In vitro activity of cyclin-dependent kinase inhibitor CYC202 (Seliciclib, R-roscovitine) in mantle cell lymphomas†

K. Lacrima1, A. Valentini1, C. Lambertini1, M. Taborelli1, A. Rinaldi1, E. Zucca2, C. Catapano1, F. Cavalli1,2, A. Gianella-Borradori3, D. E. MacCallum3 & F. Bertoni1,4*

1Experimental Oncology and2Lymphoma Unit, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland; 3Cyclacel Ltd, Dundee, Scotland, UK; 4Experimental Haematology, St Bartholomew's Hospital and The London Hospital, London, UK

Received 29 November 2004; revised 8 February 2005; accepted 8 February 2005

Background: Mantle cell lymphoma (MCL) has the worst prognosis of all B-cell lymphomas and has poor response to conventional therapy. It is characterized by the presence of a chromosomal translocation t(11;14) (q13;q32) which results in deregulated cyclin D1 expression. Since defects in cell cycle regulation and apoptosis are primary events in MCL, small-molecule inhibitors of cdks–cyclins may play an important role in the therapy of this disorder. CYC202 (Seliciclib, R-roscovitine; Cyclacel Ltd, Dundee, UK) is a purine analogue and a selective inhibitor of the cdk2–cyclin E as well as cdk7–cyclin H and cdk9–cyclin T.

Materials and methods: The activity of CYC202 was tested in four human MCL cell lines: REC, Granta-519, JeKo-1 and NCEB-1. The effect of CYC202 on the cell cycle and on apoptosis-, cell-cycle- and transcription-regulation-related proteins was assessed.

Results: The IC50 was 25 μM for REC, Granta-519 and JeKo-1 cells and 50 μM for NCEB-1 cells. CYC202 caused an accumulation of cells in the G2–M phase of the cell cycle and apoptosis. CYC202 caused down-regulation of cyclin D1 and Mcl-1 protein levels, possibly because of the inhibition of transcription elongation.

Conclusions: Our data suggest that CYC202 is an active agent in MCL. The concomitant decrease of the phosphorylated and total forms of RNA polymerase II suggests that this could be the main mechanism mediating the biological effects of CYC202 in MCL cells. The drug might represent a new therapeutic agent in this lymphoma subtype.

Key words: apoptosis, cdk inhibitor, cyclin D1, lymphoma

Introduction

Mantle cell lymphoma (MCL) accounts for approximately 8% of all non-Hodgkin’s lymphomas [1]. Despite being previously considered a low-grade indolent lymphoma, it appears to have the worst characteristics of both low- and high-grade lymphomas, i.e. incurability and rapid growth [2]. The median time to progression and survival are the shortest among all lymphoma subtypes [1]. The initiating events of MCL are thought to be caused by the t(11;14) (q13;q32) chromosomal translocation that places the cyclin D1 gene under the regulation of the immunoglobulin heavy chain (IgH) gene promoter. Additional molecular abnormalities mainly involving genes that regulate the cell cycle, such as loss of p27 protein expression, are also common. Abnormalities regulating apoptosis pathways, have also been identified, including overexpression of Bcl-2 [3]. Currently, there is no convincing evidence that any conventional chemotherapy regimen is curative [4]. Thus there is a need for new therapeutic modalities that would selectively target those pathways that are deregulated [5]. The karyotypic abnormality and the gene expression profile characteristic of MCL and involving mainly proliferation and cell-cycle-related genes make this disease an attractive candidate for therapeutic agents targeted to the cell cycle and apoptosis.

CYC202 (Seliciclib, R-roscovitine) is a purine analogue that competes with ATP for its binding site on cdks. CYC202 is selective towards cdk2–cyclin E, cdk7–cyclin H and cdk9–cyclin T1, followed by cdk2–cyclin A and cdk1–Cyclin B [6–8]. CYC202 has cytotoxic activity against a range of human cancer cell lines, as well as in tumour xenograft

*Correspondence to: Dr F. Bertoni, Experimental Oncology, Oncology Institute of Southern Switzerland, via Vincenzo Vela 6, 6500 Bellinzona, Switzerland. Tel: +41 (0)91 8200 367; Fax: +41 (0)91 8200 397; E-mail: frbertoni@mac.com

†K. Lacrima and A. Valentini contributed equally to this work.

© 2005 European Society for Medical Oncology
models [7]. Phase I clinical trials with an oral capsule formulation have been completed in patients with solid tumours [9] and phase II studies are ongoing for non-small-cell lung cancer. The aim of this work is to assess antitumour activity of CYC202 in MCL cells in vitro and to characterize the mechanisms of action of CYC202 in this disease.

Materials and methods

Cell lines

Four established human MCL cell lines (NCEB-1, REC, Granta-519 and JeKo-1) were used [10–13]. NCEB-1, JeKo-1 and REC cell lines were cultured in RPMI-1640 (Gibco Invitrogen, Basel, Switzerland), while Granta-519 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco Invitrogen). All media were supplemented with fetal calf serum (10%), gentamycin (0.1%) and l-glutamine (1%). Classic and molecular cytogenetic techniques were used to confirm the presence of the rearrangement involving the cyclin D1 (11q13) and the IgH locus (14q32) in all cell lines (data not shown).

Western blotting analysis

Cells were solubilized in lysis buffer [10 mM Tris–HCl pH 7.5, 144 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium dodecyl sulphate (SDS), 0.1% aprotinin, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride] and sonicated for 10 s. The protein content in the different samples was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Lysates (30 μg) were fractionated by SDS–PAGE using 8–15% polyacrylamide gels, based upon the expected molecular weight. The resolved proteins were blotted to a nitrocellulose membrane by semi-dry电 transfer, and the membranes were blocked for 1 h in TBS buffer (20 mM Tris–HCl pH 7.6, 137 mM NaCl) containing 5% blotting-grade non-fat milk. Membranes were incubated with primary antibodies diluted in milk with 0.1% Tween 20 overnight. The following antibodies were used: anti-cyclin D1 (clone G124–326, PharMingen, San Diego, CA, USA), anti-cdk4 (clone H-22, Santa Cruz Biotechnology, CA, USA), anti-cyclin H (clone G301–1, PharMingen), anti-cdk7 (clone 17, PharMingen), anti-cyclin B1 (SC-245, Santa Cruz Biotechnology), anti-Bcl-2 (clone N-19, Santa Cruz Biotechnology) anti-bax (Cell Signaling Technology, Beverly MA, USA), anti-Mcl-1 (clone-22, PharMingen), anti-PARP (clone F21-852, PharMingen), anti-E2F1 (clone KH95, Santa Cruz), anti-RNA polymerase II (clone 8WG16, Covance Research Groups, Berkeley, CA, USA), anti-RNA polymerase II (clone KH95, Santa Cruz), anti-tubulin (Ab-1 Oncogene, Darmstadt, Germany), anti-cyclin D1 (clone G124–326, PharMingen, San Diego, CA, USA), anti-cdk4 (clone H-22, Santa Cruz Biotechnology, CA, USA), anti-cdk7 (clone 17, PharMingen), anti-cyclin H (clone G301–1, PharMingen), anti-cdk7 (clone 17, PharMingen), anti-cyclin B1 (SC-245, Santa Cruz Biotechnology), anti-Bcl-2 (clone N-19, Santa Cruz Biotechnology) anti-bax (Cell Signaling Technology, Beverly MA, USA), anti-Mcl-1 (clone-22, PharMingen), anti-PARP (clone F21-852, PharMingen), anti-E2F1 (clone KH95, Santa Cruz), anti-RNA polymerase II (clone 8WG16, Covance Research Groups, Berkeley, CA, USA), anti-RNA polymerase II (clone KH95, Santa Cruz), anti-tubulin (Ab-1 Oncogene, Darmstadt, Germany). Membranes were washed three times in TBS for 5 min each and then incubated in TBS containing the appropriate horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Life Science, Arlington Heights, IL, USA) for 1 h. The membranes were washed three times for 5 min each in TBS with 0.1% Tween 20 and then processed for enhanced chemiluminescence detection using the manufacturer’s instructions (Amersham Life Science). Equal loading of samples was confirmed by probing for α-tubulin.

Statistics

Groups of data were compared using a paired two-sample Student’s t-test.

Results

The effect of CYC202 on viability and growth

Granta-519, NCEB-1, REC and JeKo-1 cells were assayed for cell viability after treatment with increasing concentrations of CYC202.

![Figure 1. Cytotoxic effect of CYC202 on MCL cells after 72 h exposure determined by MTT assay.](https://academic.oup.com/annonc/article-abstract/16/7/1169/167099)
CYC202 using the MTT assay. The IC\textsubscript{50} dose for each cell lines was determined after incubation with the drug for 72 h. CYC202 caused a dose-dependent decrease in cell viability in the four MCL cell lines (Figure 1). An IC\textsubscript{50} of 25 \(\mu\text{M}\) was calculated for Granta-519, REC and JeKo-1, and 50 \(\mu\text{M}\) for NCEB-1. The anti-proliferative effect of CYC202 was evaluated by measuring the growth rates of MCL cells seeded at low density and treated with a drug dose corresponding to the IC\textsubscript{50} for each cell line (Figure 2). Treatment with CYC202 caused a time-dependent inhibition of cell growth in accordance with the cell viability assay.

**CYC202-induced changes in the cell cycle**

To determine the effects of CYC202 on the cell cycle profile of MCL, the four cell lines were treated with CYC202 for 24h and 48h with the corresponding IC\textsubscript{50} drug concentrations. After 24h of treatment with the IC\textsubscript{50} dose of CYC202, an accumulation of cells in the G\(_2\)-M phase was detected in Granta-519, NCEB-1 and REC-1 cells and by 48h in JeKo-1 cells compared to controls (Figure 3). After 24 and 48h of treatment with CYC202, an increase of the sub-G\(_1\) peak, indicative of apoptosis, was evident in all the cells with the exception of Granta-519.

**CYC202-induced apoptosis in MCL cells**

The cell cycle profiles showed an increase in cells with sub-G\(_1\) DNA content after CYC202 treatment, suggestive of induction of apoptosis. To address this question directly, we measured the induction of apoptosis using more sensitive techniques, the TUNEL assay and western blotting with antibodies specific for the cleaved form of PARP. Figure 4 shows the TUNEL assay results after 72h of treatment with CYC202; apoptosis was present in all the cell lines, with the highest percentage of apoptotic cells in JeKo-1 cells and the lowest in Granta-519 cells. These data were confirmed by western blotting for the detection of the cleaved form of PARP, which showed an increase of cleaved PARP at 24h and 48h in all cell lines (Figure 5). Induction of PARP cleavage was particularly prominent in JeKo-1 cells after 24h of drug treatment.

---

**Figure 2.** Growth curves of Granta-519, NCEB-1, REC and JeKo-1 MCL cells continuously exposed to the corresponding IC\textsubscript{50} dose of CYC202. Cells were seeded on day 0 and treated with the drug on day 1. Samples were analysed daily for viability and cell number using trypan blue exclusion and an automated cell counter. The differences between control and CYC202 are all statistically significant from the 72h time point (\(P<0.05\)).
Expression of cell-cycle-related proteins

Owing to the perturbation of the cell cycle induced by CYC202, we analysed by western blotting the expression of proteins involved in the progression of the cell cycle in cells exposed to IC50 doses of the drug. First, we analysed the expression of cyclin D1 that regulates cdk4 activity and controls progression through the G1 phase. Cyclin D1 is constitutively expressed in MCL and it is believed to play a continuing role in the growth of MCL cells. Cyclin D1 protein was down-regulated after treatment with CYC202, most dramatically in the NCEB-1 cells (Figure 6). No change was observed in the cdk4 level.

To investigate the observed G2–M accumulation (Figure 3) further, cells were treated with the corresponding IC50 dose of CYC202 for 48 h and cyclin B1 protein expression was analysed. Up-regulation of cyclin B1 levels was detected in Granta-519, NCEB-1 and REC compared with the untreated cells (Figure 6). No change in the expression of cyclin B1 was detected in JeKo-1 cells, consistent with the reduced G2–M accumulation detected by flow cytometry in these cells.
Expression of apoptosis-related proteins

Flow cytometry, TUNEL assay and PARP cleavage western blotting each demonstrated that CYC202 was able to induce apoptosis in all MCL cells. Thus we examined the expression level of proteins that regulate cell survival and apoptosis in cells treated with the drug. No changes were observed in the expression of the anti-apoptotic proteins Bcl-2 and XIAP (BIRC4) or the pro-apoptotic protein Bax were observed (Figure 7). The level of the anti-apoptotic protein Mcl-1 was considerably down-regulated by treatment with CYC202 in all four cell lines.

Expression of transcription regulatory proteins

To understand the mechanism of cyclin D1 and Mcl-1 down-regulation we looked at the effects of CYC202 on the levels of total and phosphorylated RNA polymerase II and of the transcription factor E2F-1, which is known to regulate Mcl-1 expression negatively. Both RNA polymerase II and E2F1 can be affected by cdk inhibitors. Phosphorylation of the COOH-terminal domain of RNA polymerase II is required for transcription elongation and is regulated by cdk7–cyclin H and cdk9–cyclin T. The levels of both phosphorylated RNA polymerase II and total RNA polymerase II of the protein were decreased after treatment with CYC202 in all the MCL cells (Figure 8). E2F-1 was expressed in all four cell lines (Figure 8). Its level was increased in NCEB-1. Down-regulation of E2F-1 was observed in Granta, Jeko-1 and REC.
Discussion

MCL is an incurable disorder with a median overall survival of <2 years. The main genetic event underlying MCL pathogenesis is the presence of the t(11;14) (q13;q32) chromosomal translocation which causes deregulated expression of cyclin D1.

In this study we have characterized the effects of the cdk inhibitor CYC202 in human cell lines derived from MCL patients. CY202 is a synthetic cdk inhibitor with most potent activity against the cdk2–cyclin E, cdk7–cyclin H and cdk9–cyclin T complexes and with anticancer activity demonstrated in solid and haematological tumours [6–8, 15, 16]. All the MCL cell lines treated showed sensitivity to the compound at doses that are achievable in patients [9], and it was able to induce apoptosis and caused a slight accumulation of cells in the G2–M phase of the cell cycle. We observed a reduction in Mcl-1 and cyclin D1 levels after treatment with CYC202 in all four cell lines. Mcl-1 is an anti-apoptotic protein, often over-expressed in MCL [17]. It is known to be down-regulated by cdk inhibitors, such as flavopiridol and roscovitine/CYC202 [15, 18–25]. Other anti-apoptotic molecules (Bcl-2 and XIAP) were not affected by CYC202 treatment. This corroborates the notion that apoptosis induced by some cdk inhibitors might be mediated mainly by changes in Mcl-1 levels and that this can occur despite high levels of Bcl-2 [18, 23]. However, the exact mechanism of apoptosis is not clear yet. Flavopiridol and Roscovitine induce Mcl-1 down-regulation following decreased transcription [23, 26–30]. Inhibition of cdk7 and cdk9 would ultimately induce the down-regulation of the transcription. An additional mechanism for Mcl-1 down-regulation could be mediated by up-regulation of E2F-1, which directly represses Mcl-1 expression [25, 31]. In our MCL model, only NCEB-1 showed a moderate increase of E2F-1, whilst all the cell lines had a marked reduction of both total and phosphorylated forms of RNA polymerase II levels. E2F-1 might contribute to the apoptotic effect of the drug but, since MCL cells have constitutively high levels of E2F-1 [32], they might be less sensitive to E2F-1-mediated apoptosis than other cell types. The down-regulation of RNA polymerase II activity and level with consequent inhibition of transcription elongation seems to be the main mechanism for induction of apoptosis in our MCL model. Indeed, we also observed the down-regulation of cyclin D1. Both flavopiridol and CYC202 have been shown to decrease the level of cyclin D1 in other cell types [23, 26, 29, 33]. However, our data in MCL are very interesting. The cyclin D1 expression is constitutive in MCL owing to the juxtaposition of the gene to the immunoglobulin heavy-chain genes that are always transcriptionally active in B cells. The ability of CYC202 to down-regulate cyclin D1 expression in this context is very promising for

Figure 7. Changes in apoptosis regulatory proteins after CYC202 treatment. Representative western blot illustrating the expression of apoptosis-related proteins in MCL cells after 48 h exposure to CYC202 or DMSO. Tubulin was used as a control for sample loading.

Figure 8. Changes in transcription regulatory proteins after CYC202 treatment. Representative western blot illustrating the expression of RNA polymerase II and of E2F-1 in MCL cells after 24 h and 48 h exposure to CYC202 or DMSO. Tubulin was used as a control for sample loading.
the treatment of MCL patients with the drug. RNA polymerase II has recently been shown to be constitutively bound to both cyclin D1 promoter and 3' IgH regulatory regions in MCL cells [34]. The ability of CYC202 to down-regulate RNA polymerase II activity might explain its marked effect on growth and viability on MCL cells.

In conclusion, our in vitro data indicate that CYC202 is an active compound in MCL with the potential to improve the outcome of patients with this disease. A phase II study that will test the activity of CYC202 in patients with MCL is currently ongoing.

Acknowledgements

We thank our colleagues Catherine Thieblemont (France) and Eisaku Kondo (Japan) for providing two of the cell lines. This work was partially supported by Swiss Clinical Cancer Research (SAKK).

References

