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Quiescence and γH2AX in neuroblastoma are regulated by ouabain/Na,K-ATPase

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BACKGROUND: Cellular quiescence is a state of reversible proliferation arrest that is induced by anti-mitogenic signals. The endogenous cardiac glycoside ouabain is a specific ligand of the ubiquitous sodium pump, Na,K-ATPase, also known to regulate cell growth through unknown signalling pathways.

METHODS: To investigate the role of ouabain/Na,K-ATPase in uncontrolled neuroblastoma growth we used xenografts, flow cytometry, immunostaining, comet assay, real-time PCR, and electrophysiology after various treatment strategies.

RESULTS: The ouabain/Na,K-ATPase complex induced quiescence in malignant neuroblastoma. Tumour growth was reduced by >50% when neuroblastoma cells were xenografted into immune-deficient mice that were fed with ouabain. Ouabain-induced S-G2 phase arrest, activated the DNA-damage response (DDR) pathway marker γH2AX, increased the cell cycle regulator $p21^{Waf1/Cip1}$ and upregulated the quiescence-specific transcription factor hairy and enhancer of split1 (HES1), causing neuroblastoma cells to ultimately enter G0. Cells re-entered the cell cycle and resumed proliferation, without showing DNA damage, when ouabain was removed.

CONCLUSION: These findings demonstrate a novel action of ouabain/Na,K-ATPase as a regulator of quiescence in neuroblastoma, suggesting that ouabain can be used in chemotherapies to suppress tumour growth and/or arrest cells to increase the therapeutic index in combination therapies.

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Cardiac glycosides constitute a class of naturally derived compounds that bind to the ubiquitous sodium pump, Na,K-ATPase. For many years, members of this class (e.g. ouabain, digoxin, and digitoxin) have been in clinical use for the treatment of different heart diseases (Prassas and Diamandis, 2008). Interestingly, preclinical and retrospective patient data indicate that cardiac glycosides also can reduce the growth of various cancers, including breast, lung, prostate, and leukaemia (Stenkvist, 1999; Lopez-Lazaro, 2007; Mijatovic *et al*, 2007; Khan *et al*, 2009). Several signalling pathways have been proposed to account for this preferential cytotoxicity in cancer cells, including calcium (Ca^{2+}) and Apo2L/TRAIL-induced apoptosis (McConkey *et al*, 2000; Frese *et al*, 2006). The recent interest in using cardiac glycosides to treat cancers has resulted in the initiation of a number of clinical trials (Vaklavas *et al*, 2011).

The ability of cells to cycle and exit into senescence or quiescence is important for cell differentiation, tissue development, and prevention of tumourigenesis (Evan and Vousden, 2001; Liu *et al*, 2004; Lapenna and Giordano, 2009; Malumbres and Barbacid, 2009). In response to mitogens, cells overcome the G1 restriction point and commit to synthesise DNA and divide. The restriction point is regulated by the retinoblastoma protein (Rb) under the strict control of cyclin D-cyclin-dependent kinase (CDK)2 and cyclin E-CDK4 (Planas-Silva and Weinberg, 1997). These cyclin-CDK complexes phosphorylate Rb, thereby cancelling the growth-inhibitory function of Rb, to stimulate G1-S transition and S-phase progression. The CDK inhibitor $p21^{Waf1/Cip1}$ (p21) binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and causes G1 arrest in response to DNA damage (el-Deiry *et al*, 1994). p21 has also been reported to have a critical role in the transition out of the cell cycle and in maintaining cells in a quiescent state (Liu *et al*, 2009; Sang *et al*, 2008). Combinatorial therapies, in which cells are arrested in certain cell cycle phases thereby enhancing sensitivity to chemotherapy and reducing unwanted side effects, are becoming increasingly common in treating patients with cancer (Luo *et al*, 2009; Waldman *et al*, 1997). The cell signalling mechanisms that control how cells enter or exit from quiescence are not known. Slow proliferation rate and quiescence-like states in cancer cells are controlled by CDK inhibitors downstream of p53, for example p21. However, it was recently shown that a reduction in p21 *per se* was not sufficient to push arrested cells back into the cell cycle (Sang *et al*, 2008). This study identified the basic helix-loop-helix transcription factor hairy and enhancer of split1 (HES1) to be necessary for reversing the cell cycle arrest.

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Human neuroblastoma, the most common childhood solid tumour, is characterised by an extensive clinical heterogeneity ranging from spontaneous regression to extremely aggressive variants (Maris *et al*, 2007). The spontaneous regression is thought to take place through a constitutively active DNA-damage response (DDR) pathway, which is a negative regulator of cell cycle progression that may induce cellular senescence (Brodeur, 2003). Chemotherapy induces cellular responses that protect the cell from severe cellular damage, of which the activation of the DDR pathway is one such response (Downs, 2007; Bonner *et al*, 2008). DDR signal transduction senses genotoxic stress and coordinates the response into DNA repair, cell death, and/or growth arrest. The major regulators of the DDR pathway are the phosphoinositide 3-kinase (PI3K)-related protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), which phosphorylates histone H2AX on Ser 139 (γH2AX) (van Attikum and Gasser, 2009). The DDR pathway in cancer cells influences genome stability, cellular senescence and counteracts activated oncogenes and tumour progression (Bartek *et al*, 2007; Halazonetis *et al*, 2008).

The inducers of replication stress in early tumours have not yet been identified. Intriguingly, embryonic stem cells have an elevated DDR pathway basal activity (Andang *et al*, 2008), similar to the early stages of cancer, as a result of increased ion channel activity. The ion homeostasis and electrochemical gradients are critically maintained in all eukaryotic cells. The gradient is established primarily by the Na,K-ATPase through which three intracellular Na^+ ions and two extracellular K^+ ions are exchanged for every molecule of ATP hydrolysed (Kaplan, 2002). The Na,K-ATPase is a heteromer of α- and β-subunits and serves as a functional receptor for the steroid hormone ouabain, forming a signalling complex (Kaplan, 2002; Aperia, 2007). Endogenous ouabain and ouabain-like compounds are synthesised in the adrenal cortex (Huang *et al*, 2006; Bagrov *et al*, 2009), the hypothalamus (Murrell *et al*, 2005) and the placenta, (Hilton *et al*, 1996) and can serve in a local niche or as a systemic signalling molecule. Several studies have demonstrated that the ouabain/Na,K-ATPase-complex triggers signalling cascades, involving Ca^{2+}, PI3K/Akt, Ras/Raf, MAPK and/or Src (Schoner and Scheiner-Bobis, 2007). These signalling events have been shown to activate gene transcription, regulate cell growth, promote differentiation, and stimulate or protect against apoptosis (Kulikov *et al*, 2007; Desfrere *et al*, 2009; Tian *et al*, 2009; Li *et al*, 2010).

Given these observations, we investigated in this study the *in vivo* and *in vitro* role of the endogenous cardiac glycoside ouabain in regulating the cell growth of malignant neuroblastoma cells through various reported (Schoner and Scheiner-Bobis, 2007) and unreported ouabain-mediated signalling pathways. We asked whether the aggressive neuroblastoma proliferation could be reversibly or irreversibly suppressed by the treatment of cells with physiological concentrations of ouabain.

MATERIALS AND METHODS

Cell culture

The human neuroblastoma SH-SY5Y, Kelly, and SK-N-AS cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured according to the manufacturer's instructions. All cell culture reagents were from Invitrogen (Bleiswijk, Netherlands).

Xenografts and *in vivo* administration

Four- to six- week- old female NMRI *nu/nu* mice were injected subcutaneously on the right/left rear flank with 20×10^6 SH-SY5Y cells. When tumour sizes had reached approximately 0.2 ml, the mice were randomised to receive 2 mg kg^{-1} per day ouabain per oral (p.o.) or no treatment for 13 consecutive days. On a daily basis, animals were weighed and tumours were measured with digital calipers and the volume was calculated using the formula: length × width^2 × 0.44 (Tomayko and Reynolds, 1989; Wassberg *et al*, 1999). No differences in food intake, body weight, or signs of toxicity were observed between animals treated with or without ouabain. On day 13 the animals were killed and the tumours were excised, weighed, and snap frozen for further analysis. The animal experiments were approved by the regional ethics committee for animal research (N234/05) in accordance with national regulations (SFS 1988 : 534, SFS 1988 : 539 and SFS 1988 : 541).

Reagents

Reagents and concentrations used were as follows: ouabain (concentrations as indicated), 5-bromo-2′-deoxyuridine (BrdU, 10 μM), hexokinase (5 U ml^{-1}), staurosporin (1 μM) (all from Sigma, St Louis, MO, USA), nifedipine (50 μM), KN93 (5 μM), STO-609 (5 μg ml^{-1}), W-13 (15 μg ml^{-1}), H89 (10 μM), GF109203X (2.5 μM), 4-aminopyridine (1 mM), KB-R7943 (10 μM), suramin (100 μM) (all from Tocris, Bristol, UK), PP2 (10 μM, Calbiochem, Merck, Darmstadt, Germany), and U0126 (5 μM, Cell Signaling, Danvers, MA, USA).

$^{86}Rb^+$ uptake assay

Neuroblastoma SH-SY5Y cells were plated in 12-well tissue culture plates until they reached approximately 80% confluency. Cells were then incubated with PBS containing the indicated ouabain concentrations for 30 min at 37 °C. In each well ~1.5 μCi ml^{-1} $^{86}Rb^+$ was added for another 10 min. Uptake was then inhibited by 2 mM ouabain and the value at this point was taken as the maximal rate of active uptake. At the end of incubation, cells were rinsed four times in PBS containing 5 mM $BaCl_2$. Then cells were extracted with 0.3 ml of 1 M NaOH for 10 min. Samples were counted in a scintillation counter and each data point represents the average radioactivity present in four separate wells.

Electrophysiology

Electrophysiological experiments were performed on SH-SY5Y cells incubated with 50 nM ouabain in culture medium for 2 days at room temperature in ACSF containing 150 NaCl, 3 KCl, 10 Dextrose, 10 HEPES (in mM) and pH 7.3 supplemented with 3 mM $CaCl_2$ and 1 mM $MaCl_2$ using a MultiClamp 700B (Molecular Devices, Berkshire, UK). A glass pipette (6–12 MΩ, Warner Instruments, Hamden, CT, USA) was filled with an internal solution containing 10 NaCl, 10 KCl, 135 $KMeSO_4$, 2.5 MgATP, 0.3 NaGTP, 10 HEPES (in mM) and pH 7.3. Resting membrane potentials were estimated in a current clamp mode without any current injection.

Immunostaining

Cells cultured on 0.2% gelatin-coated coverslips were fixed with 4% paraformaldehyde, and blocked with 5% goat serum and 0.25% TritonX-100. Then cells were incubated with rat anti-BrdU (Abcam, Cambridge, UK) and/or rabbit anti-Ki-67 (NeoMarkers, Lab Vision, Fremont, CA, USA) primary antibodies followed by Alexa Fluor 488 goat anti-rat IgG (H + L) and/or Alexa Fluor 555 donkey anti-rabbit IgG (H + L) secondary antibodies (Invitrogen). When staining for BrdU, cells were treated with 2 M HCl for 15 min at 37 °C before staining. Nuclei were stained with TO-PRO-3 (Invitrogen). Slides were mounted using the Prolong Antifade Kit (Invitrogen) and scanned in a Carl Zeiss LSM 5 Exciter confocal microscope (Carl Zeiss, Göttingen, Germany). Images were analysed and quantified using ImageJ (NIH). Staining with only secondary antibodies was carried out as control.

Sections from xenograft tumours were incubated with primary antibodies detecting Ki-67 (NeoMarkers), active caspase-3 (R&D Systems, Abingdon, UK) or γH2AX (Ser 139, Cell Signaling). Secondary immunostaining was performed using a Superpicture Polymer detection kit (Invitrogen) with antibodies conjugated with horseradish peroxidise (HRP).

Western blot

Western blotting was performed as described elsewhere (Desfrere *et al*, 2009) with anti-Akt, phospho-Akt (Ser473), CDK1, CDK2, CDK4, cyclin A, cyclin B1, cyclin D3, cyclin E, Rb, phospho-Rb (Ser795), phospho-Rb (Ser807/811), $p21^{Waf/Cip}$ and β-actin (all from Cell Signaling), and γH2AX (Ser 139, Abcam) antibodies. The cells were lysed using modified RIPA buffer for 20 min at 4 °C. Protein concentration was determined using a BCA protein assay (Pierce, Thermo Fisher Scientific, Cramlington, UK) and equal amounts of cellular protein (~10–20 g) were separated on a 10% sodium dodecyl sulphate gel electrophoresis, followed by a transfer to a nitrocellulose membrane. Secondary antibodies were conjugated with HRP (Sigma) and films were developed with the ECL enhanced chemiluminescence system (Amersham, GE Healthcare Biosciences, Pittsburgh, PA, USA).

Comet assay

Comet assays were performed using a kit (Trevigen, Gaithersburg, MD, USA) and an Alkaline Comet Assay protocol according to the manufacturer's instructions. Data was analysed and the tail moment was calculated using the software CometScore (TriTek, Sumerduck, VA, USA).

Flow cytometry

Cell cycle analyses were performed using cells that were fixed overnight with 70% ethanol and rehydrated in PBS with RNase and propidium iodide (Sigma). Paraformaldehyde (4%) was used when cells were double stained with γH2AX (Ser 139, Upstate, Millipore, Billerica, MA, USA) and propidium iodide. Cleaved caspase-3 was stained using an apoptosis kit (BD Pharmingen, Oxford, UK). When staining for BrdU, cells were treated with 2 M HCl before adding the FITC-conjugated anti-BrdU antibody (BD Pharmingen). Membrane potential was measured with bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$, 1 μM, Invitrogen). Flow cytometry was performed on a FACScan instrument (Becton Dickinson) and data were analysed with CellQuest Pro software (Becton Dickinson) or FlowJo software (Tree Star, Ashland, OR, USA).

Real-time RT–PCR

Total RNAs were extracted from SH-SY5Y cells using RNeasy Mini Kit coupled with DNase treatment (Qiagen, Valencia, CA, USA) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Bleiswijk, The Netherlands). Resulting cDNAs were analysed in triplicates using SYBR-Green Master PCR mix (Applied Biosystems). Relative mRNA concentrations were determined by $2^{-(Ct-Cc)}$ where Ct and Cc are the mean threshold cycle differences after normalising to β2-microglobulin (B2M) values. Primers used for PCR were: B2M Fw: 5′-TTCTGGCCTGGAGGCTATC-3′, B2M Rev: 5′-TCAG GAAATTTGACTTTCCATTC-3′, HES1 Fw: 5′-GAAGCACCTCCG GAACCT-3′, and HES1 Rev: 5′-GTCACCTCGTTCATGCACTC-3′.

Data analysis

Data are presented as mean ± s.e.m. of a minimum of three experiments, unless indicated otherwise. Statistical significance was accepted at $P<0.05$ as determined by unpaired two-tailed *t*-test (GraphPad, La Jolla, CA, USA) or one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test (SigmaPlot, San Jose, CA, USA).

RESULTS

Neuroblastoma proliferation

To study the impact of the cardiac glycoside ouabain on neuroblastoma proliferation, human SH-SY5Y cells were exposed to various concentrations of ouabain and the *in vitro* incorporation of bromodeoxyuridine (BrdU) was examined. Treatment of the cells with 50–500 nM ouabain reduced BrdU incorporation dose-dependently (Figures 1A and B). The number of BrdU-positive cells was significantly lower when the ouabain concentration exceeded 50 nM (Figure 1B). Cells were also treated with BrdU for various exposure times to investigate whether ouabain caused a delay in cell cycle progression or a complete cell cycle arrest. The number of BrdU-positive cells did not increase following prolonged BrdU exposure (Supplementary Figure S1), thus suggesting that ouabain had caused complete growth arrest.

Cleaved caspase-3 was measured to verify that the observed inhibition of cell growth was not an effect of early apoptosis. These experiments demonstrated that 50 nM ouabain for 2–7 days failed to induce significant cleavage of caspase-3 in neuroblastoma cells (Figure 1C). However, when the ouabain concentration was increased 10-fold to 500 nM, substantial caspase-3 cleavage was observed (Figure 1C), similar to the response triggered by the positive control staurosporine. These data indicated that low doses of ouabain could induce growth arrest without promoting apoptosis.

Exit from the cell cycle can be irreversible, often caused by DNA damage, or reversible, as in non-dividing quiescent cells (Linke *et al*, 1996). Neuroblastoma cells were treated with ouabain for 7 days to determine whether arrested cells could remain in a non-proliferative state for an extended period of time. The vast majority of cells were negative for BrdU after 7 days with 50 nM ouabain (Figure 1D), indicating that cells had stopped proliferating. To determine whether cells were in the cell cycle, immunostaining for Ki-67 was conducted. Virtually all cells were negative for Ki-67 after 7 days of treatment with ouabain (Figure 1D), showing that the neuroblastoma cells had withdrawn from the cell cycle into the G0 phase. Thereafter ouabain was washed out and the cells were grown in ouabain-free culture medium. Two days later, on day 9, the majority of cells had efficiently resumed proliferation and re-entered the cell cycle, as shown by BrdU incorporation and Ki-67 staining (Figure 1D). This response was not unique to SH-SY5Y cells as a similar reversal of growth arrest was observed when the neuroblastoma cell lines Kelly and SK-N-AS were exposed to ouabain (Supplementary Figure S2). Increased expression of the *HES1* gene is required for quiescence to be reversible (Sang *et al*, 2008). Indeed, neuroblastoma cells treated with ouabain for 7 days had increased abundance of HES1 mRNA (Figure 1E). Together these results demonstrated that ouabain in low concentrations has an anti-proliferative effect capable of inducing quiescence in neuroblastoma cells.

Xenografted neuroblastoma

The effect of ouabain on tumour growth *in vivo* was investigated by xenografting neuroblastoma SH-SY5Y cells subcutaneously on the right/left rear flank of immune-deficient mice. Xenografted animals were treated orally with ouabain ($2\,mg\,kg^{-1}$) on a daily basis. Following this protocol, neuroblastoma growth measured as tumour volume (Figure 2A) was significantly reduced on day 5 and beyond, in animals receiving the treatment, as compared with untreated control animals. At the end of the experiment, on day 12,

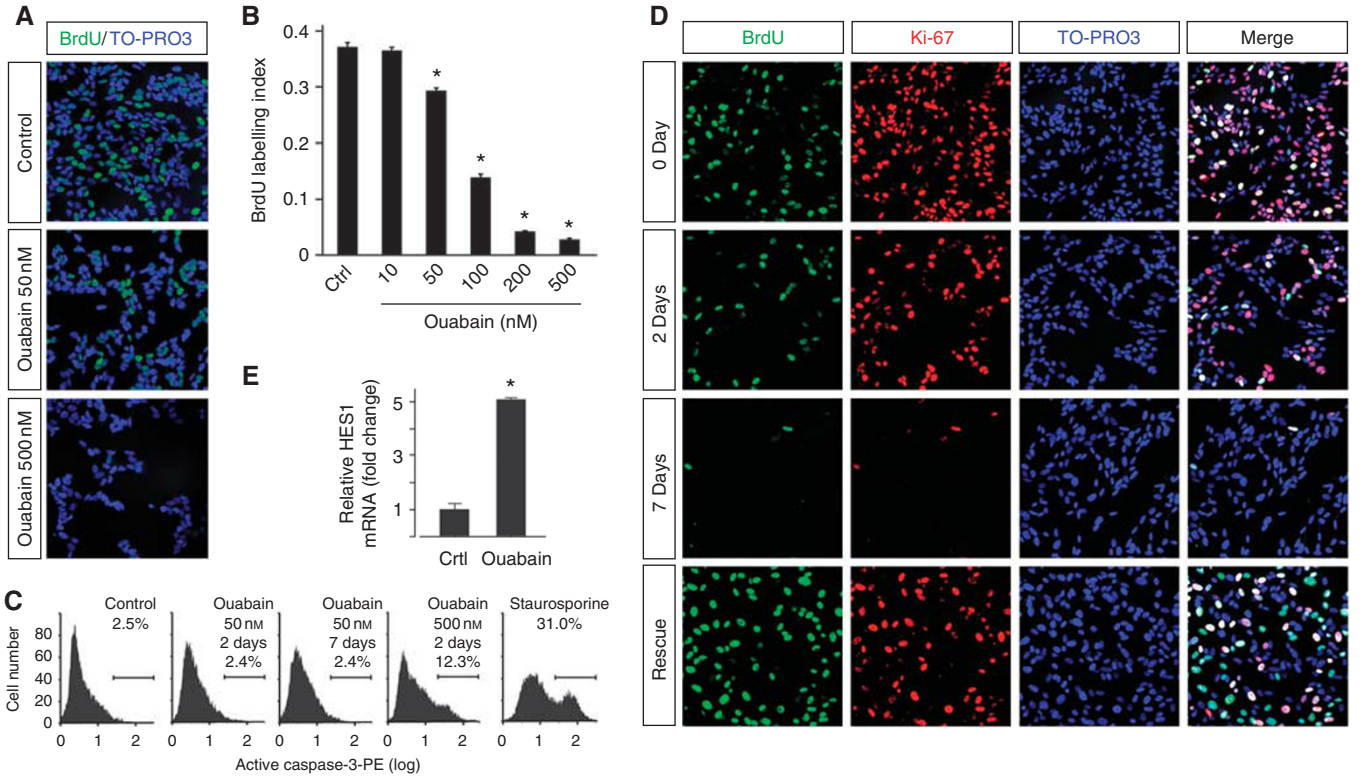

Figure 1 Ouabain induces a reversible growth arrest in neuroblastoma. (**A**) Confocal images of BrdU immunostained (green) SH-SY5Y cells treated with control, 50 or 500 nM ouabain for 2 days. Cells were pulsed with BrdU for 4 h and nuclei stained with TO-PRO-3 (blue). (**B**) Quantification of BrdU-positive cells treated with various concentrations of ouabain, as indicated. Pooled results from nine randomly selected fields-of-views from three cultures are shown. (**C**) Flow cytometric analysis of cells treated with 50 nM ouabain for 0, 2 or 7 days, or 500 nM ouabain for 2 days labelled with an antibody against active caspase-3. Cells treated with 1 μM staurosporine for 4 h were used as the positive control. (**D**) Immunostaining of BrdU (green) and Ki-67 (red) in cells treated with 50 nM ouabain for 0, 2 or 7 days. Rescued cells were treated with 50 nM ouabain for 7 days and without ouabain for 2 days. Cells were pulsed with BrdU for 6 h and nuclei stained with TO-PRO-3 (blue). (**E**) RT–PCR analysis of HES1 mRNA in cells treated with 50 nM ouabain for 7 days. *$P < 0.05$ versus control.

the tumour volume in animals receiving ouabain was significantly reduced, by 54%, compared with animals receiving no treatment ($P = 0.045$, Figure 2A). Additionally, tumour weight at autopsy on day 12 was significantly reduced in treated animals ($P = 0.019$, Figure 2B). The level of early apoptosis in tumour xenografts was analysed immunohistochemically on day 12. This analysis revealed a significant decrease of caspase-3 cleavage in treated animals ($P = 0.016$, Figure 2C). These data demonstrated that ouabain has anti-proliferative effects on tumour growth *in vivo*.

Biophysical effects

Ouabain ligand-binding to Na,K-ATPase has been shown to trigger signalling cascades both dependent on and independent of pump inhibition (Schoner and Scheiner-Bobis, 2007). To determine the pump inhibitory effect in this cell model system, ouabain at various concentrations was administered to neuroblastoma cultures and $^{86}Rb^+$-uptake was measured. $^{86}Rb^+$-uptake correlates with K^+-uptake, thus reflecting the turnover rate of the pump. This experiment showed that ouabain inhibits Na,K-ATPase in SH-SY5Y cells with an IC_{50} of 246 nM (95% CI) for ouabain (Figure 3A). An ouabain concentration of 50 nM used in subsequent experiments, inhibited active $^{86}Rb^+$ uptake by $12.7 \pm 4.4\%$ ($n = 4$). Resting membrane potential following ouabain treatment was next investigated using single cell patch clamp recordings (Figure 3B). These experiments revealed an insignificant decrease in the resting membrane potential (-35.8 ± 1.6 to -32.1 ± 1.5 mV, $n = 20$ for each group) in cells treated with ouabain for 2 days (Figure 3C). To analyse a larger population of cells ($n = 10\,000$), flow cytometry was carried out using a dye sensitive to membrane potential, $DiBAC_4(3)$. Neuroblastoma SH-SY5Y cells treated with ouabain for 1 h or 2 days displayed a continuous increase in $DiBAC_4(3)$ fluorescence intensity (Figure 3D), reflecting a decrease in membrane potential. Together these data show that low concentrations of ouabain only partially inhibit Na,K-ATPase, and this has a minor net effect on the cellular ion homeostasis.

Cell cycle phase

The cell cycle phase of ouabain-treated neuroblastoma cells was next examined. Flow cytometry analyses of propidium iodide-stained SH-SY5Y cells revealed that ouabain exposure for 2 days caused depletion of cells in G0/G1 (69 to 40%) and accumulation in S (15 to 20%) and G2/M (16 to 40%) (Figure 4A). These results together with the BrdU data suggest activation of the S-phase checkpoints in the DDR pathway. Five days later, on day 7, the majority of cells had entered into G0/G1 (82%), without showing DNA synthesis. Strikingly, very few cells were detected in the S-phase (9%). Eukaryotic cell cycle progression is dependent on regulated activities of cyclins and CDK complexes. Western blot analyses of cyclin A, B1, D3, and E, as well as CDK1, 2, and 4 showed reduced expression levels after 7 days of treatment with ouabain (Figure 4B).

The cyclin D3, CDK4 and 6 activities in the mid-late G1 phase control the G1 restriction point and activation of the cyclin E/CDK2 complex (Planas-Silva and Weinberg, 1997). Both these cyclin complexes are required for phosphorylating the tumour

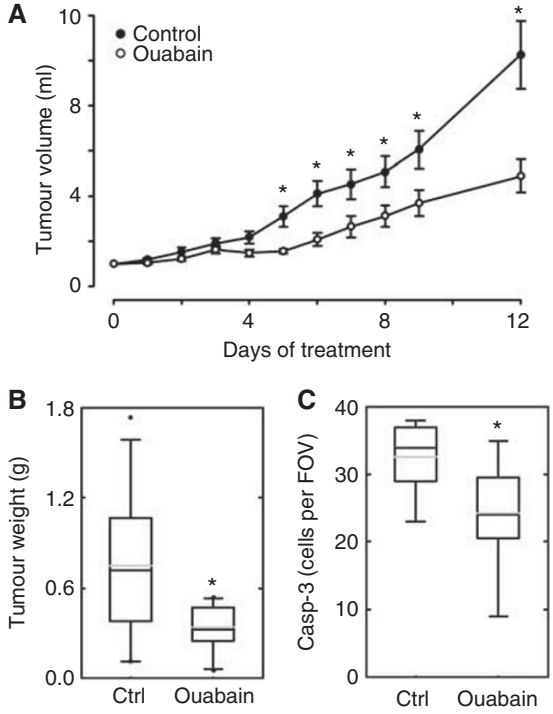

Figure 2 Ouabain inhibits the growth of neuroblastoma xenografts *in vivo* in immune-deficient mice. Xenografted neuroblastoma tumour volume (**A**) and weight (**B**) in NMRI *nu/nu* mice treated without (Ctrl) or with ouabain 2 mg kg^{-1} per day p.o. Tumour weight was measured after 13 days of treatment. (**C**) Caspase-3 (Casp-3) positive cells per field-of-view (FOV) in neuroblastoma xenografts from mice treated without (Ctrl) or with ouabain 2 mg kg^{-1} per day p.o., for 13 days. • outliers outside the 10th and 90th percentiles. *$P<0.05$ vs control.

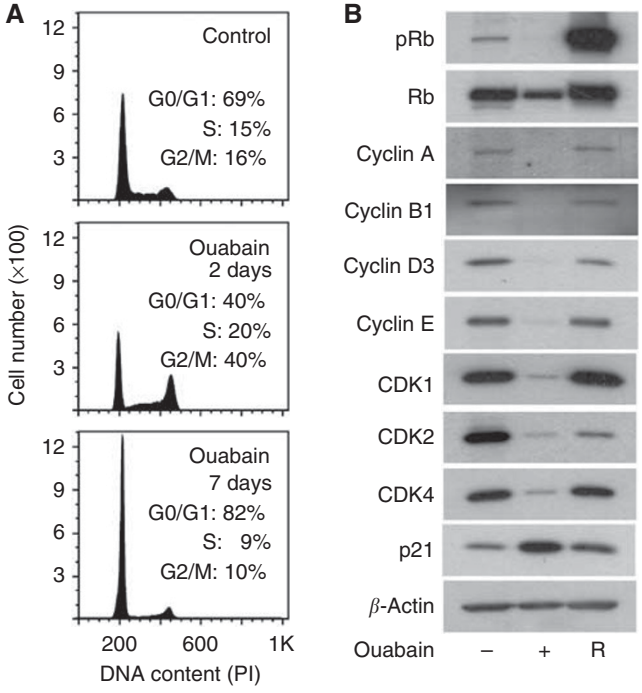

Figure 4 Ouabain arrests neuroblastoma cell cycle progression in the G1/G0 phase. (**A**) Flow cytometric recordings performed on SH-SY5Y cells treated without (Control) or with 50 nM ouabain for 2 days and 7 days stained with propidium iodide (PI). (**B**) Western blotting of cell cycle regulators pRb, Rb, cyclins A, B1, D3, E, CDK1, 2, 4, and p21 in cells treated with ouabain for 7 days. Rescued cells (R) were treated with ouabain for 7 days and without ouabain for 2 days. β-actin was used as a loading control.

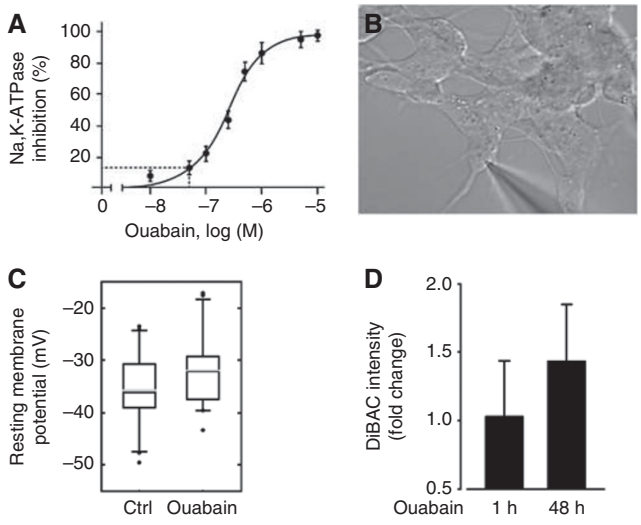

Figure 3 Pump activity and membrane polarisation in neuroblastoma treated with ouabain. (**A**) Na,K-ATPase (NKA) inhibition caused by various concentrations of ouabain-treated SH-SY5Y cells, measured as active $^{86}Rb^+$ transport. The IC_{50} for the reduction in $^{86}Rb^+$ transport was 246 nM ouabain ($n=4$). (**B**) Whole-cell patch-clamp recording of a SH-SY5Y cell. (**C**) Statistical analysis of whole-cell patch-clamp recordings with 50 nM ouabain for 2 days. (**D**) Statistical analysis of flow cytometric recordings of $DiBAC_4(3)$ loaded cells ($n=30\,000$, $N=3$) treated with 50 nM ouabain for 1 h or 2 days. • outliers outside the 10th and 90th percentiles.

suppressor protein Rb and for a commitment to replicate. Phosphorylated Rb (pRb) was attenuated in neuroblastoma cells exposed to ouabain for 7 days (Figure 4B). CDK inhibitors, such as the G0/G1 checkpoint regulator p21, are critical in enforcing long-term growth arrest, that is, quiescence or senescence (Cheng *et al*, 2000), as a response to, for example, replication stress. Immunoblot experiments demonstrated that neuroblastoma cells treated with ouabain had increased p21 (Figure 4B). The expression levels of Rb, cyclin A, B1, D3, and E as well as CDK1, 2, 4, and p21 were all rescued when ouabain was removed after 7 days (Figure 4B). In summary, these results, together with the BrdU and Ki-67 data, show that ouabain can activate a cellular programme that induces quiescence of neuroblastoma cells.

Signalling pathways

It has been previously reported that ouabain/Na,K-ATPase signal transduction elevates the cytosolic Ca^{2+} concentration to activate downstream cellular effectors (Miyakawa-Naito *et al*, 2003; Liu *et al*, 2004). The influence of Ca^{2+} signalling on ouabain-induced quiescence was therefore examined. Inhibiting L-type voltage-dependent Ca^{2+} channels with nifedipine or CaM kinases with KN93 failed to reverse the reduced BrdU incorporation caused by ouabain (Figures 5A and B). Altered expression levels of the cell cycle regulators cyclin D3, E, CDK1, 2, and 4 were partially affected by nifedipine and KN93 (Figure 5C). However, STO-609 or W-13, which block CaM kinase kinases and calmodulin, respectively, had no effect on the cell cycle regulators. Increased p21 and decreased pRb were unaffected by nifedipine, KN93, or STO-609. W-13 reduced the basal level of pRb. It has also been shown that ouabain impacts on the PI3K/Akt, Ras/Raf, MAPK and/or Src signalling cascades (Schoner and Scheiner-Bobis, 2007). The influence of

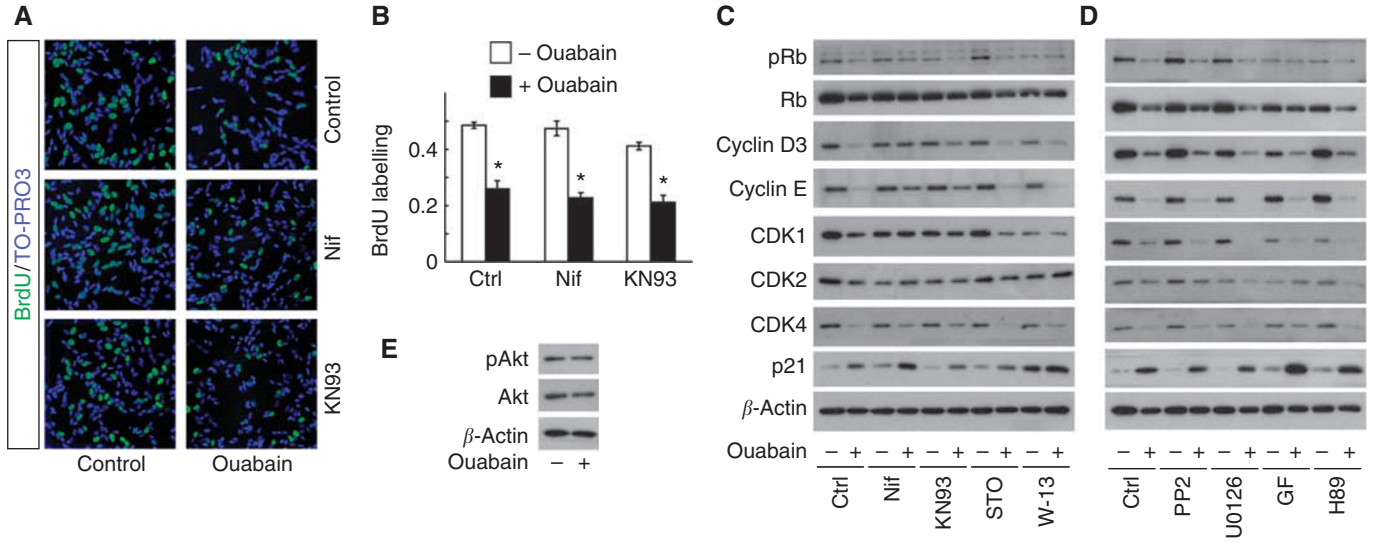

Figure 5 Cellular quiescence induced by ouabain in neuroblastoma is mediated by an alternative signalling pathway. (**A**) Immunostaining of BrdU (green) in SH-SY5Y cells treated with 50 nM ouabain together with L-type Ca^{2+} channel blocker nifedipine (Nif) or CaM kinase inhibitor KN93, respectively. (**B**) Quantification of BrdU-positive cells treated with 50 nM ouabain together with nifedipine or KN93, respectively. Pooled results from three randomly selected fields-of-view from three cultures are shown. (**C**) Effect on cell cycle regulators pRb, Rb, cyclins D3, E, CDK1, 2, 4, and p21 in cells treated with 50 nM ouabain for 2 days plus nifedipine, KN93, CaM kinase kinase inhibitor STO-609 (STO) or calmodulin inhibitor W-13, plus (**D**) Src inhibitor PP2, MEK/MAPK inhibitor U0126, PKC inhibitor GF109203X (GF), or PKA inhibitor H89. β-actin was used as a loading control. (**E**) Western blot of phosphorylated Akt (pAkt) and total Akt in cells treated with 50 nM ouabain for 2 days. $^*P<0.05$ *vs* control.

these signalling cascades on ouabain-induced cellular quiescence was next examined. Inhibiting Src with PP2, MEK/MAPK with U0126, or PKA with H89 had no effect on the altered expression levels of pRb, cyclins, CDKs, and p21 (Figure 5D). Protein kinase C blockade with GF109203X suppressed the basal level of pRb but was without effect on the other cell cycle regulators. Moreover, immunoblotting phosphorylated Akt revealed no increased activation by ouabain (Figure 5E). Inhibiting plasma membrane K^+ channels, Na^+/Ca^{2+}-exchangers or extracellular ATP signalling were likewise without effect (Supplementary Figure S3). These results suggest that the neuroblastoma quiescence induced by ouabain was activated by an, as yet, unreported signalling pathway.

Treating neuroblastoma SH-SY5Y cells with ouabain for 2 days caused accumulation in the late S-G2/M cell cycle phase, thereby suggesting activation of the DDR pathway. γH2AX is a marker for DDR pathway activity in response to replication stress and DNA damage. Flow cytometric recordings showed that γH2AX levels were rapidly increased (within 4–8 h) by ouabain (Figures 6A and B). This effect coincided with the onset of reduced BrdU incorporation (Figure 1), and therefore indicated a mechanistic connection. DNA tail comet assays revealed no overt DNA damage after 7 days of ouabain treatment (Figure 6C). In contrast, when cells were exposed to 10 μM doxyrubicin for 12 h significant DNA damage was observed. The signalling basis of ouabain-induced neuroblastoma quiescence was next investigated in an *in vivo* setting. Immunohistochemical analysis of SH-SY5Y xenograft tumours derived from mice fed daily with ouabain ($2\,mg\,kg^{-1}$) for 12 days showed augmented γH2AX activation as compared with tumours from untreated animals (Figures 6D and E). Statistical analysis showed that animals fed with ouabain had a significant increase in γH2AX (Figure 6F). Staining for the proliferation marker Ki-67 in xenografted tumours revealed that neuroblastoma cells exposed to ouabain had entered into the G0 phase (Figure 6G). Performing a statistical analysis showed a significant difference in Ki-67 staining between treated and untreated animals. In summary these data suggest that neuroblastoma quiescence *in vivo* and *in vitro* is caused by a similar signalling pathway.

DISCUSSION

The ouabain/Na,K-ATPase-complex has previously been reported to trigger signal transduction through Ca^{2+}, PI3K/Akt, Ras/Raf, MAPK, and/or Src (Schoner and Scheiner-Bobis, 2007). In human neuroblastoma cells 1–10 μM ouabain has been shown to down-regulate the anti-apoptotic proteins Bcl-2 and Bcl-XL in addition to trigger cytochrome c release and caspase-3 activation (Kulikov *et al*, 2007). The current study, however, demonstrates that neuroblastoma cells treated with 50 nM ouabain show growth arrest and tumour restraint that are regulated by a novel and heretofore unreported signalling pathway. The data demonstrate that ouabain, in a low concentration that only marginally inhibits Na,K-ATPase pump activity and membrane potential, stimulates the DDR pathway which activates γH2AX. This signalling event stimulates p21 which inhibits cyclins and CDKs, and results in dephosphorylation of Rb, which causes neuroblastoma cells to exit the cell cycle, as revealed by loss of Ki-67 expression. It has been shown that the expression of CDK inhibitors, such as p21, enforces a non-dividing senescence-like state (Sherr and Roberts, 1995; Sang *et al*, 2008). Ouabain has previously been shown to activate the mTOR pathway through p21 to slow down proliferation of human breast and prostate cancer cells (Tian *et al*, 2009). Our results, including elevated expression level of the quiescence-specific gene *HES1*, indicate that cells retain the ability to resume proliferation after extensive growth arrest. Thus, ouabain is inducing a quiescence-like state in neuroblastoma cells. The link between ouabain-binding to Na,K-ATPase and the subsequent genotoxic stress that activates DDR remains to be elucidated. It is plausible that the long-term ouabain exposure applied in the current study results in an accumulative low-level pump inhibition of Na,K-ATPase. The subsequent altered ion homeostasis can then cause replication stress that activates the DDR pathway,

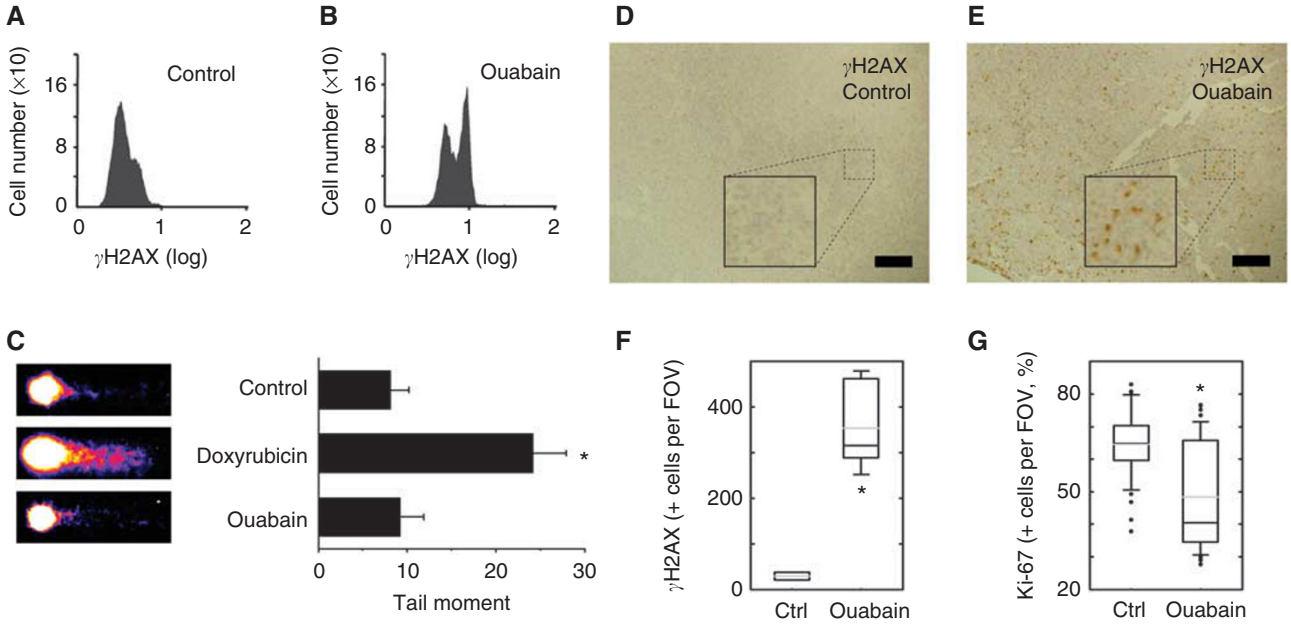

Figure 6 Xenografted neuroblastoma cells in immune-deficient mice show ouabain-mediated γH2AX activation. Flow cytometry analysis of γH2AX in SH-SY5Y cells treated with control (**A**) or 50 nM ouabain for 2 days (**B**). Comet assay for DNA damage in SH-SY5Y cell treated with ouabain for 7 days. Cells treated with 10 μM doxyrubicin for 1 h were used as the positive control. Immunostaining of γH2AX in SH-SY5Y tumours from NMRI *nu/nu* mice fed without (**D**) or with ouabain (**E**). (**F**) Statistical analysis of γH2AX phosphorylated cells per field-of-view (FOV) in xenografted cells treated without (Ctrl) or with ouabain. (**G**) Statistical analysis of Ki-67 positive cells per FOV in xenografted cells treated without (Ctrl) or with ouabain. Animals were treated with 2 mg kg^{-1} per day ouabain p.o. for 13 days. Scale bars are 200 μm. Boxed areas are zoomed fields. *$P<0.05$ *vs* control.

as previously reported for GABA (Andang *et al*, 2008). Another plausible scenario is that a pump-independent mechanism, in which Na,K-ATPase acts as a receptor and signal transducer, is triggering DDR. Further studies are required to determine each step in the signalling cascade by which ouabain is inducing quiescence in neuroblastoma cells.

As is ubiquitously expressed, we speculate that endogenous ouabain has a developmental role in modulating cell cycle progression. Such a universal cell signalling mechanism could regulate cell growth in general and explain the elevated circulating levels of ouabain and ouabain-like factors during pregnancy and in newborn infants (Schoner and Scheiner-Bobis, 2007; Bagrov *et al*, 2009). Ouabain is synthesised in the brain and adrenal glands (Schoner and Scheiner-Bobis, 2007), the environment where most neuroblastoma tumours reside (Maris, 2010; Park *et al*, 2010). It is conceivable that endogenous ouabain has a role in the spontaneous regression of neuroblastoma, thought to be modulated by the DDR pathway (Brodeur, 2003). The concentration of endogenous ouabain in the developing embryonic human nervous system (with or without neuroblastoma) is unknown, but is predicted to be within the subnanomolar-to-nanomolar range (Schoner and Scheiner-Bobis, 2007; Bagrov *et al*, 2009). Furthermore, in the nervous system, there are multiple Na,K-ATPase α-subunit isoforms that each have cell-type-specific and developmental-specific expression patterns (Wetzel *et al*, 1999; Richards *et al*, 2007), as well as different ouabain affinities (Kim *et al*, 2007; Richards *et al*, 2007). These spatial and temporal expression patterns of various Na,K-ATPase α-subunit isoforms remain largely unknown but may have an important role during development and in mediating ouabain-induced signalling. Indeed, ouabain has previously been shown to stimulate dendritic growth in cortical neurons (Desfrere *et al*, 2009). Perturbed ouabain/Na,K-ATPase signal transduction could therefore be an inducing factor of neuroblastoma in children.

The major drawback of cancer chemotherapy is systemic toxicity and drug resistance. This has led to extensive research towards reducing unwanted side effects and increasing the actual drug activity (Tyagi *et al*, 2002). To meet these demands, combination chemotherapies using compounds with known mechanisms of action that increase the therapeutic index of the clinical anticancer drug have received growing attention (Millikan *et al*, 2001). We speculate that ouabain, which arrests proliferating neuroblastoma cells first in S-G2/M and then in G0, in combination with other chemotherapy, could improve chemosensitivity for more efficient tumour eradication. Such alternative entry points into the cell cycle constitute an interesting target for therapeutic interventions. Supporting the hypothesis that ouabain could act as a potent combination drug are previous reports of elevated DDR pathway activity leading to reduced proliferation and chemoresistance in cancer cells (Bartkova *et al*, 2005; Bao *et al*, 2006). Attenuating the protective function of the DDR pathway may cause irreversible DNA damage to ouabain-treated cancer cells.

This study demonstrates that the endogenous cardiac glycoside ouabain can induce quiescence in neuroblastoma cancer cells. Xenografting neuroblastoma into immune-deficient mice revealed that the ouabain/Na,K-ATPase-complex suppresses tumour growth *in vivo*. Ouabain-arrested cells showed activation of γH2AX and upregulation of the quiescence-specific gene *HES1*. Upon removal of ouabain, cells resumed proliferation and reversed the levels of p21, cyclins, CDKs, and pRb, without showing overt DNA damage. These results reveal a novel function of ouabain/Na,K-ATPase as a putative tumour suppressor inducing quiescence in malignant neuroblastoma.

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