways in normal human keratinocytes has already been reported [27,28] and is probably linked to the regulation of epidermal differentiation. Furthermore, the formation of a complex between these two kinases, with inhibition of ERK 1/2 by p38, is possible [27,38]. However, in our experiments, such an inhibition of ERK 1/2 was not observed at the lower concentrations (5 and 10 μM) of CYC202, contrarily to the activation of p38 MAPK. Although further studies are required, we suggest that CYC202 may alter the keratinocyte physiology at least partly through its interactions with the MAP kinases.

In conclusion, our study provides the first information about the effects of the CDK inhibitor CYC202 on normal human epidermal keratinocytes, including effects on cell proliferation and signaling, on viability and activation of apoptosis, on induction of a stress response and epidermal differentiation. Regardless of the popularity of this compound as a potent anti-cancer agent, it seems that cycling cells exhibit similar sensitivities to CYC202. In this regard, the relative selectivity of the compound towards tumour cells and its cytotoxicity towards different normal cells of the human body need to be carefully assessed in order to be confronted to the results obtained during clinical trials.

Acknowledgements

The authors wish to thank Dr. B. Bienfait (Clinique St. Luc, Bouge-Namur) for providing normal skin samples. The excellent technical assistance of F. Herphelin and D. Van Vlaender is also gratefully acknowledged. GA holds a fellowship from the University of Namur (FUNDP) and RJ holds a fellowship from the FRIA. This study was supported in part by Medical University, Sofia Grant 5/2003 to VM and by FRFC Grant No. 2.4506.01 to YP.

References

[1] McKay IA, Leigh IM. Altered keratinocyte growth and differentiation in psoriasis. Clin Dermatol 1995;13:105–14.
[2] Van de Kerkhof PC, Van Erp PE. The role of epidermal proliferation in the pathogenesis of psoriasis. Skin Pharmacol 1996;9:343–54.
[3] Meijer L. Chemical inhibitors of cyclin-dependent kinases. Trends Cell Biol 1996;6:393–7.
[4] Fischer PM, Gianella-Borradori A. CDK inhibitors in clinical development for the treatment of cancer. Expert Opin Investig Drugs 2003;12:955–70.
[5] Knockaert M, Greengard P, Meijer L. Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol Sci 2002;23:417–25.

[6] McClue SJ, Blake D, Clarke R, Cowan A, Cummings L, Fisher PM, et al. In vitro and in vivo antitumor properties of the cyclin-dependent kinase inhibitor CYC202 (*R*-roscovitine). Int J Cancer 2002;102: 463–8.
[7] Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 1997;243:527–36.
[8] Alessi F, Quarta S, Savio M, Riva F, Rossi L, Stivala LA, et al. The cyclin-dependent kinase inhibitors olomoucine and roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 kinase activity. Exp Cell Res 1998;245:8–18.
[9] Mgbonyebi OP, Russo J, Russo IH. Roscovitine induces cell death and morphological changes indicative of apoptosis in MDA-MB-231 breast cancer cells. Cancer Res 1999;15:1903–10.
[10] Meijer L, Raymond E. Roscovitine and other purines as kinase inhibitors: from starfish oocytes to clinical trials. Acc Chem Res 2003;36:417–25.
[11] Cook PW, Pittelkow MR, Shipley GD. Growth factor-independent proliferation of normal human neonatal keratinocytes: production of autocrine- and paracrine-acting mitogenic factors. J Cell Physiol 1991;146:277–89.
[12] Poumay Y, Pittelkow MR. Cell density and culture factors regulate keratinocyte commitment to differentiation and expression of suprabasal K1/K10 keratins. J Invest Dermatol 1995;104:271–6.
[13] Lowry OJ, Rosebrough NJ, Farr AL, Randall RJ. Protein measurements with Folin phenol reagent. J Biol Chem 1951;193:265–75.
[14] Schwab M, Alitalo K, Varmus HE, Bishop JM, George D. A cellular oncogene (c-Ki-ras) is amplified, over expressed and located with karyotypic abnormalities in mouse adrenocortical tumor cells. Nature 1983;303:497–501.
[15] De Potter IY, Poumay Y, Squillace KA, Pittelkow MR. Human EGF receptor (HER) family and heregulin members are differentially expressed in epidermal keratinocytes and modulate differentiation. Exp Cell Res 2001;271:315–28.
[16] Laborda J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. Nucleic Acids Res 1991;19:3998.
[17] Stoll SW, Kansra S, Peshick S, Fry DW, Leopold WR, Wielsen JF, et al. Differential utilization and localization of ErbB receptor tyrosine kinases in skin compared to normal and malignant keratinocytes. Neoplasia 2001;3:339–50.
[18] Wille Jr JJ, Pittelkow MR, Shirpley GD, Scott RE. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum free medium: clonal analyses, growth kinetics and cell cycle studies. J Cell Physiol 1984;121:31–44.
[19] Ljungman M, Paulsen MT. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. Mol Pharmacol 2001;60(4):785–9.
[20] Whittaker SR, Walton MI, Garrett MD, Workman P. The cyclin-dependent kinase inhibitor CYC202 (*R*-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase pathway. Cancer Res 2004;64:262–72.
[21] Eckert RL, Efimova T, Dashti SR, Balasubramanian S, Deucher A, Crish JF, et al. Keratinocyte survival, differentiation, and death: many roads lead to mitogen-activated protein kinase. J Investig Dermatol Symp Proc 2002;7(1):36–40.
[22] Mitev V, Miteva L. Signal transduction in keratinocytes. Exp Dermatol 1999;2:96–108.
[23] Praskova M, Kalenderova S, Miteva L, Poumay Y, Mitev V. Dual role of protein kinase C on mitogen-activated protein kinase activation and human keratinocyte proliferation. Exp Dermatol 2002;11(4):344–8.
[24] Mitev V, Le Panse R, Coulomb B, Miteva L, Houdebine LM. Epidermal growth factor stimulates mitogen-activated protein kinase by a PKC-dependent pathway in human keratinocytes. Biochem Biophys Res Commun 1995;208(1):245–52.
[25] Eckert RL, Efimova T, Balasubramanian S, Crish JF, Bone F, Dashti S. p38 Mitogen-activated protein kinase on the body surface: function for p38 delta. J Invest Dermatol 2003;120:823–8.
[26] Deacon K, Mistry P, Chernoff J, Blank JL, Patel R. p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. Mol Biol Cell 2003;14:2071–87.
[27] Efimova T, Broome AM, Eckert RL. A regulating role for p38 delta MAPK in keratinocyte differentiation: evidence for p38 delta–RK1/2 complex formation. J Biol Chem 2003;278(36):34277–85.
[28] Jans R, Atanasova G, Jadot M, Poumay Y. Cholesterol depletion upregulates involucrin expression in epidermal keratinocytes through activation of p38. J Invest Dermatol 2004;123(3):564–73.
[29] Garmyn M, Mammone T, Pupe A, Gan D, Declercq L, Maes D. Human keratinocytes respond to osmotic stress by p38 map kinase regulated induction of HSP70 and HSP27. J Invest Dermatol 2001;117:1290–5.
[30] Guay J, Lambert H, Gingras-Breton G, Lavoie J, Huot J, Landry J. Regulation of actin dinamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 1997;110:357–68.
[31] Niwa M, Hotta K, Kanamori Y, Hatakeyama D, Hirade K, Katayama M, et al. Involvement of p38 mitogen-activated protein kinase in heat shock protein 27 induction in human neutrophils. Eur J Pharmacol 2003;466:245–53.
[32] Wong JW, Shi B, Farboud B, McClaren M, Shubamoto T, Cross CE, et al. Ultraviolet B-mediated phosphorylation of the heat shock protein HSP27 in human keratinocytes. J Invest Dermatol 2000;115(3):427–34.
[33] Missero C, Calautti E, Eckner R, Chin J, Li HT, Livingston DM, et al. Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. Proc Natl Acad Sci 1995;92:5451–5.
[34] Malgrange B, Knockaert M, Belachew S, Nguyen L, Moonen G, Meijer L, et al. The inhibition of cyclin-dependent kinases induces differentiation of supernumerary hair cells and Deiters' cells in the developing organ of Corti. FASEB J 2003;17(14):2136–8.
[35] Chen YN, Sharma S, Ramsey T, Jiang L, Martin MS, Baker K, et al. Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. Proc Natl Acad Sci 1999;96:4325–9.
[36] Wesierska-Gadek J, Gueorguieva M, Horky M. Dual action of cyclin-dependent kinase inhibitors: induction of cell cycle arrest and apoptosis: a comparison of the effects exerted by roscovitine and cisplatin. Pol J Pharm 2003;55:895–902.
[37] Verdaguer E, Jorda E, Canudas A, Jimenez A, Pubill D, Escubedo E, et al. Antiapoptotic effects of roscovitine in cerebellar granule cells deprived of serum and potassium: a cell cycle-related mechanism. Neurochem Int 2004;44:251–61.
[38] Zhang H, Shi X, Hampong M, Blanis L, Pelech S. Stress-induced inhibition of ERK1 and ERK2 by direct interaction with p38 MAP kinase. J Biol Chem 2001;276:6905–8.
[39] Alvi AJ, Austen B, Weston VJ, Fegan C, Maccallum D, Gianella-Borradori A, et al. A novel CDK inhibitor, CYC (*R*-roscovitine), overcomes the defect in p53-dependent apoptosis in B-CLL by down-regulation of genes involved in transcription regulation and survival. Blood 2005;105(11):4484–91.
[40] Zarubin T, Han J. Activation and signalling of the p38 MAP kinase pathway. Cell Res 2005;15(1):11–8.
[41] Ozaki I, Tani E, Ikemoto H, Kitagawa H, Fujikawa H. Activation of stress-activated protein kinase/c-Jun NH_2-terminal kinase and p38 kinase in calphostin C-induced apoptosis requires caspase-3-like proteases but is dispensable for cell death. J Biol Chem 1999;274 (9):5310–7.
[42] Lee SR, Lo EH. Interactions between p38 mitogen-activated protein kinase and caspase-3 in cerebral endothelial cell death after hypoxia–reoxygenation. Stroke 2003;34(11):2704–9.