Dev Genes Evol
DOI 10.1007/s00427-014-0465-0

ORIGINAL ARTICLE

Inhibitors of the p53-Mdm2 interaction increase programmed cell death and produce abnormal phenotypes in the placozoon *Trichoplax adhaerens* (F.E. Schulze)

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Received: 25 October 2013 / Accepted: 17 January 2014

Abstract Recent identification of genes homologous to human p53 and Mdm2 in the basal phylum Placozoa raised the question whether the network undertakes the same functions in the most primitive metazoan organism as it does in more derived animals. Here, we describe inhibition experiments on p53/Mdm2 interaction in *Trichoplax adhaerens* by applying the inhibitors nutlin-3 and roscovitine. Both inhibitors had a strong impact on the animals' survival by significantly increasing programmed cell death (cf. apoptosis, measured via terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay). Treatment with roscovitine decreased cell proliferation (visualized by means of bromodeoxyuridine incorporation), which is likely reducible to its function as cyclin-dependent kinase inhibitor. Obvious phenotypic abnormalities have been observed during long-term application of both inhibitors, and either treatment is highly lethal in *T. adhaerens*. The findings of this study suggest a conserved role of the p53/Mdm2 network for programmed cell death since the origin of the Metazoa and advocate the deployment of Placozoa as a model for p53, apoptosis, and possibly cancer research.

Keywords Placozoa · p53 · Mdm2 · Nutlin-3 · Roscovitine

Communicated by Volker G. Hartenstein

Electronic supplementary material The online version of this article (doi:10.1007/s00427-014-0465-0) contains supplementary material, which is available to authorized users.

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Introduction

Trichoplax adhaerens, the only described species of the basal phylum Placozoa, offers peculiar opportunities to investigate complex mechanisms such as the control of programmed cell death at the base of the metazoan tree of life (cf. Schierwater et al. 2009a, b; Osigus et al. 2013). Sequencing of the *Trichoplax* genome in 2008 revealed a surprisingly high diversity of protein coding genes, which look like an apparent discrepancy to its extremely simple bauplan (Srivastava et al. 2008; de Jong et al. 2009; Eitel et al. 2013; Ringrose et al. 2013).

The tumor suppressor p53 and its ubiquitin ligase Mdm2 have lately been shown to be conserved from Placozoa to human (Lane et al. 2010). In more derived ("higher") animals, p53 has been shown to have a protective function that ensures the integrity of a cell. In case of cellular stress or DNA damage, the protein is able to induce the transcription of target genes important for repair mechanisms or to provoke entrance into the apoptotic pathway (for review cf. Levine and Oren 2009; Vousden and Prives 2009). The negative regulator of p53, Mdm2, is antagonizing p53 in case of normal conditions (Manfredi 2010). In invertebrates, protein homologues to Mdm2 have yet been identified for seven species only (Momand et al. 2011). This observation may support the hypothesis of an ancient Mdm2-independent p53 modulation (Momand et al. 2011; Muttray et al. 2010). p53 is indispensable for the maintenance of tissue fitness, which is for example mirrored by the fact that 50 % of all known human tumors are connected to a p53 deregulation (Joerger and Fersht 2007). Hence, the p53/Mdm2 interplay is an area of intense research. It is particularly noteworthy that Mdm2 is missing in well-known invertebrate model systems like *Caenorhabditis* or *Drosophila* (Jin et al. 2000; Derry et al. 2001; Lane and Verma 2012). This information pleads for a derived function of the network in these organisms, which likely evolved an

Published online: 13 February 2014

Mdm2-independent way of p53 regulation or do even possess alternative p53-independent mechanisms to control apoptosis. The lack of Mdm2 in *Caenorhabditis* and *Drosophila* makes these species ill-suited to be appropriate models for p53 research regarding the negative regulation by Mdm2 (Lane and Verma 2012; Momand et al. 2011).

Knowledge on the presence of p53 and Mdm2 in Placozoa has so far been based on sequence analyses only (Lane et al. 2010). Here, we describe the first experimental approach to unravel functions of p53/Mdm2 interaction in the primitive animal *T. adhaerens*. The application of two inhibitors, nutlin-3 and roscovitine, was used to address the question whether these proteins undertake functions in Placozoa that are homologous to the ones known in more derived animals. Nutlin-3 is a cis-imidazoline that chemically obstructs the p53/Mdm2 interface and thus their interaction (Vassilev et al. 2004). The purine roscovitine, commonly known as cyclin-dependent kinase (CDK) inhibitor, has also been shown to have an effect on Mdm2 expression on mRNA and protein level (Lu et al. 2001). Both inhibitors cause imbalances in the p53/Mdm2 network by accumulation of p53 (Brown et al. 2009). Besides observations of treated *Trichoplax* individuals via light microscopy, cell proliferation and programmed cell death during treatment were monitored by means of bromodeoxyuridine (BrdU) incorporation and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays. The results of our experiments clearly show that the p53/Mdm2 interplay has a fundamental impact on the progression of programmed cell death in the placozoon *T. adhaerens*.

Material and methods

Animal material

T. adhaerens (haplotype 1, the "Grell" strain) was cultured as previously described (Schierwater and Kuhn 1998; Schierwater 2005, and full details of culturing conditions will be reported in Schierwater et al. 2014, in prep). The animals were fed ad libitum with the cryptomonad *Pyrenomonas helgolandii* and *Chlorella salina* (chlorophyta, both strains were obtained from the SAG Göttingen, Germany). Algae were cultured in artificial seawater (ASW, 35‰ salinity, 50:50 mixture of Red Sea Salt, Red Sea Fish Pharm Ltd., Eilat Israel and Reef Crystals, Aquarium Systems, Sarrebourg, France) and were fed with Prov Medium (National Center for Marine Algae and Microbiota, East Boothbay, ME, USA) following the manufacturer's instructions. One milliliter of each steady state algae culture with maximum density was mixed with 48 ml ASW (35‰ salinity). The solution was used for culturing and for all experiments described below. Experiments were performed with randomly chosen individuals of all sizes that were preliminary checked for not showing phenotypic irregularities (as such may result, e.g., from degeneration). Animals were transferred into experimental containers by means of Pasteur pipettes.

Inhibitor treatment

The inhibitors were dissolved in dimethyl sulfoxide (DMSO, Merck Darmstadt, Germany) and were diluted in ASW up to 10 μM (nutlin-3, racemic, Calbiochem, USA) and 20 μM (roscovitine, Calbiochem, USA). Control experiments were performed with 0.1 % DMSO in ASW and ASW only. Animals were kept in one-well glass chamber slides (one-well cell culture chamber, 10 mm^2 surface area, Sarstedt, Nümbrecht, Germany; 2 ml volume) for daily counting and microscopic observations. The ASW containing the appropriate concentration of inhibitor and food solution, respectively, was changed every 48 h to avoid osmotic stress due to evaporation of water. Four individual experiments have been performed.

For subsequent BrdU or TUNEL staining as described below, the previous application of inhibitors was done in glass culture dishes (14 cm in diameter, 50 ml volume, approximately 50 individuals per dish) in the same concentrations as used before.

The BrdU assay

For the detection of cell proliferation after inhibitor treatment, animals were treated each with roscovitine (20 μM), nutlin-3 (10 μM), or DMSO (0.1 % control) for 72 h. Individuals were then incubated in ASW containing BrdU (50 ng/ml; Sigma, Steinheim, Germany) for 4 h. After fixation in Lavdowsky fixative (ethanol/Tris-buffered saline (TBS: pH 7.5, 0.3 M NaCl, 0.2 M Tris HCl)/acetic acid/formaldehyde, 11/11/1/2, all ingredients from Roth, Karlsruhe, Germany) for 1 h at room temperature, the samples were permeabilized in TBS (pH 7.5) containing 0.5 % Tween-20 (henceforth termed TBST; Roth, Karlsruhe, Germany) for 12 h at 4 °C. The tissue was further permeabilized by proteinase K digestion (4 ng/ml; Roth, Karlsruhe, Germany) in TBST for 5 min. After stopping the reaction with glycine (1 mg/ml; Roth, Karlsruhe, Germany), two more washes with TBST were performed. Animals were then rinsed in 2 N HCl (in TBST; Roth, Karlsruhe, Germany) for 30 min, and after further three washing steps in TBST, the tissue was quenched with 3 % H_2O_2 (Roth, Karlsruhe, Germany). Samples were washed afterwards with TBST two more times and then blocked in 0.1 % bovine serum albumin (BSA; Sigma, Steinheim, Germany) in TBST for 30 min. For detection of incorporated BrdU, a horseradish peroxidase labeled polyclonal sheep BrdU antibody (Abcam) was diluted 1:100 in TBST + BSA (0.1 %), and samples were incubated for 3 h at room temperature. The detection of the antibody was performed using the

Thyramide Signal Amplification Kit #23 (TSA®; Invitrogen, Eugene, OR, USA) following the manufacturer's protocol. Nuclei were subsequently counterstained with 4′,6-diamino-2-phenylindole (1× DAPI, 300 nM; Roth, Karlsruhe) in TBST for 10 min, and after two terminal washes in TBS, samples were mounted with Vectashield (Vector Labs, Burlingame, CA, USA) for microscopy.

The TUNEL assay

Animals were treated with inhibitors, fixed, and permeabilized as described above. Dying cells were then labeled using the ApopTag ® red in situ kit (Chemicon International Melbourne, Australia) following the manufacturer's instructions. Subsequently, nuclei were counterstained with 1× DAPI in TBST for 10 min as described for BrdU staining, and samples were mounted on slides with Vectashield.

Microscopy

All microscopic pictures were made with a Zeiss Axiovert 200M (Carl Zeiss, Göttingen, Germany) connected to a digital camera (Axio Cam MRn, Carl Zeiss, Göttingen, Germany). Zeiss filter sets used for fluorescence pictures were 02 (DAPI) and 25 (Alexa Fluor 546). Pictures were modified using the Adobe Photoshop Elements 8.0 program to increase contrast only. Animal sizes and amount of proliferative or respectively dying cells have been estimated utilizing the ImageJ software version 1.44. The proportions of proliferating (BrdU) and dying cells (TUNEL) have been estimated by counting all nuclear DAPI signals as well as all BrdU and TUNEL-positive cells.

Statistics

To reveal differences in animal and population sizes after inhibitor treatment, each control (ASW and 0.1 % DMSO) was compared to the experiments (nutlin-3 or roscovitine) independently by means of a *t* test analyses (two-sided conducted in Excel® Microsoft Office® 2007, cf. Online Resource Table 1b). For analyses of dying or proliferative cells, respectively, only the DMSO control was used (no ASW control was performed).

Results

Both inhibitors have a strong impact on the animals' physiology

Long-term application of the inhibitors led to severe stress for the animals, which was observed after application of nutlin-3 as well as roscovitine. The overall number of 10 μM nutlin-3-treated animals was significantly reduced after 3 days ($p<0.05$) compared to DMSO and ASW controls. The 20 μM roscovitine-treated animal population significantly decreased even after 4 days ($p<0.05$). Treatment was lethal to all individuals after 11 days maximum (cf. Fig. 1a, b). The death of treated individuals was generally preceded by a decrease in size (Figs. 1b and 2). This size decrease was immediately significant the day after the first treatment ($p<0.05$ for nutlin-3 treatment and $p<0.01$ for roscovitine application; cf. Figs. 1b and 2).

A certain proportion (up to 21.4 and 19.6 % in case of nutlin-3 for roscovitine, respectively) of individuals reproducibly showed distinct changes in body shape within the first 6 days of treatment (Fig. 3). In three out of four experiments, the expression of fringed hunches at the animals' margin happened after application of both inhibitors, respectively, and holes in the center of animals were observed in two of four experiments with roscovitine (Fig. 3a–c). Control experiments and lower concentrations of inhibitors did not result in any of such shape changes (data not shown).

Inhibitor treatment increases programmed cell death, and roscovitine has an impact on cell proliferation

Besides the abnormal phenotypic effects resulting from inhibitor treatment, outcomes of BrdU and TUNEL staining indicate that treatment with nutlin-3 and roscovitine significantly affects programed cell death (Fig. 4). After 72 h of nutlin-3 treatment, the average amount of dying cells was increased by a factor of 9.4 compared to the DMSO control. Roscovitine treatment for the same interval resulted in an increase of dying cells by a factor of 5.1 compared to the control. Both values differ significantly from the DMSO control (cf. Fig. 4a, b, $p<0.001$). Nutlin-3 treatment in contrast did not significantly affect cell proliferation. Although average cell proliferation rate in each approach was equally reduced by ~10 % after application of the particular inhibitors, only the decrease caused by roscovitine was statistically significant (cf. Fig. 4c, d, $p<0.05$).

Discussion

Nutlin-3 and roscovitine have been known to induce the accumulation of p53 in a cell, leading to p53-induced cell death (cf. Brown et al. 2009). Despite intense research efforts, many functions of the vertebrate p53/Mdm2 interaction and their targets still remain unknown. Thus, knowledge of the network in different animal phyla, especially in lower metazoans, may provide crucial insights from evolutionary perspectives. So far, known functions of p53 in invertebrates are related not only to the protection of genome integrity in early embryos as well as in germ line cells but also to the regulation of stem cell proliferation and development (Jin et al. 2000; Jessen-Eller et al. 2002; Pankow and Bamberger 2007; Pearson and Sanchez Alvarado 2010).

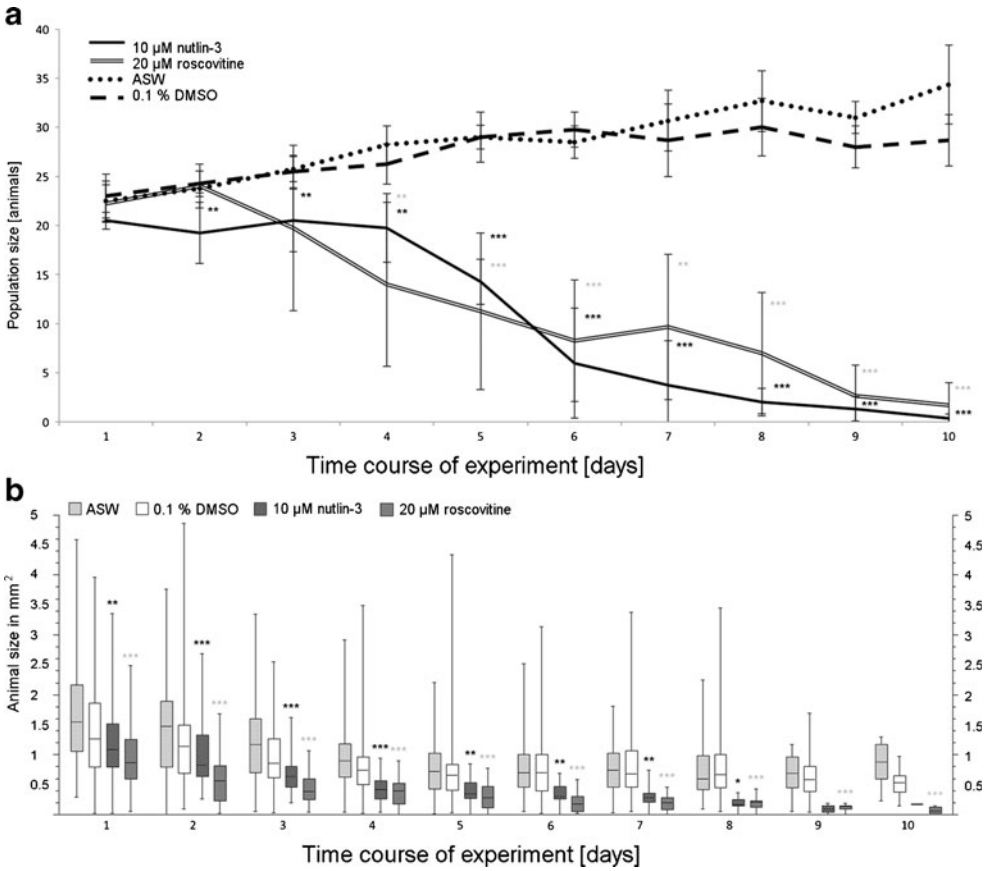

Fig. 1 Time course of population size and animal sizes after inhibitor treatment. The application of 10 µM nutlin-3 and 20 µM roscovitine was lethal within 11 days of treatment. All animals treated with the inhibitors were dead at day 11 (not indicated here). The number of animals decreases over time and significantly differs from the controls (ASW and 0.1 % DMSO) after 3 days of nutlin-3 treatment and 4 days after initial roscovitine application (**a**). An overall reduction of size was observed directly after the initial treatment for both inhibitors (**b**). *Whiskers* mark minimum and maximum body size, the *box* represents the *upper*, and the *lower quartile* and the *horizontal line* indicate the median. *Asterisks* mark significances for nutlin-3 (*black*) and roscovitine (*gray*) treatments. *$p<0.05$; **$p<0.01$; ***$p<0.001$. For raw data and statistics, see Online Resource Table 1 and 2

Inhibition of p53/Mdm2 interaction triggers programmed cell death and is lethal in Placozoa

Nutlin-3 is known to bind to the p53-binding pocket of the Mdm2 protein and thereby prevent its role as a p53 antagonist (Vassilev et al. 2004). This may also be the case in Placozoa, particularly, since the p53-binding pocket of Mdm2 is structurally conserved in the Placozoa, and the *Trichoplax* Mdm2 protein has even been shown to be able to bind to human p53 in vitro (cf. Lane et al. 2010; Lane and Verma 2012). The

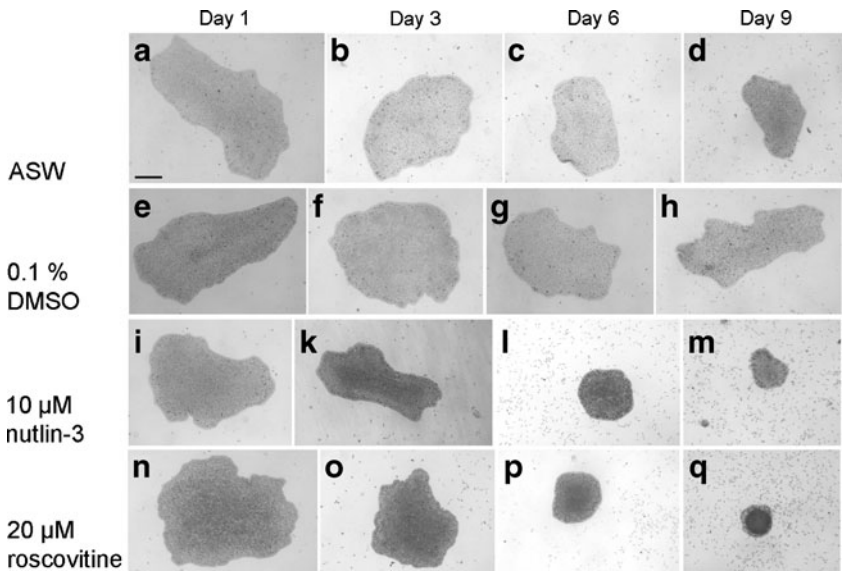

Fig. 2 Phenotypic changes after inhibitor treatment. Light microscopy of animals treated with 10 µM nutlin-3 (**i**–**m**) and 20 µM roscovitine (**n**–**q**) as well as ASW only (**a**–**d**) and 0.1 % DMSO (**e**–**h**). Day 1 (**a**, **e**, **i**, **n**), day 3 (**b**, **f**, **k**, **o**), day 6 (**c**, **g**, **l**, **p**), and day 9 (**d**, **h**, **m**, **q**) after initial treatment. ASW and DMSO control animals do not show phenotypic conspicuities, whereas nutlin-3 and roscovitine treatments were accompanied by a reduction of size. *Size bar* in **a** marks 100 µm for all pictures

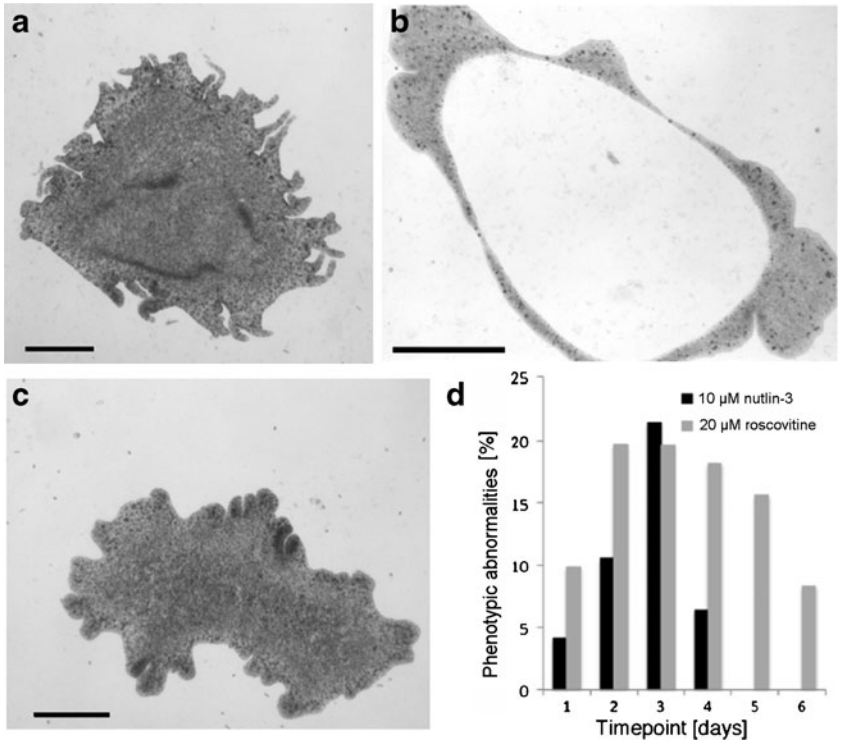

Fig. 3 Phenotypic changes after inhibitor treatment. Abnormalities were observed within the first 6 days of treatment with both inhibitors: fringed hunches and central holes after roscovitine treatment (**a** day 2 and **b** day 3) and fringes only after nutlin-3 application (**c** day 2). The overall percentages of animals with phenotypic changes (**d**) affirm an impact of inhibitor treatment on the animals' physical appearance. However, the observed effects turned out to be not significant ($p>0.05$ for all experiments). The *size bar* marks 100 µm (**a**, **c**) and 200 µm (**b**). For raw data and statistics on phenotypes, see Online Resource Table 3

observed increase of programmed cell death in nutlin-3-treated animals could be caused by p53 accumulation in line with p53-induced programmed cell death.

The usage of roscovitine in the laboratory mainly addresses questions concerning its function as a cyclin-dependent kinase (CDK) inhibitor but its role in downregulation of Mdm2 has

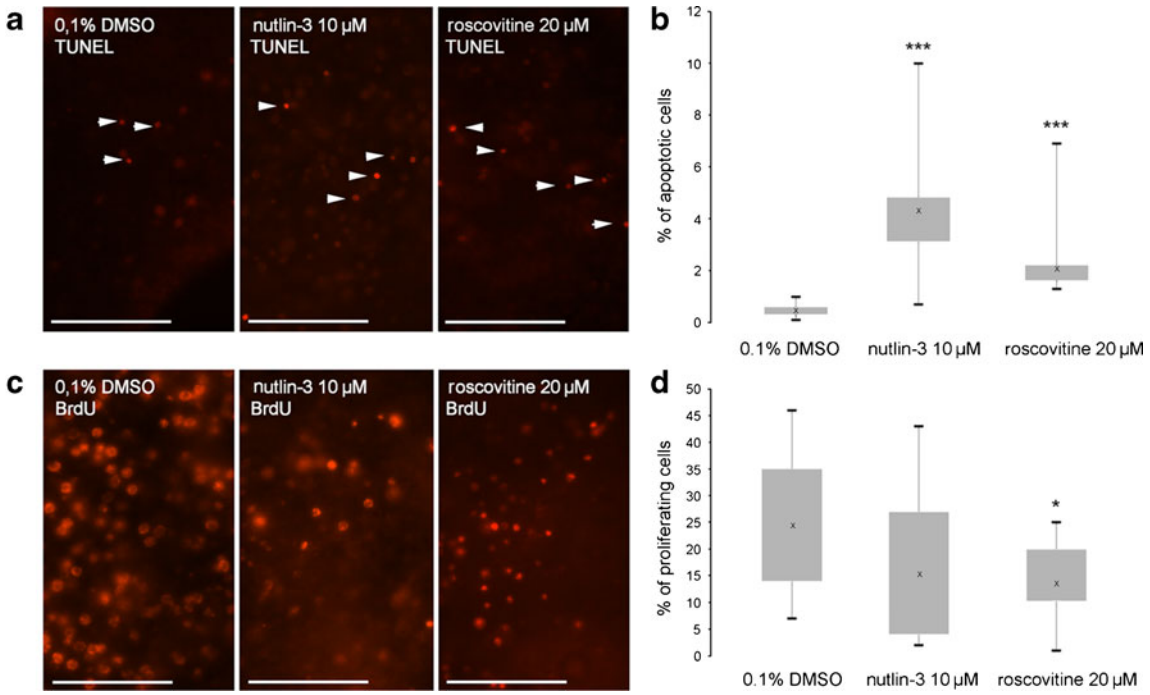

Fig. 4 TUNEL and BrdU staining 72 h after inhibitor application. Dying (TUNEL, *upper panel*) and proliferating cells (BrdU, *lower panel*). Programmed cell death was highly significantly increased after treatment with either 10 µM nutlin-3 and 20 µM roscovitine ($p<0.001$), whereas proliferation was significantly decreased ($p<0.05$) after treatment with 20 µM roscovitine only. *Boxplot* shows maximum and minimum (*whiskers*), *upper* and *lower quartile* (*box*) and the average (*cross*). *Asterisks* mark significant deviations from the DMSO control. The *size bars* marks 20 µm. For raw data and statistics, see Online Resource Table 4

previously been demonstrated (Meijer et al. 1997; Lu et al. 2001). Augmentation of the programmed cell death due to roscovitine treatment of *Trichoplax* individuals can be a result of both CDK as well as Mdm2 inhibition.

Roscovitine treatment affects cell proliferation, and both inhibitors produce abnormal phenotypes

Nutlin-3 treatment of *Trichoplax* individuals did not affect cell cycle progression as monitored by the BrdU assay. This stands in contrast to the effect triggered by roscovitine application: treatment with roscovitine caused a significant decrease in cell proliferation. This effect could be related to its function as CDK inhibitor since roscovitine treatment is known to cause cell cycle arrest (cf. Meijer et al. 1997). The amount of cell proliferation events differs significantly between *Trichoplax* individuals, depending on the animals' developmental stage (e.g., swarmer, before/after fission) and size (unpublished data). This explains the high variance of BrdU signal (Fig. 4c, d) and leads us to the speculation that an average reduction of signal (~10 %) may point to an effect on cell cycle progression induced by both inhibitor treatments. The reduction of body size during long-term inhibitor treatment further supports the assumption that cell proliferation is affected by either inhibitor treatments. ASW and DMSO controls also showed a decrease in size which, however, likely is a consequence of suboptimal culturing conditions during the experiment and differs significantly from the treatments.

Abnormal phenotypes have been observed after treatment with both inhibitors, whereas the effect caused by roscovitine was stronger. The described phenotypes provide strong evidence for a disturbance of the animals' development: the enlarged margin, respectively, the hole in the body center, suggests an imbalance in the control of central to marginal tissue growth ratio which is usually tightly regulated in placozoans (Schwartz 1984). It may be the case that the inhibitor treatment also has an influence on stem cell proliferation that was not detectable in our experiments, as proliferation of epithelial cells is frequent and possibly masks signal from cells with a lower proliferation rate. Altered stem cell proliferation as a consequence of inhibitor treatment could explain the enlargement of the animals' margin since placozoan stem cells are known to be located in the area close to the margin and control the animals' growth and division rate (Jakob et al. 2004).

The overall results of this study provide evidence for a p53/Mdm2 network in Placozoa that has similar functions to the ones found in more derived animals, particularly vertebrates, which are also involved in developmental processes. Future studies on p53 and Mdm2 in Placozoa will help to understand this network from an evolutionary and possibly ancestral point of view that might nicely complement research on higher organisms, including investigations that could help encourage research on human malignancies.

Acknowledgments K. vdC thanks the Evangelische Studienwerk Villigst e.V. for a PhD fellowship and the Boehringer Ingelheim Fonds for a travel grant. We are grateful to Dr. Ismail M. Hanif for providing the inhibitors and for coming up with the initial idea for the experiments. We thank two anonymous reviewers for their valuable comments that improved the manuscript.

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