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Novel indolylmaleimide acts as GSK-3β inhibitor in human neural progenitor cells

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ABSTRACT

The Wnt pathway is involved in cellular processes linked to either proliferation or differentiation. Therefore small molecules offer an attractive opportunity to modulate this pathway, whereas the key enzyme GSK-3β is of special interest. In this study, non-symmetrically substituted indolylmaleimides have been synthesized and their ability to function as GSK-3β inhibitors has been investigated in a human neural progenitor cell line. Among the newly synthesized compounds, the substance IM-12 showed a significant activity in several biological tests which was comparable or even outplayed the effects of the known GSK-3β inhibitor SB-216763. Furthermore the treatment of human neural progenitor cells with IM-12 resulted in an increase of neuronal cells. Therefore we conclude that indolylmaleimides act via the canonical Wnt signalling pathway by inhibition of the key enzyme GSK-3β.

1. Introduction

Wnt signalling is connected to important cellular processes such as cell polarity, cell death, proliferation, self-renewal and morphogenic movements.[1] The involvement of Wnt signalling in neural stem cell differentiation includes numerous aspects such as migration,[2] synaptogenesis,[3] axon guidance[4] and neural induction.[5]

Wnts constitute a family of 19 secreted glycoproteins which are activators of at least three pathways: one canonical (β-catenin-dependent) and two non-canonical pathways (Wnt-planar (PCP) and Wnt/Ca^{2+}-pathway).[6] The canonical pathway is mainly characterized by the stabilization of β-catenin in the cytosol. In an inactive state, β-catenin is degraded by a complex formed of Adenomatous Polyposis Coli protein (APC), Axin and Glycogen synthase kinase-3β (GSK-3β).[7] Activation of the pathway induces the decay of the degradation complex, stabilizing β-catenin which then translocates into the nucleus where it binds to the T-cell factor (TCF)/lymphoid enhancer factor (LEF)-complex and regulates the transcription of Wnt-specific target genes.[8,9] The inhibition of the β-catenin degradation complex can be achieved in two ways: either by the binding of a Wnt protein to a complex of frizzled receptor and low-density lipoprotein receptor related protein (LRP) or by the direct inhibition of GSK-3β. So far, several pharmacological GSK-3 inhibitors have been described in the literature. The mechanism of inhibition varies from ATP-competition, as in the case with paullones, arylindolylmaleimides or indirubins, to Mg^{2+}-competition with lithium or beryllium ions.[10,11] Notably, GSK-3 plays a role in several diseases, such as diabetes,[12] Alzheimer's disease,[13] or bipolar disorders,[14] which makes it an attractive pharmacological target.

Another interesting aspect is the influence of canonical Wnt signalling on several processes linked to proliferation and differentiation of neural precursor cells. The absence of basic fibroblast growth factors (bFGF) enhances neuronal differentiation of neural precursor cells by canonical Wnt signalling.[15] Wnt 1, Wnt 3a and Wnt 5a (non-canonical) regulate proliferation and differentiation of neural precursor cells during dopaminergic neuronal development in the fetal ventral midbrain.[16] GSK-3 deletion strongly inhibits neurogenesis.[17] The influence of both, canonical and non-canonical Wnt signalling is stage and cellular-context dependent. Because of the manifold applications for new GSK-3β inhibitors, we decided to synthesize new small molecules, focusing in particular on their application in neurodegenerative diseases. Several drugs have been extensively characterized in this regard. A key

Abbreviations: bFGF, basic fibroblast growth factor; DAPI, 4′,6-diamidin-2′-phenylindolyldihydrochloride; DMEM, dulbecco's modified eagle medium; EGF, epidermal growth factor; ELISA, enzyme linked immunoabsorbent assay; KP, Kenpaullone; mAb, monoclonal antibody; NPC, neural progenitor cell; RIPA, radio immunoprecipitation assay; SB21, SB-216763; SV40, simian virus 40.

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substance is the GSK-3β inhibitor SB-216763 (SB-21, Fig. 1) which is an indolylmaleimide derivative that acts competitively with ATP and is generally specific to GSK-3β.[18] These attributes make SB-216763 an interesting lead structure for new active compounds which may inhibit GSK-3β as well.

The synthesized derivates are characterized with regards to their inhibitory potential on GSK-3β and the evolving effect on Wnt signalling in human neural progenitor cells (hNPCs). In this study, we used the human NPC line ReNcell VM (Millipore, USA) to investigate the biological function of the newly synthesized substances. Notably, this cell line can differentiate into neurons, astrocytes, and oligodendrocytes within a few days.[19,20] Beside this, the cell line shows a fast proliferation and can be cultured easily which makes it an appropriate model system to test the influence of GSK-3 inhibitors on neuronal differentiation. Furthermore only few studies deal with the differentiation of human neuronal progenitor cells. Following from a previous communication on selected catalytic and stoichiometric synthesis of non-symmetrically substituted 4-indolylmaleimides,[21] we here describe in detail chemical and biological data showing the effect on Wnt signalling on human NPCs. As a major result, one of the new substances showed significant biological effects on Wnt signalling in the same range as the known GSK-3β inhibitor SB-216763.

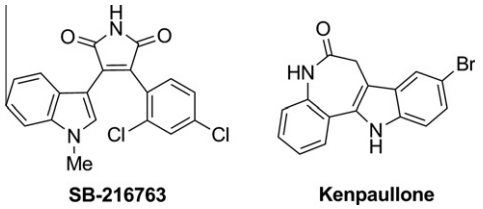

Figure 1. Chemical structures of the known GSK-3β inhibitors SB-216763 and Kenpaullone.

2. Results

2.1. Synthesis of substituted 4-indolylmaleimides

Indolylmaleimides (IM) 1–7 (Fig. 2) have been prepared by $Pd(OAc)_2$/cataCXium®A-catalyzed carbonylation of 3-bromo-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-maleimide with carbon monoxide in the presence of alcohols or amines at 90–115 °C.[21] Thus, novel 3-alkoxycarbonyl- and 3-aminocarbonyl-4-indolylmaleimides were obtained in 25–70% yield. Alternatively, new 4-amino-3-indolylmaleimides 8–15 (Fig. 2) have been synthesized in good yields (69–91%) via stoichiometric amination of the same 3-bromo-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-maleimides with corresponding amines.[21]

2.2. Treatment of ReNcell VM with SB-216763, Kenpaullone and indolylmaleimides increases the amount of total β-catenin

Initially, we investigated whether or not the application of SB-216763 or Kenpaullone to hNPCs could augment the level of total β-catenin. Thus, cells were cultivated under proliferation conditions until 70% confluence before differentiation was induced. The drugs were diluted in differentiation medium at appropriate concentrations. To determine the adequate time point for further studies, total cell extracts were harvested over 48 h and the amount of total β-catenin was measured using an ELISA specific for human total β-catenin. As expected, the change to differentiation condition resulted in an increase of β-catenin. This effect was potentiated by the addition of Kenpaullone or SB-216763 to the medium. Since the maximum of β-catenin accumulation is

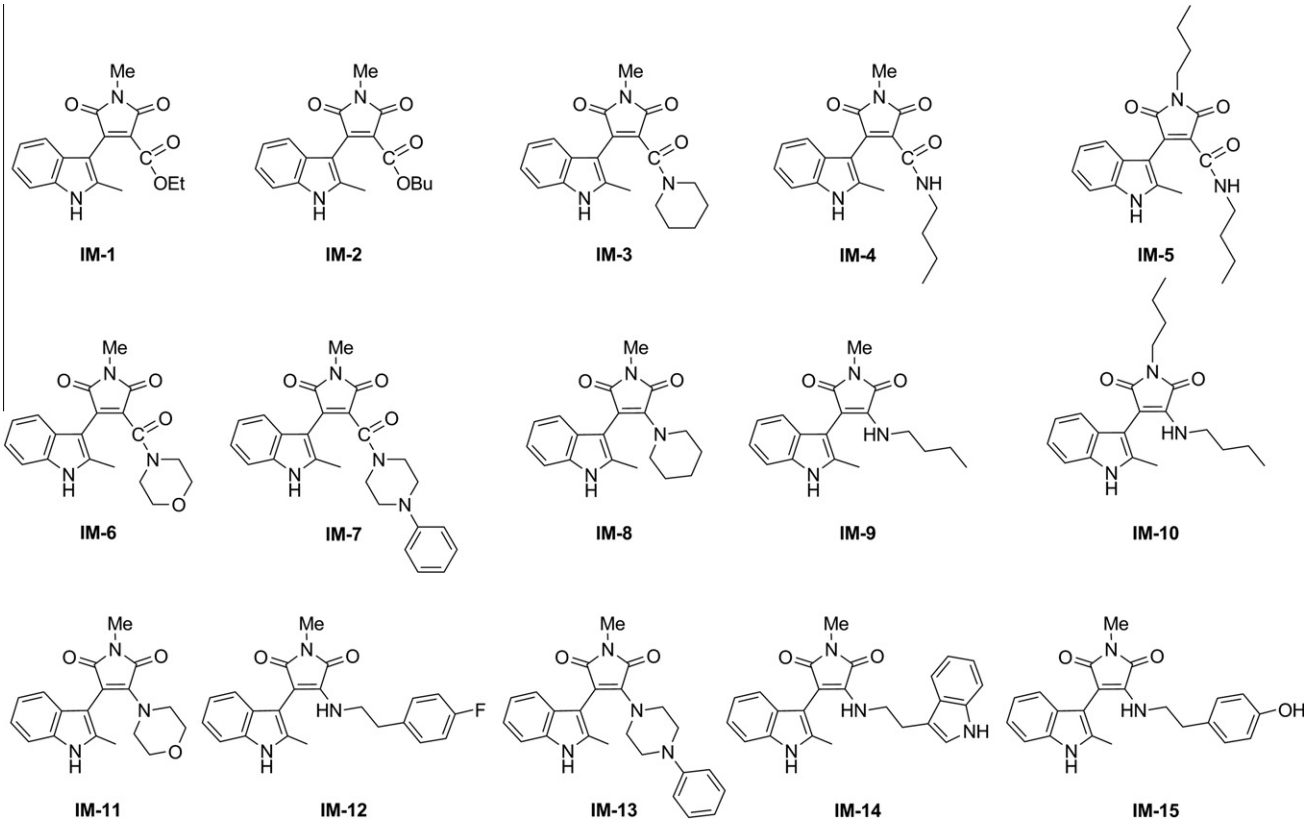

Figure 2. Chemical structures of various non-symmetrically substituted indolylmaleimides tested for biological activity.

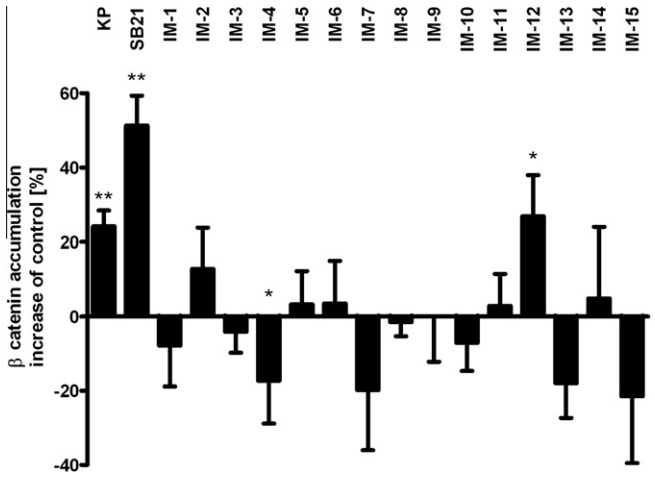

Figure 3. Total β-catenin (β-catenin specific ELISA-detection) in hNPCs after treatment with indolylmaleimides 1–15. Cells were cultured for 2 h under differentiation conditions in the presence of known GSK-3β inhibitors and derivatives based on SB-216763. Concentrations were 1 μM for Kenpaullone (KP) and 3 μM for SB-216763 and the derivatives. IM-12 increased β-catenin levels by 27% ± 11%, whereas other compounds were not able to significantly enhance β-catenin accumulation. Data were normalized to DMSO control and represent mean ± SEM (N = 4–8, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at $^{*}p$ <0.05 or $^{**}p$ <0.01.

observed after 2 h further experiments were performed within this time frame (data not shown).

A panel of novel substances was tested with regard to their ability as activators of canonical Wnt signalling using ELISA test assay. As shown in Figure 3, the established GSK-3β inhibitors Kenpaullone and SB-216763 significantly increased the β-catenin level by about 30 ± 7% and 50 ± 8%, respectively. Among the novel indolylmaleimides only IM-12 increased β-catenin significantly in the same range like the control compounds (27 ± 11%) with no significant difference to SB-216763. Therefore, IM-12 was selected as a lead structure for the synthesis of a small chemical library (Fig. 4). Indolylmaleimides 16–19 were prepared (79–97% yield) to investigate the effect of substituents on the phenyl ring. No augmentation of the β-catenin accumulation compared to IM-12 was observed (Fig. 5). Next, indolylmaleimides 20–22 with a different substitution pattern on the indol ring were synthesized (68–97% yield) and tested as well. Again, these compounds did not show the same impact as the lead compound in this series (Fig. 5).

2.3. Characterization of IM-12

As our experiments revealed only IM-12 as a hit, we further characterized this compound in different biological assays. The effect of IM-12 concentration on β-catenin accumulation was examined. As shown in Figure 6A, IM-12 enhances the β-catenin amount most at a concentration of 3 μM (Fig. 6A), whereas higher concentrations displayed no further effect compared to control cells. Furthermore, we analyzed the combination of IM-12 with SB-216763 to test for any additive effects: SB-216763 (3 μM) was tested with different concentrations of IM-12 (0.1–10 μM). As shown in Figure 6B no additive effect to SB-216763 was not observed. Interestingly, the combination of 3 μM SB-216763 and 10 μM of IM-12 depleted the β-catenin level in a significant way, whereas 3 μM SB-216763 together with lower concentrations of indolylmaleimide **12** (0.1–5 μM) showed no effect (Fig. 6B).

2.4. Inhibition of GSK-3β by IM-12

To prove that the IM-12-driven β-catenin accumulation is caused by GSK-3β inhibition, a GSK-3β activity assay as well as

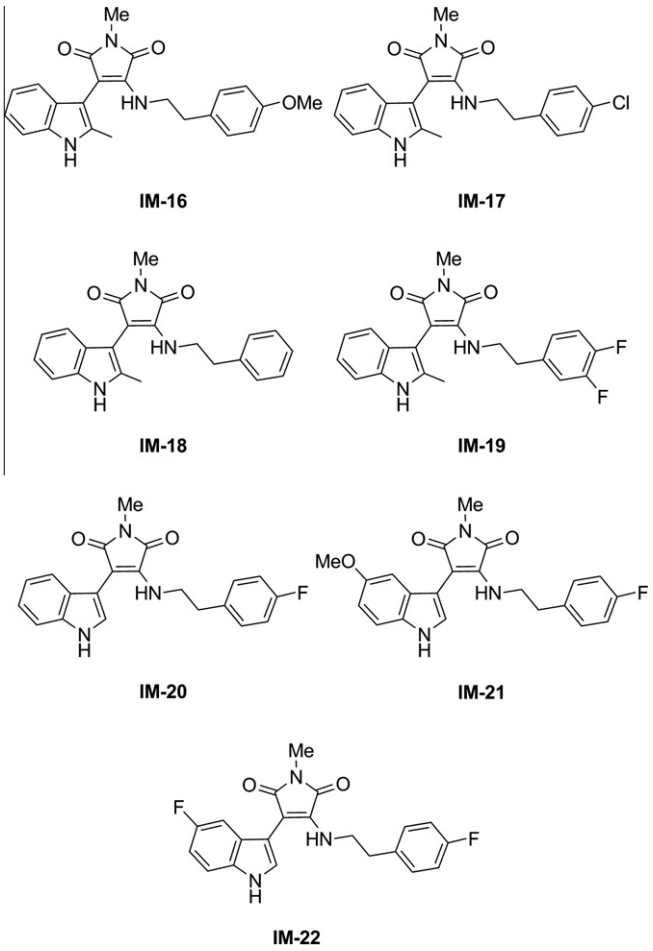

Figure 4. Small chemical library based on IM-12.

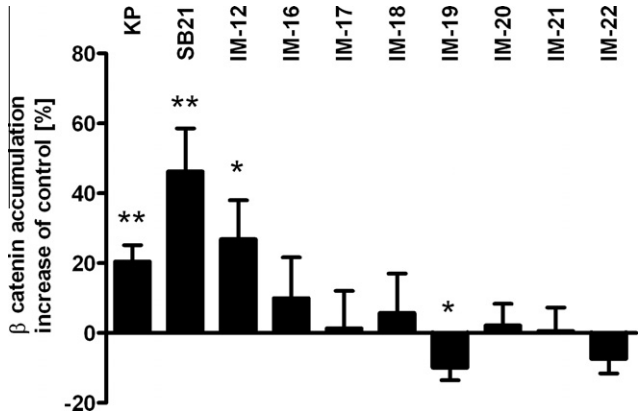

Figure 5. Total β-catenin in ReNcell VM cells after treatment with derivatives of IM-12. Cells were cultured for 2 h under differentiation conditions in the presence of known GSK-3β inhibitors and derivatives based on IM-12. Concentrations were 1 μM for Kenpaullone (KP) and 3 μM for SB-216763 and derivatives. Derivatives of IM-12 were unable to induce the same enhancing effect on β-catenin accumulation as IM-12. Data were normalized to DMSO control and represent mean ± SEM (N = 3–4, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at $^{*}p$ <0.05 or $^{**}p$ <0.01.

an in vitro-binding assay was performed. An IC_{50} was determined in a luminometric system and revealed in IC_{50} of 92 nM for SB-216763, which is slightly higher to the given literature value of 34 nM.[18] Interestingly, IM-12 showed a bell-shaped dose–response relationship, whereas the calculated IC_{50} was 53 nM (Fig. 7A and

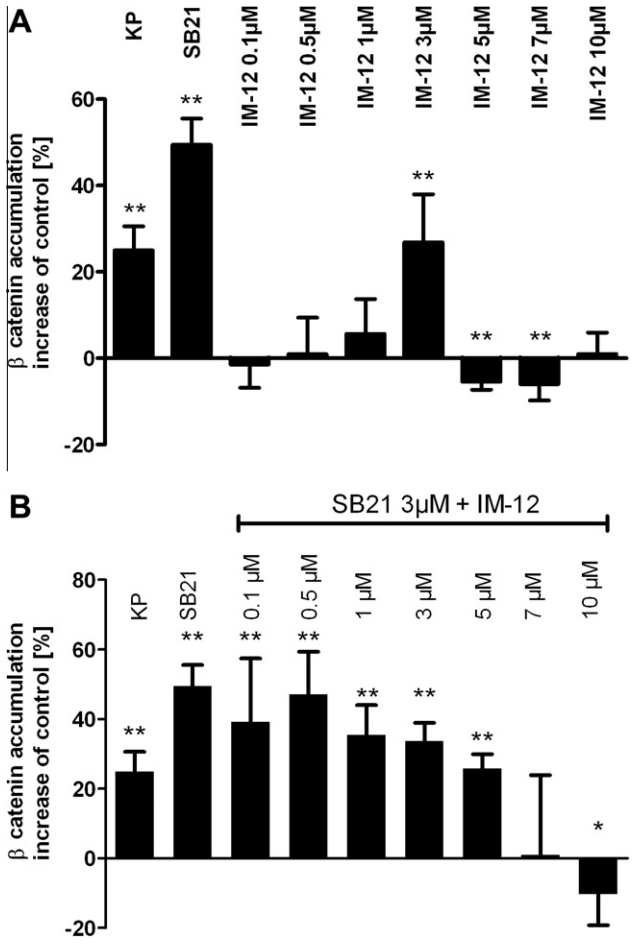

Figure 6. (A) Total β-catenin in ReNcell VM after treatment with different concentrations of IM-12. IM-12 augmented β-catenin accumulation significantly at a concentration of 3 µM. Lower or higher concentration had no further effect. (B) Total β-catenin in ReNcell VM after treatment with combinations of SB-216763 (3 µM) and IM-12 (0.1–10 µM). The combination of SB-216763 and IM-12 resulted in no additive effect to SB-216763. Cells were cultured for 2 h under differentiation conditions in the presence of IM-12. Concentrations were 1 µM for Kenpaullone (KP), 3 µM for SB-216763 (SB-216763) and variable for IM-12. Data were normalized to DMSO control and represent mean ± SEM ($N = 3–10$, each done in triplicates). Values were significantly different between drug treated and DMSO treated control cells at $^{*}p < 0.05$ or $^{**}p < 0.01$.

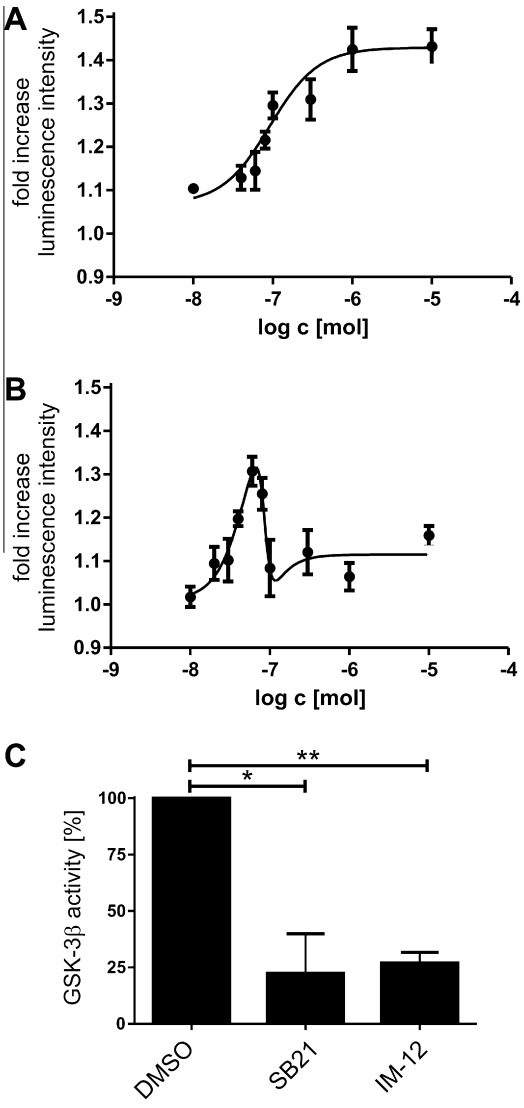

Figure 7. Inhibition of GSK-3β by SB-216763 and IM-12. IC_{50}-value for GSK-3β inhibition by SB-216763 (A) and IM-12 (B) were determined in a kinase activity assay. Luminescence signal is inversely correlated with kinase activity. Data were normalized to control and represent mean ± SEM ($n = 3–8$) (C) For in vitro-binding assay, ReNcell VM were differentiated in the presence of compounds or DMSO for 2 h. GSK-3β was extracted by immunoprecipitation and its activity was measured by radioactive phosphorylation of a specific substrate. The known GSK-3β inhibitor downregulated GSK-3β activity to 22 ± 18%. IM-12 acted in the same range and inhibited GSK-3β to a remaining activity of 27 ± 5%. Data represent mean ± SEM ($N = 3$). Values were significantly different between drug treated cells and DMSO treated control cells at $^{*}p < 0.05$ and $^{**}p < 0.01$.

B). As data of enzymatic inhibitory studies are not comparable to the situation in cellular models, the inhibition of GSK-3β by IM-12 in ReNcell VM cells was investigated. Cells were treated for 2 h with SB-216763 or IM-12. GSK-3β was extracted with immunoprecipitation and activity was measured by radioactive phosphorylation of a specific GSK-3 substrate. SB-216763 could inhibit GSK-3β to a remaining activity of 22 ± 18%, IM-12 acted in the same range and inhibited GSK-3β to 27 ± 5% (Fig. 7C) at a concentration of 3 µM.

2.5. IM-12 attenuates cell proliferation

As Wnt signalling is also involved in cell proliferation,[22,23] we analyzed whether IM-12 and SB-216763 have an influence on the proliferation of human NPCs. ReNcell VM cells were seeded in a defined number and were cultivated for 24 h under proliferation conditions. SB-216763 and IM-12 were diluted in proliferation medium to a final concentration of 3 µM and were applied to the cells, which were exposed to the drug-supplemented media during the whole experiment. The cell number was measured after every 24 h.

The cells showed an exponential growth curve (Fig. 8A) with a doubling time of 19.8 ± 0.6 h (Fig. 8B). In contrast, treatment of the cells with SB-216763 and IM-12 increased the cell doubling time significantly to 24.4 ± 0.7 h and 26.5 ± 0.9 h, respectively. The numbers of SB-216763 and IM-12 treated cells were significantly reduced after 72 h compared to the number of DMSO treated control cells (Fig. 8A). This effect seemed not be cytotoxic as the cell viability was not influenced (data not shown).

2.6. Nuclear accumulation of β-catenin

The stabilization and translocation of β-catenin to the nucleus after inhibiting GSK-3β offer an additional opportunity to test the potency of potential GSK-3β inhibitors as Wnt-modulating substances. First, we treated ReNcell VM cells with SB-216763 and

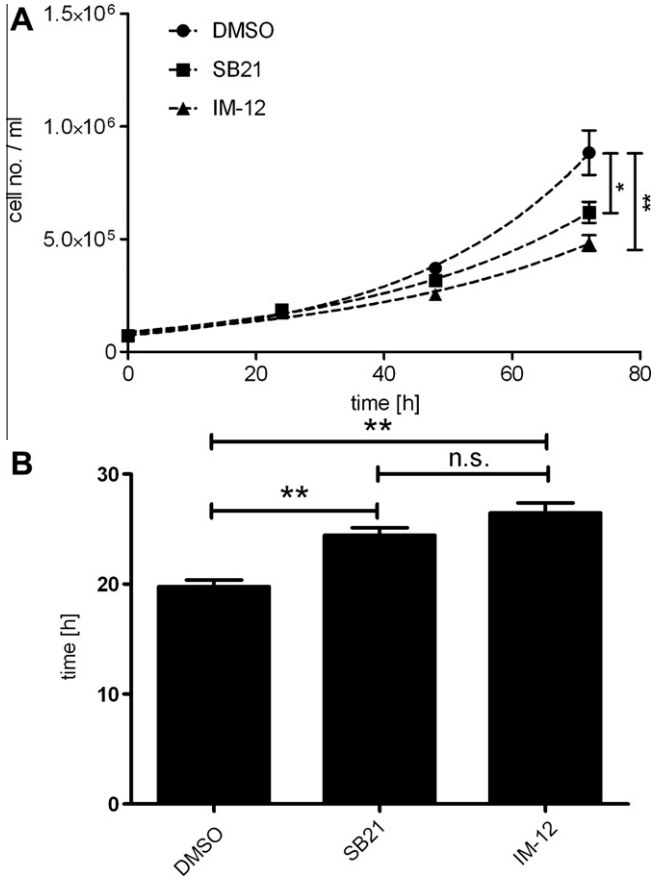

Figure 8. (A) Growth curve of treated ReNCell VM cells. Cells were seeded at a defined cell density and were cultivated in the presence of DMSO, SB-216763 or IM-12. The cell number was determined every 24 h. (B) Doubling time of ReNcell VM. Doubling times were calculated with the data from growth curves in Figure 7A. The conditioning of proliferating cells with SB-216763 or IM-12 resulted in a significant increase of the cell doubling time. Concentrations were 3 μM for SB-216763 and IM-12. After 72 h of treatment, SB21 and IM-12 significantly reduced the cell proliferation. Data represent mean ± SEM ($N = 4$, each done in triplicates). Values were significantly different between drug treated cells and DMSO treated control cells at $^{*}p < 0.05$ and $^{**}p < 0.01$.

IM-12 to prove the ability of the substances to induce accumulation of β-catenin. As in our hands, the cells did not show a clear accumulation of β-catenin which was most likely due to the growth pattern of the cells (data not shown), we used ST14A cells in a second approach. ST14A cells have been described previously as a model for visualizing nuclear accumulation of β-catenin.[24] ST14A cells were treated with SB-216763 and IM-12 to investigate whether GSK-3 inhibition results in a nuclear β-catenin translocation. Prior experiments revealed that the β-catenin accumulation is observed best after 6 h of differentiation (data not shown). At the start of differentiation, SB-216763 and IM-12 were added to the media. The treatment with SB-216763 was followed by an accumulation of β-catenin around the nucleus, (Fig. 9B, left panel) which was not observed in DMSO treated control cells (Fig. 9A, left panel). Cells which were treated with IM-12 showed an enrichment of β-catenin around the nucleus in the same extent as SB-216763 (Fig. 9C, left panel). The accumulation of β-catenin was confirmed by a line-scan of the fluorescence intensity (F 568) of the β-catenin staining. An example is shown in Figure A-C (right panel). The white lines indicate the position of the line-scans, whereas the arrows indicate the border of the cytosol (C) and the nucleus (N). One can observe an increase of the fluorescence intensity in the peri-nuclear region of cells treated with SB-216763 or IM-12 but not in DMSO treated cells.

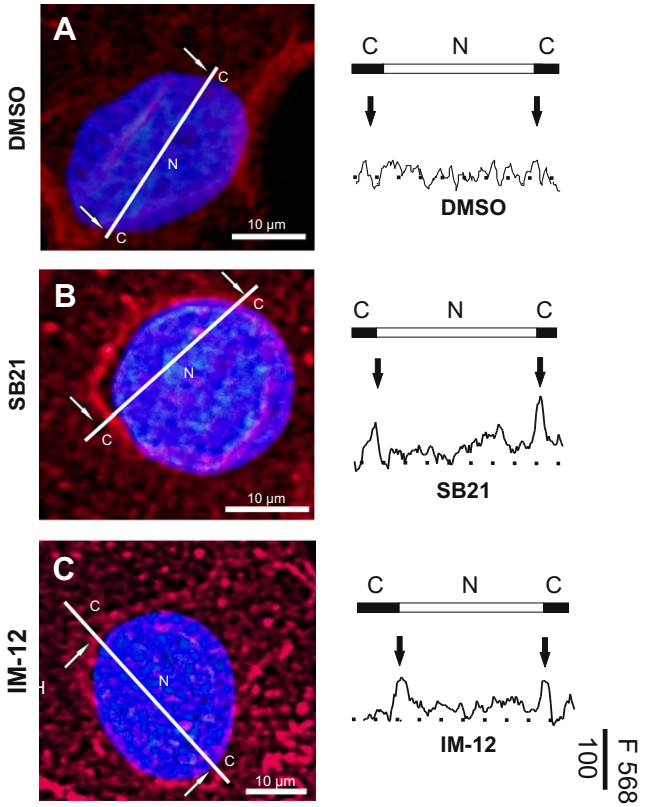

Figure 9. GSK-3 inhibitors elevate nuclear β-catenin accumulation after 6 h of differentiation. ST14A cells were stained for β-catenin (red) and DAPI (blue). Left panel: (A) In DMSO treated cells no β-catenin accumulation was observed In contrary, in SB-216763 (B) or IM-12 (C) treated cells, an accumulation of β-catenin around the nucleus was observed. Right panel: The peri-nuclear accumulation is also visible in a line-scan detecting the fluorescence intensity (F568) of the cytosol (C) and nucleus (N). Arrows indicate the peri-nuclear accumulation of β-catenin. The scale bar indicates a size of 10 μm.

2.7. Influence on TCF-activity

Next we investigated the TCF-activity of IM-12 treated hNPCs. ReNcell VM cells were transfected with TOPFlash, FOPFlash (a control vector which contains a mutant TCF binding site) or the combination of TOPFlash or FOPFlash with pCAGGS-S33Y, a vector containing a stabilized form of β-catenin. Twenty-four hours after transfection, cultivation conditions were changed from proliferation to differentiation. With the start of differentiation, 3 μM SB-216763 or IM-12 was added to the media. After 24 h of differentiation, a luciferase assay was performed (Fig. 10). The co-transfection of TOPFlash with pCAGGS-S33Y showed a 4.6-fold induction of TCF-activity compared to FOPflash, which confirms our findings, that the cell line ReNcell VM is able to act canonically. Then we investigated, whether SB-216763 and IM-12 can mimic the effect of stabilized β-catenin or not. Interestingly, treatment of TOP-transfected cells with SB-216763 did not lead to TCF-induction compared to control cells, whereas IM-12 resulted in an increase (Fig. 10). When cells transfected with TOP and pCAGGS-S33Y were conditioned with SB-216763, the TCF-induction was 55% higher than the induction in control cells. When cells were treated with IM-12, TCF-activity was significantly increased by 270% compared to controls (Fig. 10, $p < 0.05$).

2.8. Influence on neuronal differentiation

To investigate the impact of IM-12 on neuronal differentiation, the expression of βIII tubulin positive cells (βIIItub^{+}) was measured. As an example a βIII tubulin staining of proliferating

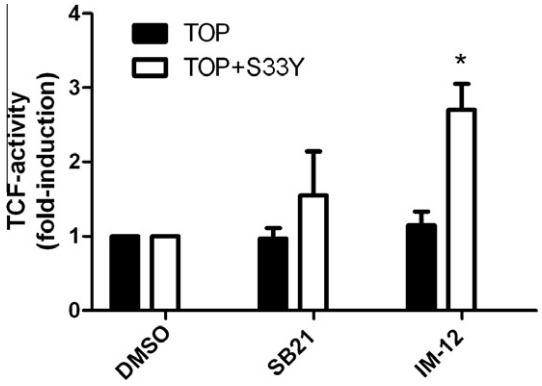

Figure 10. TCF-activity of ReNcell VM after treatment with SB-216763 and IM-12. Cells were transfected with TOP/FOP plasmids or co-transfected with pCAGGS-S33Y (TCF reporter gene assay SuperTOPflash). Twenty-four hours after transfection, conditions were changed from proliferation to differentiation with addition of small molecules. TCF-activity was measured after 24 h of differentiation. Ratio TOP/FOP was normalized to control cells. Unlike SB-216763, IM-12 increased TCF-activity compared to DMSO control cells. This effect was even stronger when cells were co-transfected with pCAGGS-S33Y. Still SB-216763 was not able to induce TCF-activity in a significant way compared to DMSO control cells. Data represent mean ± SEM ($N = 3$, each done in triplicates). Values were significantly different between drug treated cells and DMSO treated control cells at $^{*}p < 0.05$.

(Fig. 11A) and differentiated cells (Fig. 11B) is shown. Upon differentiation the number of βIIItub^{+} cells is increased as shown by flow cytometry (Fig. 11A and B). For flow cytometry ReNcell VM cells were differentiated for 3d under the influence of either DMSO, SB-216763 or IM-12. Proliferating cells showed a very small amount of βIIItub^{+} cells (0.07 ± 0.02%), which was most likely due to spontaneous differentiation (Fig. 11A and C). After 3 days of differentiation 2.0 ± 0.3% cells were positive for βIII tubulin under control conditions (Fig. 11B and C). The level of βIIItub^{+} cells was nearly doubled by SB-216763 and increased up to 3.2 ± 0.4% (Fig. 11C). Cells treated with IM-12 showed a higher increase up to 3.7 ± 0.7% (Fig. 11C), although the difference was not significant to cells treated with SB-216763 but significant to control cells.

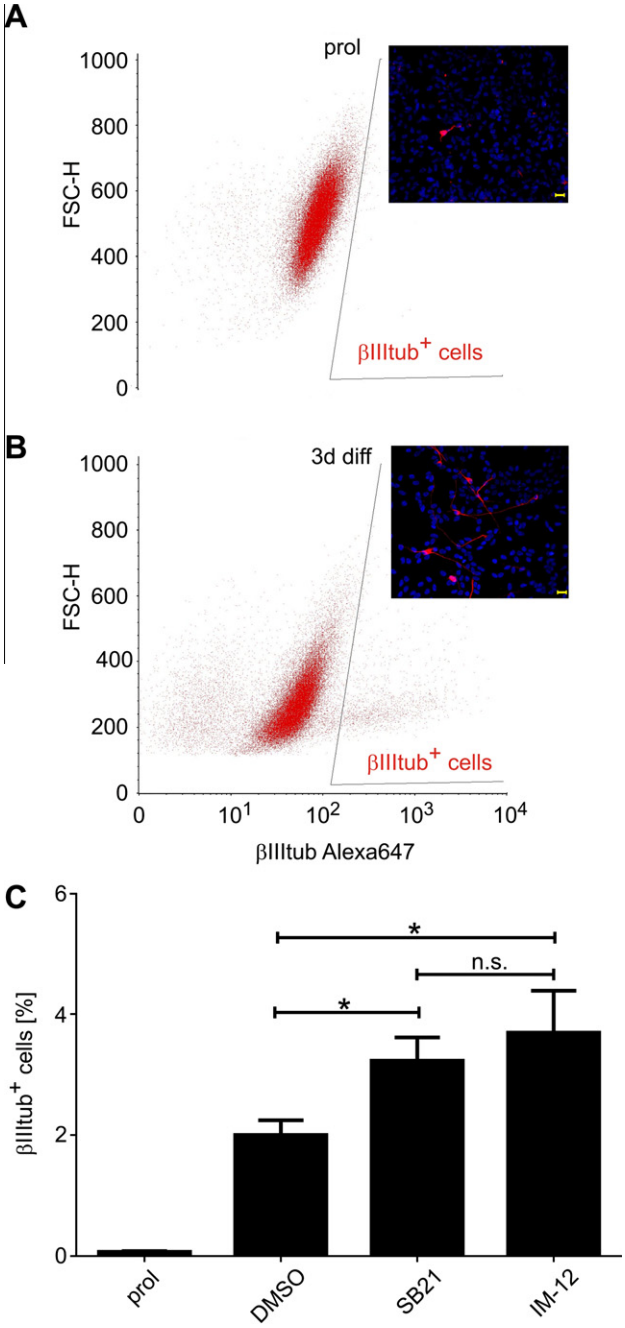

Figure 11. Influence of SB-216763 (SB-21) and IM-12 on neuronal differentiation. ReNcell VM cells were differentiated in presence of the compounds or DMSO. Expression of βIII tubulin was determined by immunocytochemistry (insert A and B, scale bar indicates 20 μm) and quantified by flow cytometry (A and B). After 3 days of differentiation 2.0 ± 0.2% βIIItub^{+} cells were detected under control conditions. The treatment of the cells with SB-216763 or IM-12 resulted in an increase of βIIItub^{+} cells up to 3.2 ± 0.4 % and 3.7 ± 0.7%, respectively. Data represent mean ± SEM ($N = 6$). $^{*}p < 0.05$.

3. Discussion

GSK-3β has been shown to be involved in several diseases. Continuous activation of β-catenin is often linked to cell proliferation and tumour growth.[25] Neurofibrillary tangle deposits are formed as a consequence of GSK-3β activation in Alzheimer disease brains.[26] Therefore, the inhibition of GSK-3β is an attractive target for pharmaceuticals. To test novel active compounds in vitro, the choice of a suitable cellular model system is important. GSK-3β is mainly located in the brain and expressed primarily in neurons.[27,28] It has been described previously that ventral midbrain precursors from non-human vertebrates can respond to a treatment with the GSK-3 inhibitors Kenpaullone and indirubine-3-monoxime (I3M) by stabilization of β-catenin[16] whereas valproic acid induces GSK-3β inhibition and β-catenin accumulation in rat NPCs.[29]

SB-216763 is selective to GSK-3.[30] Thus, conditioning of HEK293-cells with SB-216763 resulted in cytosolic β-catenin accumulation. In cerebellar granule neurones, neuroprotective effects were observed.[31] Our experiments show an upregulation of β-catenin in human NPCs after treatment with established GSK-3 inhibitors and the novel compounds (Fig. 3) and furthermore a nuclear translocation in ST14A cells (Fig. 8).

In our study, analysing the biological activity of novel non-symmetrically substituted indolylmaleimides, we can demonstrate that IM-12 enhances the β-catenin accumulation significantly. This result may be ascribed to the amine moiety, which is an additional hydrogen bonding motif. In addition, it has been reported that biologically active substances often benefit from the presence of fluorine substituents due to improved metabolic stability, bioavailability and protein–ligand interactions of the fluorinated compounds.[32] Thus, the substitution with one or more fluorine atoms,[33] and more specifically, the incorporation of the 4-fluorophenethylamine unit,[34] has led to an increased biological activity of small molecule therapeutics. In contrast, the indolylmaleimides

IM-1, 4, 7, 10, 13, and 15 slightly decreased the β-catenin accumulation (Fig. 3). Indolylmaleimides IM-16–22 did not show a further enhancement of β-catenin accumulation compared to IM-12 (Fig. 5).

Our experiments revealed a concentration of 3 μM as the optimal concentration to give the highest effect on β-catenin accumulation whereas other concentrations showed no further difference in β-catenin-increase compared to control cells. In vitro-binding assay of GSK-3β showed that IM-12 acted in the same range as SB-216763 and downregulated the activity of GSK-3β to 27%. Coghlan et al.[18] reported an IC_{50}-value of 34 nM for SB-216763, which was 96 nM in our study. The IC_{50} for GSK-3β inhibition of IM-12 was 53 nM, whereas interestingly a bell-shaped dose–response relationship was observed. These date match to the influence of different IM-12 concentrations on β-Catenin accumulation, where concentrations higher than 3 μM show a rapid decrease. For this experiment, an IC_{50}-value of 3.8 μM for IM-12 was determined. The difference between the IC_{50} for cellular and enzymatic inhibitory-assays can be explained by the fact that an enzymatic inhibitory-assay with a recombinant enzyme is much more sensitive than a cellular system in which many other unknown factors of metabolic and biochemical pathways are involved, however the cellular assay might be of more relevance for the prediction of the biological consequence of the given drug.

Combinations of SB-216763 with various concentrations of IM-12 showed no additive effects on the β-catenin accumulation compared to SB-216763 alone. In contrast, 3 μM of SB-216763 in addition with 10 μM IM-12 significantly reduced the β-catenin accumulation. Previous experiments in our group showed that SB-216763 in concentrations equal or higher than 5 μM reduces cell proliferation in a significant manner (data not shown). It seems that higher concentrations of SB-216763 or IM-12 have a negative or even toxic effect on the cells. IM-12 and SB-216763 could act in a very similar way whereby the combination of both substances show negative effects at lower combined than single concentrations. Further studies will focus on these effects. The data regarding the accumulation of β-catenin driven by small molecules are in contrast to the induction of TCF-activity as one would expect that a high rate of β-catenin accumulation results in high TCF-activity. Treatment of ReNcell VM results in a more potent TCF-activity than with SB-216763. Several factors could be responsible for this. IM-12 could also prime the nuclear shuttling of β-catenin or the kinetic of TCF-activity could be influenced by both substances in a different way.

In addition, our studies showed an inhibition of cell proliferation after treatment with canonical Wnt-activators. The doubling time of the human NPCs was significantly increased compared to control experiments. This is conflictingly described in the literature. For example, Hirsch et al.[22] described that treatment with SB-216763 did not result in any significant effect on proliferation in murine neonatal NPCs. On the other hand, Adachi et al.[35] observed an enhancing effect on proliferation of murine progenitor cells from the subventricular zone when treated with the GSK-3 inhibitor R3303544, which is structurally very similar to SB-216763. Murine NPCs from telencephalon reacted with increasing cell proliferation in the presence of SB-216763.[23] Inhibition of cell proliferation by SB-216763 has also been reported in colon cancer cell lines.[36] They monitored shrinking of tumours in mice which were formed by human SW480-cells after the mice were treated with SB-216763 or AR-A014418, another GSK-3β inhibitor, respectively.

Our experiments revealed an increase in cell proliferation when cells were cultured in the presence of growth factors (FGF-2, EGF, referred as control conditions) whereas the additional treatment with GSK-3 inhibitors SB-216763 and IM-12 decreases cell growth (Fig. 8A). This is in contrast to the data of Shimizu et al.[23] as they reported that FGF-2 enhanced proliferation via activating PI3K and inhibitory phosphorylation of GSK-3β and that SB-216763 partly mimicked this effect. As this is the first study on human NPCs it is possible that SB-216763 and its action on Wnt signalling has a different function in human neural cells. Interestingly, the results resemble those described for cancer cell lines, which could be driven by the fact that ReNcell VM cells are immortalized with c-Myc.

As the data, regarding cellular proliferation and the influence of canonical Wnt, are very contradictory, we wanted to know how differentiation in human neural progenitor cells is impaired by GSK-3β inhibitors. Activation of canonical Wnt signalling by Wnt3a could enhance neuronal differentiation of mNPCs.[22] In contrast, SB-216763 has been shown to decrease the number of $\beta III tub^+$ cells in mNPCs.[23] The authors concluded from their data that the inhibition of differentiation by the inhibitor of GSK-3β is mediated by Notch signalling. Conditioning of hNPCs with SB-216763 resulted in our studies in an increase of $\beta III tub^+$ cells, which could be mimicked by IM-12. It is important to investigate components of canonical Wnt signalling other than GSK-3β activity to evaluate the Wnt-specifity of new GSK-3β inhibitors due to the fact that GSK-3β is involved in many other cellular pathways and has numerous other substrates including enzymes or transcription factors.[12] This needs to be further investigated.

4. Conclusion

Aim of this study was to investigate the ability of newly synthesized indolylmaleimides to act as GSK-3β inhibitors. The effects of the new compounds were tested in several cellular assays and compared to the known GSK-3β inhibitor SB-216763. We successfully demonstrated that one of the new compounds, namely IM-12 inhibited GSK-3β and subsequently increased β-catenin concentration significantly in hNPCs. In addition we monitored a nuclear accumulation of β-catenin after having conditioned the cells with SB-216763 as well as with IM-12. The observed levels of TCF-induction induced by IM-12 was higher in comparison to SB-216763. Furthermore IM-12 promotes the neuronal differentiation of human neural progenitor cells. In summary, our results demonstrate, that the novel indolylmaleimide IM-12 acts as GSK-3β inhibitor resulting in the activation of downstream components of canonical Wnt signalling and has an adjacent positive impact on the neuronal differentiation in human neural progenitor cells.

5. Methods

5.1. Reagents and chemistry

DMSO, Kenpaullone, SB-216763 were purchased from Sigma Aldrich (Taufkirchen, Germany). Stock solutions were prepared by dissolving all substances in pure DMSO. All reactions were performed using standard Schlenk technique (argon). ^1H and ^{13}C NMR spectra were recorded on a Bruker AV 300/AV 400 spectrometer at 25 °C. Chemical shifts (δ) are given in ppm and refer to the residual non-deuterated solvent as the internal standard (DMSO-d_6: 2.50/39.51). Gas chromatography was performed on a Hewlett–Packard HP 6890 chromatograph with a HP5 column. IR spectra were recorded on a Nicolet Magna 550. Mass spectroscopy was performed on a 5973 Network Mass Selective Detector from Agilent Technologies. Chemicals were purchased from Fluka, Aldrich, or Strem and used as received.

The synthesis of 3-bromo-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-maleimide has been described previously.[37]

5.1.1. General procedure for palladium-catalyzed aminocarbonylations using autoclave technique

Reactions were carried out in a 300 mL autoclave of the 4560 series from *Parr Instruments*® containing an alloy plate to hold six 4 mL glass vials. 3-Bromo-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-

maleimide (79.8 mg, 0.25 mmol) and a magnetic stir bar were filled in each vial. The vials were closed with a septum equipped with an inlet needle and flushed with argon several times. A stock solution (2.03 mL) prepared from $Pd(OAc)_2$ (2.81 mg, 0.5 mol %), cataCXium® A (13.45 mg, 1.5 mol %), base (0.75 equiv), and solvent (20 mL) was transferred to each vial via syringe. Subsequently, the adequate amount of the corresponding amine was added. The vials were placed in the alloy plate which was transferred into the autoclave under argon atmosphere. First, the locked autoclave was flushed with carbon monoxide. Then, the autoclave was pressurized with 5–15 bar of CO at room temperature and the reaction was performed at 90–115 °C for 16 h. Afterwards, the autoclave was cooled to room temperature and vented to discharge the excess of carbon monoxide. Finally, reaction solutions were filtered and the solvent was removed in vacuum. The crude products were purified by column chromatography on silica gel.

5.1.1.1. 3-(4-Fluorophenylethylamino)-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (IM-12). To a stirred solution of 3-bromo-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-maleimide (480 mg, 1.5 mmol), in anhydrous 1,4-dioxane (15 mL), subsequently, triethylamine (0.42 mL, 3 mmol) and 4-fluorophenethylamine (0.9 mL, 6 mmol) were added at room temperature under argon atmosphere. The reaction mixture was refluxed for 20 h. The solvent was then removed in vacuum and the crude product was purified by column chromatography (hexane/EtOAc) on silica gel. Yield 540 mg, 95%; mp 187 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.15 (br s, 1H), 7.42 (t, J = 6.3 Hz, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.08–7.00 (m, 1H), 6.99–6.91 (m, 1H), 6.90–6.80 (m, 2H), 6.48–6.39 (m, 2H), 3.18–2.95 (br m, 2H), 2.92 (s, 3H), 2.48–2.30 (br m, 2H), and 2.23 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.1 (C=O), 167.7 (C=O), 160.6 (d, ^1J (C,F) = 241 Hz), 144.4, 135.4, 135.2, 134.5 (d, J = 3.2 Hz), 130.0 (d, J = 8.2 Hz, 2CH), 120.4 (CH), 118.9 (CH), 118.2 (CH), 114.7 (d, J = 21.2 Hz, 2CH), 110.7 (CH), 101.2, 90.6, 43.9 (CH_2), 34.9 (CH_2), 23.5 (CH_3), and 12.3 (CH_3). MS (EI, 70 eV): m/z (%) = 377 (100) $[M]^+$. HRMS (EI): calcd for $C_{22}H_{20}FN_3O_2$: 377.15396; found: 377.15334 $[M]^+$. IR (ATR): ν_{max} = 3359, 3269, 3048, 2921, 2877, 1747, 1690, 1641, 1617, 1603, 1554, 1532, 1510, 1451, 1390, 1336, 1288, 1246, 1233, 1220, 1180, 1159, 1130, 1103, 1082, 1025, 987, 832, 787, 744, 706, 674, and 659 cm^{-1}.

5.1.1.2. 3-(4-Methoxyphenylethylamino)-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (IM-16). Yield 95%; mp 154 °C (dec.). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.27 (br s, 1H), 7.37–7.32 (m, 1H), 7.26–7.21 (m, 1H), 7.16–7.05 (m, 2H), 6.71–6.65 (m, 2H), 6.59–6.53 (m, 2H), 5.35 (br s, 1H), 3.73 (s, 3H), 3.29–3.14 (br m, 2H), 3.08 (s, 3H), 2.47 (t, J = 7.4 Hz, 2H), and 2.23 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.8 (C=O), 168.3 (C=O), 158.2, 143.8, 135.3, 135.1, 130.0, 129.7, 129.4 (2CH), 121.4 (CH), 120.0 (CH), 118.7 (CH), 113.9 (2CH), 110.5 (CH), 101.5, 92.8, 55.2 (OCH_3), 44.5 (CH_2), 35.5 (CH_2), 23.9 (CH_3), and 12.6 (CH_3). MS (EI, 70 eV): m/z (%) = 389 (100) $[M]^+$. HRMS (EI): calcd for $C_{23}H_{23}N_3O_3$: 389.17339; found: 389.173173 $[M]^+$. IR (ATR): ν_{max} = 3358, 3268, 3047, 2919, 2872, 2837, 1747, 1693, 1640, 1614, 1556, 1531, 1511, 1444, 1389, 1334, 1301, 1288, 1242, 1178, 1131, 1110, 1084, 1035, 10009, 985, 954, 928, 877, 861, 830, 810, 787, 782, 749, 702, 673, and 658 cm^{-1}.

5.1.1.3. 3-(4-Chlorophenylethylamino)-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (IM-17). Yield 91%; mp 198–200 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.14 (s, 1H), 7.40 (t, J = 6.3 Hz, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.10–7.00 (m, 3H), 6.98–6.91 (m, 1H), 6.42 (d, J = 8.2 Hz, 2H), 3.17–2.97 (br m, 2H), 2.92 (s, 3H), 2.46–2.30 (br m, 2H), and 2.23 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.1 (C=O), 167.7 (C=O), 144.4, 137.4, 135.4, 135.2, 135.0, 130.6, 130.0 (2CH), 127.9 (2CH), 120.4 (CH), 118.9 (CH), 118.2 (CH), 110.7 (CH), 101.1, 90.6, 43.7 (CH_2), 35.0 (CH_2), 23.5 (CH_3), and 12.2 (CH_3). MS (EI, 70 eV): m/z (%) = 393 (85) $[M]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{22}H_{21}ClN_3O_2$: 394.13168; found: 394.13194. IR (ATR): ν_{max} = 3356, 3263, 3051, 2923, 2875, 2852, 1745, 1691, 1640, 1617, 1555, 1533, 1492, 1452, 1410, 1389, 1334, 1290, 1243, 1233, 1203, 1177, 1130, 1109, 1091, 1045, 1016, 986, 955, 942, 923, 862, 825, 813, 780, 744, 685, and 659 cm^{-1}.

5.1.1.4. 1-Methyl-3-(2-methyl-1*H*-indol-3-yl)-4-(phenylethylamino)-1*H*-pyrrole-2,5-dione (IM-18). Yield 97%; mp 200 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.16 (br s, 1H), 7.41 (t, J = 6.4 Hz, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.22 (d, J = 7.6 Hz, 1H), 7.08–6.99 (m, 4H), 6.98–6.91 (m, 1H), 6.46–6.37 (m, 2H), 3.18–2.97 (br m, 2H), 2.92 (s, 3H), 2.47–2.31 (br m, 2H), and 2.24 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.2 (C=O), 167.8 (C=O), 144.4, 138.3, 135.4, 135.2, 130.0, 128.2 (2CH), 128.1 (2CH), 126.0 (CH), 120.4 (CH), 118.9 (CH), 118.2 (CH), 110.6 (CH), 101.2, 90.5, 44.0 (CH_2), 35.9 (CH_2), 23.5 (CH_3), and 12.3 (CH_3). MS (EI, 70 eV): m/z (%) = 359 (100) $[M]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{22}H_{21}N_3O_2$: 360.17065; found: 360.17125. IR (ATR): ν_{max} = 3347, 3250, 3056, 3026, 2935, 2879, 1747, 1693, 1639, 1615, 1554, 1532, 1452, 1387, 1354, 1339, 1279, 1248, 1230, 1181, 1129, 1109, 1093, 1029, 1009, 1000, 982, 922, 789, 781, 739, 695, 674, and 659 cm^{-1}.

5.1.1.5. 3-(3,4-Difluorophenylethylamino)-1-methyl-4-(2-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (IM-19). Yield 79%; mp 200 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.18 (br s, 1H), 7.43 (t, J = 6.3 Hz, 1H), 7.33 (d, J = 7.9 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.15–6.91 (m, 3H), 6.39–6.31 (br m, 1H), 6.23 (t, J = 9.7 Hz, 1H), 3.19–2.96 (br m, 2H), 2.91 (s, 3H), 2.45–2.33 (br m, 2H), and 2.23 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.1 (C=O), 167.7 (C=O), 148.9 (dd, ^1J (C,F) = 245 Hz, ^3J (C,F) = 12.6 Hz), 147.9 (dd, ^1J (C,F) = 243 Hz, ^3J (C,F) = 12.6 Hz), 144.3, 136.1 (dd, J = 3.8 Hz, J = 5.8 Hz), 135.4, 135.1, 129.8, 124.9 (dd, J = 3.3 Hz, J = 6.3 Hz, CH), 120.4 (CH), 119.0 (CH), 118.2 (CH), 117.0 (d, J = 1.9 Hz, CH). 116.7 (d, J = 1.9 Hz, CH), 110.7 (CH), 101.1, 90.7, 43.6 (CH_2), 34.7 (CH_2), 23.5 (CH_3), and 12.2 (CH_3). MS (EI, 70 eV): m/z (%) = 395 (100) $[M]^+$. HRMS (ESI): calcd for $[M+H]^+$ $C_{22}H_{19}F_2N_3O_2$: 396.15181; found: 396.15197. IR (ATR): ν_{max} = 3352, 3261, 3059, 2926, 2877, 1747, 1694, 1642, 1615, 1555, 1515, 1453, 1433, 1388, 1354, 1339, 1283, 1210, 1181, 1117, 1091, 1010, 1002, 985, 944, 869, 814, 781, 738, 700, 671, and 657 cm^{-1}.

5.1.1.6. 3-(4-Fluorophenylethylamino)-4-(1*H*-indol-3-yl)-1-methyl-1*H*-pyrrole-2,5-dione (IM-20). Yield 68%; mp 206 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.28 (br d, J = 1.6 Hz, 1H), 7.44 (d, J = 8.0 Hz), 7.42–7.34 (m, NH overlapped, 1H), 7.37 (d, J = 7.6 Hz, 1H), 7.31 (d, J = 2.5 Hz, 1H), 7.16–7.08 (m, 1H), 7.04–6.96 (m, 1H), 6.93–6.83 (m, 2H), 6.56–6.47 (m, 2H), 3.24–3.11 (br m, 2H), 2.92 (s, 3H), and 2.46–2.35 (br m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.3 (C=O), 167.7 (C=O), 160.6 (d, ^1J (C,F) = 241 Hz), 143.4, 135.6, 134.5 (d, J = 3.1 Hz), 130.0 (d, J = 7.9 Hz, 2CH), 128.5, 126.2 (CH), 121.2 (CH), 119.5 (CH), 119.0 (CH), 114.7 (d, J = 20.9 Hz, 2CH), 111.5 (CH), 104.0, 91.3, 44.2 (CH_2), 34.9 (CH_2), and 23.5 (CH_3). MS (EI, 70 eV): m/z (%) = 363 (92) $[M]^+$. HRMS (EI): calcd for $[M]^+$ $C_{21}H_{18}FN_3O_2$: 363.13910; found: 363.138559. IR (ATR): ν_{max} = 3313, 3052, 2924, 2871, 1754, 1694, 1647, 1613, 1541, 1455, 1438, 1388, 1359, 1340, 1318, 1284, 1244, 1218, 1178, 1158, 1102, 1056, 1020, 1009, 984, 932, 878, 853, 823, 783, 766, 751, 737, and 669 cm^{-1}.

5.1.1.7. 3-(4-Fluorophenylethylamino)-4-(5-methoxy-1*H*-indol-3-yl)-1-methyl-1*H*-pyrrole-2,5-dione (IM-21). Yield 82%; mp 198 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.14 (br d, J = 1.7 Hz,

1H), 7.39–7.34 (m, NH overlapped, 1H), 7.34 (d, J = 8.5 Hz, 1H), 7.27 (d, J = 2.5 Hz, 1H), 6.93–6.83 (m, 3H), 6.77 (dd, J = 2.4 Hz, J = 8.7 Hz, 1H), 6.57–6.49 (m, 2H), 3.70 (s, 3H), 3.25–3.13 (br m, 2H), 2.92 (s, 3H), and 2.47–2.39 (br m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.4 (C=O), 167.8 (C=O), 160.7 (d, ^1J (C,F) = 241 Hz), 153.5, 143.4, 134.5 (d, J = 2.9 Hz), 130.8, 130.0 (d, J = 8.0 Hz, 2CH), 129.0, 126.8 (CH), 114.7 (d, J = 20.7 Hz, 2H), 112.3 (CH), 111.5 (CH), 103.8, 101.0 (CH), 91.5, 55.3 (CH_3), 44.3 (CH_2), 35.1 (CH_2), and 23.5 (CH_3). MS (EI, 70 eV): m/z (%) = 393 (84) $[M]^+$. HRMS (EI): calcd for $[M]^+$ $C_{22}H_{20}FN_3O_3$: 393.14832; found: 393.148384. IR (ATR): ν_{max} = 3320, 3114, 3069, 3002, 2932, 2883, 1758, 1697, 1648, 1615, 1583, 1550, 1509, 1480, 1452, 1440, 1387, 1356, 1335, 1290, 1241, 1213, 1183, 1158, 1129, 1108, 1054, 1029, 1017, 982, 922, 851, 830, 801, 779, 757, 721, and 666 cm^{-1}.

5.1.1.8. 3-(5-Fluoro-1H-indol-3-yl)-4-(4-fluorophenylethylamino)-1-methyl-1H-pyrrole-2,5-dione (IM-22). Yield 97%; mp 213 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.38 (br d, J = 1.7 Hz, 1H), 7.47–7.38 (m, 3H), 7.10 (dd, J = 2.5 Hz, J = 10.0 Hz, 1H), 7.00–6.85 (m, 3H), 6.61–6.52 (m, 2H), 3.26–3.14 (m, 2H), 2.91 (s, 3H), and 2.47–2.37 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 172.2 (C=O), 167.6 (C=O), 160.7 (d, ^1J (C,F) = 241 Hz), 156.9 (d, ^1J (C,F) = 232 Hz), 143.5, 134.5 (d, J = 3.0 Hz), 132.3, 130.0 (d, J = 7.9 Hz, 2CH), 128.7 (d, J = 9.9 Hz), 128.2 (CH), 114.8 (d, J = 21.0 Hz, 2CH), 112.6 (d, J = 9.8 Hz, CH), 109.5 (d, J = 26.1 Hz, CH), 104.4 (d, J = 4.7 Hz), 104.1 (d, J = 23.3 Hz, CH), 90.7, 44.2 (CH_2), 34.8 (CH_2), and 23.5 (CH_3). MS (EI, 70 eV): m/z (%) = 381 (67) $[M]^+$. HRMS (EI): calcd for $[M]^+$ $C_{21}H_{17}F_2N_3O_2$: 381.12833; found: 381.128368. IR (ATR): ν_{max} = 3308, 3057, 2924, 2866, 1753, 1696, 1649, 1623, 1601, 1581, 1540, 1510, 1486, 1459, 1388, 1357, 1339, 1283, 1215, 1191, 1160, 1145, 1110, 1097, 1051, 1017, 980, 933, 855, 829, 810, 794, 780, 757, 745, and 670 cm^{-1}.

5.2. Cell culture

The ReNcell VM cell line is derived from the human ventral midbrain of embryonic week 10 and immortalized by v-myc transduction[38] and was obtained by Millipore (Billerica, USA). ReNcell VM cells were cultivated essentially as described previously.[38]

Briefly, cells were cultured on laminin (Trevigen, Gaithersburg, MD, USA) coated flasks or 6-well plates in cultivation media, consisting of DMEM/F12 supplemented with B27 media supplement, GlutaMax™, gentamycin and heparin sodium salt (all Invitrogen, Karlsruhe, Germany). For proliferation conditions, the media contained additional 20 ng/ml epidermal growth factor (EGF; Roche, Mannheim, Germany) and 10 ng/ml basic fibroblast growth factor (bFGF; Roche). At the stage of approximately 70% confluency, the cells were passaged.

Cells were differentiated by using a standard differentiation protocol, where the differentiation is induced by withdrawal of growth factors.[19,20]

ST14A cells, a conditionally immortalized rat embryonic (E14) striatal progenitor cell line with a temperature-dependent expression of an immortalizing oncogene, have been described before.[39] The cells were obtained from embryonic day 14 (E14) rat striatum and were transfected with the temperature-sensitive mutant tsA58U19 of the SV40 large T antigen.[40] Cells were cultured in flasks and chamber slides at 33 °C for proliferation and 39 °C for differentiation, respectively.

Drugs were applied in cell culture media with the initiation of differentiation for the indicated times.

5.3. Proliferation assay

To measure viable cells, 50–100 µl of cell suspension was analyzed using CASY technology (Innovatis, Reutlingen, Germany) with the appropriate program. ReNcell VM cells were seeded at a defined cell number and proliferated for 24 h. Then the medium was changed to proliferation medium with added substances at indicated concentrations. The cell number was determined every 24 h. Cells were exposed to the added drugs during the whole experiment, whereas the media was changed every 24 h.

5.4. Protein determination

The amount of protein in ReNcell VM cells extracts was determined as described earlier.[41] In brief, the BCA protein assay reagent kit (Thermo Scientific, Rockford, USA) was used. Cells were lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Roche) and centrifuged for 5 min at 15,000 rpm. The supernatant was used for further experiments. Two hundred microlitres BCA reaction solution were added to 10 µl of total cell protein and incubated for 1 h at 37 °C. The absorbance was measured at 570 nm with a plate photometer (Tecan, Crailsheim, Germany).

5.5. ELISA

The stabilization of β-catenin was measured using the human total β-catenin ELISA DuoSet IC system (R&D Systems, Wiesbaden, Germany) according to the manufacturer's recommendation. Fifteen microgram of total cell lysate were used. The optical density was measured with a plate photometer (Tecan) at 450 nm with a wavelength correction at 570 nm.

Cells were differentiated 24 h after seeding for 2 h. The substances were added at indicated concentrations with the start of differentiation.

5.6. GSK-3β activity assay

IC_{50} of new synthesised compound IM-12 to GSK-3β was determined by a luminometric GSK-3β activity assay. This method has been described by Baki et al. (2007) and gives comparable IC_{50} values to radioactive detection.[42] Briefly, compounds were tested in different concentrations diluted in assay buffer containing final concentrations of: 4 mM MOPS pH 7.2; 0.4 mM EDTA; 1 mM EGTA; 2.5 mM β-glycerophosphate; 4 mM $MgCl_2$; 40 µM BSA; 0.05 mM DTT. Four microlitres of diluted compounds were added to 25 µM pGS-2 peptide substrate (Millipore, Billerica, USA), 20 ng recombinant GSK-3β (R&D systems) and 1 µM ATP (Cell Signaling, Boston, USA) to a total assay volume of 40 µl. The enzymatic reaction was stopped after 30 min of incubation at 30 °C by adding 40 µl KinaseGlo (Promega, Madison, USA). The luminometric signal was allowed to stabilise for 10 min and the measured with a Glomax® 96 Microplate Luminometer (Promega).

5.7. GSK-3β in vitro kinase assay

ReNcell VM were differentiated at 80% confluency for 2 h. The test substances were added with the start of differentiation at indicated concentrations.

Cells were lysed in RIPA buffer, supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) and centrifuged for 5 min at 15,000 rpm. The supernatant was used for further experiments. Immunoprecipitation of GSK-3β was performed with a specific mouse monoclonal anti GSK-3β [G8] antibody (Abcam, Cambridge, UK) with 5 µg/sample for 2 h at 4 °C. The bound protein was precipitated with Protein A/G-Plus agarose-beads (10 µl beads per sample; Santa Cruz Biotechnology, Santa Cruz, USA). GSK-3β kinase activity was measured in a reaction mixture containing final concentrations of: 4 mM MOPS pH 7.2; 0.4 mM EDTA; 1 mM EGTA; 2.5 mM β-glycerophosphate; 4 mM $MgCl_2$;

40 μM BSA; 0.05 mM DTT. 10 μg/sample pGS-2 peptide substrate (Millipore) was used.

The assay was initiated by the addition of a mixture of unlabelled ATP (final concentration: 50 μM) and [γ-^{32}P] ATP (Hartmann Analytic, Braunschweig, Germany; 4 μCi per sample). Following 20 min incubation at 37 °C the assay was stopped by a short centrifugation at 10,000*g* and spotting the supernatant onto phosphocellulose discs. The filter mats were washed once in 1% acetic acid and three times in H_2O. Afterwards, pGS-2 peptide-associated γ-^{32}P radioactivity bound to the phosphocellulose was quantified by Cerenkov counting.

5.8. Luciferase reporter gene assay

For the analysis of TCF-dependent transcription ReNcell VM cells were transfected with p12xSuperTOPFlash vector or p8xFOPflash and renilla luciferase vector phRL-TK (kindly provided by Randal T. Moon) with the Nucleofection system (Lonza, Cologne, Germany), according to the manufacturer's recommendation. As a positive control, cells were co-transfected with the vector pCAGGS-S33Y containing a stabilized form of β-catenin. Twenty-four hours after transfection, proliferation conditions were changed to differentiation conditions. With the start of differentiation, drugs were added at indicated concentrations. The activity of the firefly luciferase and constitutively expressed Renilla luciferase were measured using the luminometer LB 9508 (Berthold, Bad Wildbad, Germany) and the Dual Luciferase Assay Kit (Promega).

5.9. Immunocytochemistry

ReNcell VM cells were cultured and differentiated as recently desribed.[20] Shortly, ReNcell VM were seeded on cover slips and proliferated for 3 days and subsequently differentiated by withdrawal of growth factors. Cells were fixed with 4% PFA in 0.1% PBS for 15 min, at time points 0 h and 3 days, whereas 0 h was the time point of the induction of differentiation. Afterwards the cells were treated with blocking solution containing 0.4% Triton X-100 for 30 min at room temperature. Primary antibody for βIII tubulin (Santa Cruz, 1:500 mouse monoclonal) was incubated for 1 h as well as the secondary antibody (Alexa Fluor 568 goat anti rabbit, 1:1000, Molecular Probes). Probes were sealed with mounting medium containing DAPI (1.5 μg/ml, Vector Labs, USA) as a marker for cell nuclei. Microscopic analysis was performed using the Keyence Biozero system (Keyence, Neu-Isenburg, Germany).

ST14A cells were cultured in 8-well chamber-slides under proliferation conditions. With the change to differentiation conditions, a media change was performed and the drugs were added at indicated concentrations to the media. At time points 0 and 6 h, the cells were fixed with 4% paraformaldehyde in 0.1% PBS for 10 min. After washing, unspecific binding sides were blocked with PBS containing 0.3% Triton X-100 and 5% goat serum for 30 min. Cultures were incubated with the primary antibody for 30 min at room temperature. β-Catenin specific antibody (mouse mAb, Santa Cruz, Heidelberg, Germany) was diluted 1:200 in PBS with 1% goat serum. The slides were washed with PBS and then incubated with the secondary antibody (Alexa Fluor 568 goat anti-mouse antibody, diluted 1:100 in PBS with 1% goat serum) for 30 min at room temperature, was used. After washing with PBS, the cells were covered with mounting medium (Vector Labs, Burlingame, CA, USA) containing DAPI (1.5 μg/ml) for nuclear staining. Microscopic analysis was performed using the Keyence Biozero microscope (Keyence).

5.10. FACS analysis

The influence of SB-216763 and IM-12 on the neuronal differentiation of ReNcell VM cells was determined by FACS analysis. Cells were stained for βIII tubulin and the total number of positive cells was measured. Therefore cells were cultured in 6-well plates up to 80% confluency and subsequently differentiated for 3 days. Medium supplemented with substances was changed every 24 h. For FACS analysis, cells were trypsinised and centrifuged at 100*g* at rt for 5 min, washed with PBS without Ca^{2+} and Mg^{2+} and fixed with 1% PFA in PBS for 15 min. Cells were then resuspended in washing buffer (PBS + 0.5% BSA + 0.02% Na-azide) and stored at 4 °C in the dark. For the staining cells were centrifuged and resuspended in saponin buffer (PBS + 0.03% saponin + 0.5% BSA + 0.02% Na-azide) containing βIII tubulin antibody (Santa Cruz, 1:100 mouse monoclonal) and incubated for 2 h at rt. Afterwards cells were washed and incubated with the secondary antibody (Alexa Fluor 647 goat-anti-mouse, 1:1000, Molecular Probes) for 1 h in saponin buffer. Cells were washed twice with saponin buffer and resuspended in wash buffer for analysis. Measurement was done using FACSCalibur (BectonDickinson, San Jose, USA) in combination with Cell Quest Pro software.

5.11. Statistical analysis

All results are shown as mean ± SEM of data. Statistical analysis was performed with a Student's *t*-test. $p < 0.05$ was considered to indicate statistically significance using Excel (Microsoft, USA) and Prism5 (GraphPad Prism. Inc., USA).

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