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# R-roscovitine (CYC202) alleviates renal cell proliferation in nephritis without aggravating podocyte injury

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*Background.* Cyclin-dependent kinase (CDK) inhibition is a new therapeutic approach to proliferative glomerulonephritides. CDK2 is required for $G_1$/S transition and DNA synthesis and is inhibited by CYC202 (R-roscovitine). Since podocytes express CDK2 in nephritis and since loss of podocytes contributes to glomerulosclerosis, the rationale of the present study was to test whether CDK2 inhibition is safe in instances of podocyte injury.

*Methods.* Rats with passive Heymann nephritis, a model of membranous glomerulonephritis, were treated (day 3 to 30) with vehicle, low (25 mg/kg/day), or high (50 mg/kg/day) doses of CYC202.

*Results.* On day 27, blood pressure was normal in nephritic controls and was dose-dependently reduced by CYC202. Urinary albumin excretion did not differ between the groups on days 9, 16, 23, and 30. To investigate podocyte injury, we assessed the glomerular de novo expression of desmin, which was markedly up-regulated in almost all passive Heymann nephritis glomeruli but was not significantly different between the three groups. No tubulointerstitial de novo expression of desmin or alpha-smooth muscle actin (α-SMA), or tubulointerstitial monocyte/macrophage infiltration was noted in any group. Biologic activity of CYC202 was evident in the form of a dose-dependent decrease in the number of glomerular and tubulointerstitial mitotic figures as compared to vehicle alone. Glomerular immunostaining for cyclin D1, a marker for $G_0$ to $G_1$ transition, was significantly decreased in CYC202 treated groups at day 9.

*Conclusion.* Whereas inhibition of CDKs by CYC202 reduced intrarenal cell proliferation in passive Heymann nephritis it did not aggravate podocyte damage, suggesting that this novel therapeutic approach is safe in renal diseases characterized by podocyte injury.

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Glomerulonephritides and systemic immunologic diseases account for 20% to 25% of terminal renal failures in most Western countries. Many progressive immune-mediated glomerular diseases are characterized by mesangial proliferative changes and would thus potentially benefit from therapy with a cell cycle inhibitor. However, all of these glomerular diseases, as well as metabolic glomerular diseases such as diabetic nephropathy, appear to advance to glomerular scaring through secondary podocyte damage [1–3]. Other progressive glomerulonephritides do not exhibit mesangial proliferative changes but rather primary podocyte injury. A classic disease of this group is membranous glomerulonephritis, where an (auto) immune process leads to podocyte damage without significant changes inside the glomerular tuft. Primary or secondary podocyte damage determines the rate of glomerular and subsequent renal tubulointerstitial scaring [3].

The particular role of the podocyte as a final common pathway of glomerular scaring has traditionally been explained by the terminal differentiation of these cells and the assumption that a damaged podocyte can not be replaced, resulting in areas of bare glomerular basement membrane (GBM), which then fuse with the outer Bowman's capsule and thereby generate a first adhesion between glomerular tuft and capsule. Such adhesions are viewed as the starting point of segmental and ultimately global glomerulosclerosis [3, 4].

Recently, the view of the podocyte as a terminally differentiated cell has been challenged. Observations of rare, yet definitive podocyte DNA synthesis and mitoses in glomerulonephritis, and bi- (multi-) nucleation rather than increased cell numbers, suggest that injured podocytes in principle possess the ability to replicate but instead engage in a defective cell cycle with acytokinetic mitosis [5]. Normal and/or injured podocytes contain high levels of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 [6, 7], which would prevent cell cycle progression. However, they have also been demonstrated to express CDK2, a promoter of cell cycle progression,

during passive Heymann nephritis [7], a model of human membranous glomerulonephritis in rats [8]. This model is therefore of particular use to study the effects of CDK2 inhibition in podocyte disease, given the concern that CDK2 inhibition might impair the little adaptive response that podocytes can exhibit and thereby aggravate the development of glomerulosclerosis. However, CDK2 inhibition in passive Heymann nephritis might also be beneficial. Very recent data show that engagement of podocytes in the cell cycle may alter their adhesive properties in such a way that they are lost in the urine [with all the potential consequences described above (i.e., denudation of basement membrane and adhesion formation)] [9]. In this scenario, it might even be beneficial to prevent the podocyte from engaging in the cell cycle. The aim of our study was therefore to determine the effects of currently employed therapeutic doses of a CDK2 inhibitor, R-roscovitine (CYC202), in passive Heymann nephritis. CYC202, a purine analogue, inhibits the activity of CDK2 but also of other CDKs, including CDK1 (cdc2), CDK5, CDK7, and CDK9, by binding to their adenosine triphosphate (ATP) binding pocket [10]. However, the affinity of CYC202 to the different CDKs is variable with inhibition constants $(IC)_{50}$ of 0.1 μmol/L for CDK2/cyclin E, 0.7 μmol/L for CDK2/cyclin A, 0.5 μmol/L for CDK7/cyclin H, 0.84 μmol/L for CDK9/cyclin T1, and 2.7 μmol/L for CDK1/cyclin B [11].

## METHODS

### Experimental design

Male rats (Sprague-Dawley, Charles River Wiga GmbH, Sulzfeld, Germany), weighing 180 to 210 g at the start of the experiment, were used in the experiments. Rats were housed in cages under conditions of constant temperature (22°C) and humidity (50%), with a 12-hour dark/light cycle. The animals had free access to tap water and standard rat chow. Passive Heymann nephritis was induced on day 0 by intravenous injection of 0.3 mL of sheep anti-Fx1A antibody per rat, prepared as described previously [8]. All animal experiments were approved by the local review boards.

Treatment with CYC202 was started on day 3, in order to avoid interference with the induction phase of passive Heymann nephritis, and continued until day 30. Treatment consisted of once-daily oral gavage of CYC202 until day 10. CYC202 was dissolved in 30 mmol/L HCl and stirred with a magnetic stirrer until dissolution. After day 10, treatment was switched to daily intraperitoneal injections. The vehicle used here was dimethyl sulfoxide (DMSO) (ICN Biomedicals, Aurora, OH, USA). Three groups of rats were studied: group I, nephritic untreated group ($N = 10$) (animals received vehicle alone); group II, nephritic low dose treatment group ($N = 10$) (animals were treated once daily with 25 mg CYC202/kg/day); and group III, nephritic high dose treatment group ($N = 10$) (animals were treated once daily with 50 mg CYC202/kg/day).

To determine urinary albumin excretion, 24-hour urine collections were performed in metabolic cages on days 9, 16, 23, and 30 after disease induction.

Renal biopsies for histologic evaluation were obtained on day 9 by intravital biopsy and during postmortem on day 30 after disease induction. The thymidine analogue 5-bromo-2′deoxyuridine (BrdU) (100 mg/kg body weight) (Sigma Chemical Co., St. Louis, MO, USA) was injected intraperitoneally at 4 hours before sacrifice on day 30. All rats were sacrificed under isoflurane anesthesia and blood was collected by puncture of the vena cava inferior 5 hours after administering the last dose of CYC202. Kidneys were harvested and prepared for histologic examination.

### Renal morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution [12] and embedded in paraffin. Four micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin.

Using a 400-fold magnification of the PAS-stained sections, the total number of mitoses within the glomerular tuft (extrapolated to mitoses per 100 glomerular cross sections) was counted. To determine this, between 20 and 100 glomerular profiles were evaluated per specimen on day 9 and between 100 and 190 glomerular profiles were evaluated on day 30.

In addition, the number of mitoses in the tubulointerstitium was evaluated using a grid composed of 100 fields at a magnification of 400-fold, so that every field corresponded to an area of 0.0625 $mm^2$. One hundred grid fields in the renal cortical tubulointerstitium were analyzed and mean counts per kidney were obtained.

Electron microscopy was performed following standard protocols [13]. Blocks of renal tissue (1 $mm^3$) were fixed in a solution of 2% formaldehyde and 2.5% glutaraldehyde according to Karnovsky's method, with cacodylate buffer (0.2 mol/L, pH 7.4). After fixation, the samples were dehydrated and embedded in epoxy resin (glycide ether 100) (Serva, Heidelberg, Germany). Sections were cut and stained with toluidine-blue for light microscopy prescreening. Ultrathin sections were then cut at 80 to 100 nm, stained with uranyl acetate and lead citrate, and viewed and photographed in a Philips TEM 400 transmission electron microscope. For evaluation of slit numbers per length of GBM, images were obtained from random areas, enlarged 13,000-fold and the number of filtration-slits per GBM length was measured.