

## Glucocorticoids Augment Survival and Proliferation of Tumor Cells

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**Abstract.** *Background:* Glucocorticoids are widely used for cancer patients, although they can reduce the efficacy of anticancer treatment. *Materials and Methods:* We characterized non-apoptotic actions of glucocorticoids on tumor cell lines, primary tumor cells and an *in vivo* model, together with molecular signaling studies. *Results:* Glucocorticoids enhanced cell proliferation in 9/17 cell lines and significantly promoted tumor cell proliferation in a preclinical mouse model of lung carcinoma. 65/139 primary acute childhood leukemia samples were glucocorticoid resistant. Both dexamethasone and prednisolone increased *in vitro* survival in 21/65 samples from glucocorticoid resistant primary leukemia revealing a completely new action of glucocorticoids. Dexamethasone-induced proliferation was mediated by glucocorticoid receptor and activated the proliferation signaling pathways of protein kinase B/AKT and p38 mitogen-activated protein kinase. *Conclusion:* Our data suggest that restriction of the use of glucocorticoids during anticancer treatment might improve the outcome of patients with solid tumors.

Glucocorticoids (GCs) such as dexamethasone and prednisone/prednisolone are widely applied in cancer patients during antitumor therapy. In leukaemia and lymphoma, GCs are used as anticancer drugs due to their antitumor effect in hematopoietic malignancies (1, 2). In

contrast, solid tumors are completely resistant to GC-induced apoptosis and thus GCs are given to alleviate adverse effects (3-5).

The adjuvant use of GCs during therapy of solid tumors is under debate, since GCs have been shown to undermine the efficacy of chemotherapy both *in vitro* as well as in certain clinical trials of solid tumors (2, 6-8). As early as the 1950s, GCs were shown to promote metastasis formation in solid tumors (9, 10).

In normal cells, such as fibroblasts and erythroid progenitor cells, GCs were shown to induce proliferation (11, 12). In contrast, only few studies exist on the growth-promoting off-target effects of GCs on tumor cells; these studies are restricted to single tumor entities, including astrocytoma (13), glioma (14) and Kaposi's sarcoma (15), and lack signaling data.

As numerous patients with cancer receive GCs during their treatment and as the safe use of GCs represents an important clinical issue during anticancer therapy, we aimed here to characterize the anti-apoptotic and pro-proliferative phenotypes induced by GCs across different tumor cell lines and primary tumor cells, together with the respective intracellular signaling mechanisms.

### Materials and Methods

*Ethics statement.* The study was approved by written consent by Ethikkommission des Klinikums der Universität of Ludwig Maximilians University, Munich (068-08) and was carried out according to the Declaration of Helsinki.

*Cell cultures and treatments.* A498, A549, CADO, HeLa, KELLY, LNCaP, MCF-7 and RH-30 cells were freshly obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany); A172, CALU-6, MDA-MB-231, U118, U373 and U2OS cells were freshly obtained from the American Type

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*Key Words:* Tumor cell proliferation, glucocorticoids, glucocorticoid receptor, p38MAPK, AKT.

Culture Collection (ATCC; Manassas, VA, USA). Cell lines were cultured fewer than six months after receipt from the Collections. SHEP cells were kindly provided by S. Fulda (University Children's Hospital, Ulm, Germany) and RD and RMS-13 cells by R. Kappler (Ludwig Maximilians University, Munich, Germany). Cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium or Dulbecco's Modified Eagle Medium (DMEM) High Glucose (Gibco, Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Pan Biotech GmbH, Aidenbach, Germany) and 2 mM glutamine (Gibco). Primary leukemia blasts were obtained from children (n=139) treated for acute leukemia at the Ludwig Maximilians University's children's hospitals during 2002 to 2009. Samples were isolated using Ficoll Isopaque (GE Healthcare, München, Germany). Samples were used which contained at least 90% leukemia cells by morphology and immunphenotyping. Primary cells were cultured in RPMI-1640 medium (Gibco) supplemented with 20% FCS (Pan Biotech GmbH), 1 mM sodium pyruvate, 50  $\mu$ M  $\alpha$ -thioglycerol (both Sigma), 2 mM glutamine, 6 mg/l insulin, 3 mg/l transferrin, 4  $\mu$ g/l selenium (ITS), 1% penicillin-streptomycin (all from Gibco) and 1% gentamycin (Biochrom AG, Berlin, Germany). Dexamethasone, prednisolone, RU486 (all from Sigma), paclitaxel, protein kinase B (AKT) Inhibitor VIII, SB202190, SB203580, p38 mitogen-activated protein kinase (MAPK) Inhibitor (all from Merck KGaA, Darmstadt, Germany) were used. For all experiments, cells were seeded in a range from  $5\text{-}8 \times 10^4$  cells/well in 96-well plates, except for western blots, where the cells were seeded at  $10^6$  cells/well in 6-well plates. Experiments were performed under starvation conditions without FCS, except for RH-30 cells where 10% FCS was used. Control cultures were treated with phosphate buffered saline (PBS), except for experiments with dimethylsulfoxid (DMSO)-soluble substances where DMSO was used. Dexamethasone was used at  $10^{-5}$  M, if not indicated differently, in order to be below reported peak plasma concentrations in humans ( $2 \times 10^{-5}$  M) (16).

For western blots, the following primary antibodies were used: anti-glyceraldehyd-3-phosphat-dehydrogenase (GAPDH) (Thermo Fisher Scientific, Bonn, Germany); anti-glucocorticoid receptor (N3RC1) (BD Biosciences); anti-p-AKT, anti-AKT, anti-p-N3RC1, anti-p-glycogen synthase kinase 3 (GSK3), anti-GSK3, anti-p-heat shock protein (Hsp27), anti-Hsp27, anti-p-p38-MAPK and anti-p38-MAPK (all from New England Biolabs GmbH, Frankfurt, Germany).

*Measurement of tumor cell growth, proliferation and apoptosis.* Cellscreen (Innovatis AG, Bielefeld, Germany) was used according to the manufacturer's instructions to perform time-lapse microscopy repeatedly. The proliferation rate of dexamethasone treated cells was calculated as [(density of dexamethasone treated cells after 24 hours divided by density of dexamethasone treated cells at start) divided by (density of control cells after 24 hours divided by density of control cells at start) multiplied by 100]. Cell lines were positive for GC-induced proliferation (YES), if at least 3/5 experiments showed an increase in proliferation by dexamethasone treatment to at least 120% that of control cells; otherwise, the cell line was classified as no. The *xCELLigence* System (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions and measured changes in the electrical impedance. A dimensionless parameter termed Cell Index (CI) was derived as a relative change in measured electrical impedance to represent cell status and was calculated using the RTCA software 1.1 (Roche). Cell Proliferation

ELISA BrdU (Roche) was performed according to the manufacturer's instructions; bromdesoxyuridin (BrdU) was added for 4 hours. For determination of absolute cell numbers, BD TruCount Tubes (BD Biosciences, Heidelberg, Germany) and CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) to measure cellular ATP content were used according to the manufacturer's instructions. Apoptosis was measured by propidium iodide staining of fragmented DNA for tumor cell lines or forward side scatter analysis for primary leukemia cells in FACSscan (BD Biosciences) as published elsewhere (17). Patient-derived leukemia cells were harvested after 24 hours, if spontaneous apoptosis of untreated cells had reached 25% or more; otherwise cells were harvested after 48 hours. Specific apoptosis was calculated as [(apoptosis of treated cells) minus (apoptosis of untreated cells)] divided by [100 minus (apoptosis of untreated cells)] multiplied by 100}.

*Western blot analysis.* Western blots were performed as described elsewhere (18). Quantification was performed using the AIDA Image Analyzer software (Raytest GmbH, Straubenhardt, Germany). One representative experiment out of at least three independent experiments is presented; GAPDH served as loading control.

*RNA interference.* To knockdown N3RC1, a N3RC1 specific siRNA was used (siRNA ID #s6187; Applied Biosystems, Darmstadt, Germany). Cells were transfected with siRNA at a final concentration of 25 nM using Lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. In the case of RH-30, transfection was repeated after 24 hours. AllStars negative siRNA AF 488 (Qiagen, Düsseldorf, Germany) was used as control sequence.

*Quantitative real time polymerase chain reaction (RT-PCR).* Total RNA from cell lines was isolated using the QIAGEN RNeasy Mini Kit (Qiagen) and cDNA was generated using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR was performed using the Universal ProbeLibrary (UPL) and LightCycler 480 Real-Time PCR System (all from Roche). The ProbeFinder software selected an optimal combination of a UPL probe (probe #62 for *hN3RC1* and #73 for *hHPR1*) and a gene-specific primer set. The primer pairs used for the amplification of target mRNAs were: N3RC1: forward: 5'-GGGTGG AGATCATATAGACAATCAA-3', reverse: 5'-ACATGCAGGGTA GAG TCATTCTC-3'; human hyperparathyroidism 1 (hHPR1): forward: 5'-TGACCTTGATTTATTTGCA TACC-3', reverse: 5'-CGAGCAAGACGTTTCAGTCCT-3' (MWG Biotech AG, Ebersberg, Germany). Basic relative quantification of N3RC1 with HRPT1 as reference gene was carried out using LightCycler 480 software 1.5.0 (Roche) with the  $\Delta\Delta C_T$  method. Expression of control siRNA-treated samples were set to a value of 1 and normalized relative ratios are depicted in the graphs.

*Animal trial.* Animals were treated under the actual institutional guidelines as approved by the Regierung von Baden-Württemberg (35-9185.81/6-119/02). CALU-6 cells ( $5 \times 10^6$ /animal) were suspended in 200  $\mu$ l PBS and injected *s.c.* into the right flank of 8 to 10 week-old Swiss *nu/nu* mice (Charles River Laboratories, Wilmington, MA, USA). Dexamethasone was dissolved in ethanol at a concentration of 1 mg/ml. It was further diluted with 0.1% bovine serum albumin (BSA) to a final concentration of 5  $\mu$ g/ml.

Animals were treated *s.c.* with 0.2 ml/20 g body weight (corresponding to 50 µg/kg/day) on days 0-4, 7-11 and 14-18. The group size for dexamethasone treatment was eight animals, and for the placebo (PBS with 0,1 % BSA und 0,5 % ethanol) group nine animals. Tumor size was determined with calipers on day 4, 11, 14, 18, 21 and 25 and mean values±standard error are indicated. All animal trials were approved by the Bavarian and Baden-Württemberg Federal Government and animal care was in accordance with institution guidelines.

**Statistical analysis.** For all cell line experiments, data are presented as the mean±SEM of at least three independent experiments. When more than two groups were compared, one way RM ANOVA was used. Whenever a difference was detected by ANOVA, or for comparison of two groups, paired Student two-tailed *t*-test was applied to isolate statistical differences. The animal trial was evaluated using the two-sample equal variance, two-tailed Student *t*-test. Significance was accepted at  $p < 0.05$  and is indicated in the figures by an asterix.

## Results

GCs are under suspicion of exerting anti-apoptotic and pro-proliferative effects on solid tumor cells (2, 8, 19). In the present study, we aimed to characterize non-apoptotic effects of GCs on different tumor cells, including primary, patient-derived leukemia cells, and carry out signaling studies.

Firstly, we studied cell lines from solid tumors which all displayed known resistance to GC-induced apoptosis (data not shown). In accordance with published results (6, 20), dexamethasone reduced apoptosis induction by paclitaxel in the mammary carcinoma cell line MDA-MB-231 in our hands (Figure 1A).

To our surprise, and beyond the known inhibition of apoptosis, microscopy images suggested a higher number of living tumor cells in wells treated with dexamethasone compared to controls (Figure 1A, lower left compared to upper left). Indeed, dexamethasone significantly increased the density of living MDA-MB-231 cells and other cell lines as measured using time-lapse microscopy followed by computer-based analysis (Figure 1B), and using electrical impedance (data not shown).

**Dexamethasone enhances tumor cell proliferation both *in vitro* and *in vivo*.** To quantify dexamethasone induced tumor cell proliferation, we screened a panel of 17 tumor cell lines of different origins, including epithelial, neuroectodermal and mesenchymal cells. Dexamethasone enhanced tumor cell proliferation in nine out of 17 cell lines from solid tumors, notably in 4 out of 5 neuroectodermal tumor cell lines (Figure 1, Table I). When the proliferation rate of untreated cells was set to 100%, dexamethasone augmented the proliferation rate on average to 140% in these nine cell lines. Thus, dexamethasone increased tumor cell proliferation in numerous cell lines derived from solid tumors from all three different germ layers.

To verify that stimulation with dexamethasone led to an increased number of living tumor cells, we determined absolute cell densities using a FACS-based technique and calibration beads. Here, dexamethasone induced a significant increase of tumor cell numbers (Figure 2A, left). Additionally, dexamethasone augmented BrdU incorporation corresponding to increased DNA production (Figure 2A, right). These data confirm that dexamethasone induced the formation of new tumor cells and thus tumor cell proliferation.

Dexamethasone enhanced proliferation over a wide range of different concentrations covering several orders of magnitude (data not shown). The pro-proliferative effect remained constant over prolonged periods of time *in vitro* (data not shown). In line with this, dexamethasone enhanced the metabolic cell activity as measured by increased ATP consumption. When a second GC, prednisolone, was tested on cell lines, it induced proliferation in the same tumor cell lines as dexamethasone did (data not shown).

For the *in vivo* trial, we chose a lung carcinoma cell line as GCs were shown to impair therapy outcome in patients with lung cancer (7). Most importantly, dexamethasone markedly enhanced the size of subcutaneous tumors grown in nude mice (Figure 2B) confirming the proliferation-promoting effect of dexamethasone *in vivo*. These data further prove that dexamethasone induced proliferation is continuous over a long period of time.

Taken together, these data show dexamethasone induced proliferation in cell lines from different solid tumor types *in vitro* and in a preclinical lung carcinoma model *in vivo*.

**Dexamethasone-induced proliferation is mediated by the glucocorticoid receptor.** Next, we searched for intracellular signaling molecules mediating the pro-survival and pro-proliferative effects of dexamethasone. For these studies, we used both the epithelial mammary carcinoma cell line MCF-7 (Figures 3 and 4) and the mesenchymal rhabdomyosarcoma cell line RH-30 (data not shown) as two unrelated cell lines of different origins.

For intracellular signalling, GCs can activate the N3RC1, but GCs can also signal independently of the N3RC1 (21). We found rapid and prolonged phosphorylation of the N3RC1 on Ser<sup>211</sup> as a marker for nuclear activation of the N3RC1 (Figures 3A, data not shown). Dexamethasone activated the N3RC1 over a concentration range of several orders of magnitude (Figure 3B). Phosphorylation of N3RC1 was followed by rapid degradation of the N3RC1 protein.

Addition of the N3RC1 antagonist RU486 completely abrogated dexamethasone induced proliferation (Figure 3C). Transfection of siRNA directed against N3RC1 mRNA led to a significant decrease of both N3RC1 mRNA and protein levels (Figure 3D). In the absence of N3RC1, dexamethasone was unable to increase cell proliferation (Figure 3E), proving

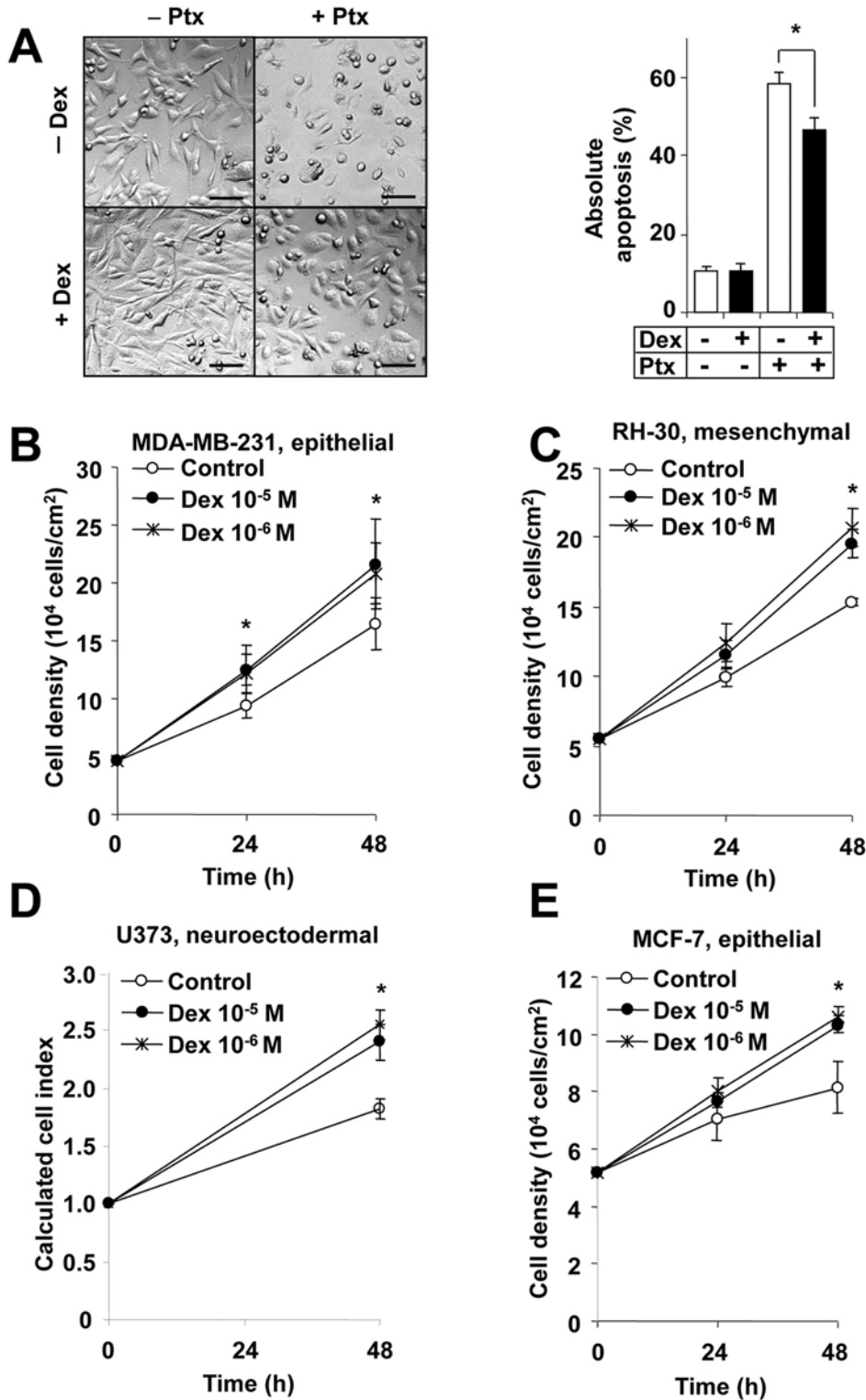


Figure 1. Dexamethasone (Dex) inhibits Paclitaxel (Ptx)-induced apoptosis of MDA-MB-231 cells and induces tumor cell growth. A: MDA-MB-231 cells were treated with or without Dex ( $10^{-5}$  M) for 1 hour followed by paclitaxel ( $10^{-6}$  M) for another 96 hours. Representative light microscopy images were taken after 48 hours (left). Scale bars 0.1 mm. Apoptosis was measured by FACScan after 96 hours by propidium iodide staining of fragmented DNA (right). The means of four independent experiments  $\pm$  standard error are shown; \* $p < 0.01$  using paired t-test. B-E: Tumor cell lines of different origins were treated with Dex ( $10^{-5}$  M,  $10^{-6}$  M) for the time periods indicated and tumor cell growth was measured using time-lapse microscopy. The means of at least three independent experiments  $\pm$  standard error are shown; \* $p < 0.05$  using paired t-test.

Table I. The pro-proliferative effects of dexamethasone (Dex) on cell lines from different types of solid tumor.

Name	Tumor type	Origin	GC-induced proliferation
CALU-6	Lung carcinoma	Epithelial	Yes
MCF-7	Mammary carcinoma	Epithelial	Yes
MDA-MB-231	Mammary carcinoma	Epithelial	Yes
A498	Renal cell carcinoma	Epithelial	No
A549	Lung carcinoma	Epithelial	No
HELA	Cervical carcinoma	Epithelial	No
LNCaP	Prostatic carcinoma	Epithelial	No
CADO	Ewing's sarcoma	Mesenchymal	Yes
RH30	Rhabdomyosarcoma	Mesenchymal	Yes
U2OS	Osteosarcoma	Mesenchymal	No
RMS-13	Rhabdomyosarcoma	Mesenchymal	No
RD	Rhabdomyosarcoma	Mesenchymal	No
A172	Glioblastoma	Neuroectodermal	Yes
SHEP	Neuroblastoma	Neuroectodermal	Yes
U118	Glioblastoma	Neuroectodermal	Yes
U373	Glioblastoma-astrocytoma	Neuroectodermal	Yes
KELLY	Neuroblastoma	Neuroectodermal	No

The 17 cell lines of different tumor type and origin were screened for the effect induced by Dex ( $10^{-5}$  M,  $10^{-6}$  M). Tumor cell growth was measured by repetitive microscopy and impedance analysis for 48h. Cell lines were classified as 'Yes' or 'No' for the presence of glucocorticoid-induced proliferation according to the criteria described in the Materials and Methods. Representative original data are shown in Figure 1B.

that dexamethasone induced proliferation is mediated by the N3RC1. In line with these data, LNCaP cells, which do not express the N3RC1 (22), did not exhibit dexamethasone induced tumor cell proliferation (Table I). In sum, these data suggest that the N3RC1 mediates dexamethasone induced tumor cell proliferation.

*Dexamethasone activates AKT and p38-MAPK.* In normal cells, dexamethasone was shown to activate proliferation signaling pathways such as protein kinase B/AKT and p38-MAPK cascade (23-25). Accordingly, dexamethasone induced phosphorylation of protein kinase B/AKT in MCF-7 cells, followed by phosphorylation of its downstream target GSK3. Similarly, p38-MAPK was phosphorylated, as was its downstream target HSP27 (Figure 4A). Nuclear factor kappa B (NF- $\kappa$ B) and extracellular signal-regulated kinases (Erk) 1/2 were not activated (data not shown). In contrast to phosphorylation of N3RC1, activation of these proteins was delayed and started 2-4 hours after stimulation suggesting indirect signaling.

To inhibit AKT or p38-MAPK, we used kinase inhibitors similar to those which are currently being investigated in early clinical studies. The applied AKT inhibitors are reported to be pleckstrin homology (PH) domain-dependent and to block stimulated phosphorylation and activation of AKT(26). p38-MAPK inhibitors used all bind to the ATP pocket of the kinase itself and block phosphorylation and activation of downstream signaling molecules.

When activity of either AKT or p38-MAPK was reduced by these inhibitors, proliferation by dexamethasone was abrogated (Figure 4B and data not shown), suggesting an important role of both AKT and p38-MAPK for dexamethasone induced proliferation in MCF-7 cells. These data show that dexamethasone induced proliferation can be inhibited by direct interference with survival and proliferation signaling pathways activated by dexamethasone.

*Dexamethasone enhances survival of primary, patient-derived leukemia cells.* Next, we extended our studies to hematopoietic tumor cells. To exclude cell line artifacts caused by prolonged culture of cell lines *in vitro*, we used fresh tumor cells from children with acute leukemia.

A total of 139 primary leukemia samples were obtained from diagnostic bone marrow puncture of children with newly diagnosed acute leukemia or with relapse before onset of treatment and cells were stimulated *in vitro* directly after isolation. In contrast to cells from solid tumors and in accordance with the important role of glucocorticoids during anti-leukemia therapy, the majority of primary leukemia cells underwent apoptosis upon stimulation with dexamethasone, and with prednisolone (53% and 58%, respectively; shown as negative survival in Figure 5A, and data not shown). In apoptosis-sensitive primary leukemia cells, we did not detect further anti-apoptotic effects of GCs.

Nevertheless, and in accordance with published results (27), 65/139 (47%) primary samples were resistant towards

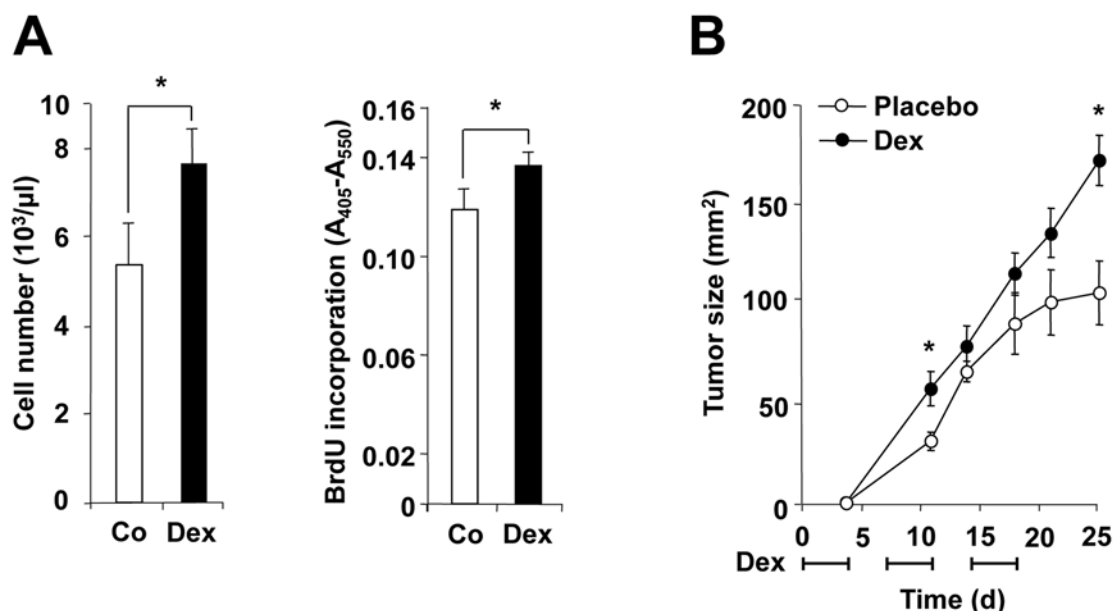


Figure 2. Dexamethasone (Dex) enhances tumor cell growth both *in vitro* and *in vivo*. A: CALU-6 cells were treated with Dex ( $10^{-5}$  M) for 24 hours. Absolute cell count was determined by FACScan using TruCount Tubes (left); DNA synthesis was measured by BrdU incorporation (right). The means of five independent experiments  $\pm$  standard error are shown; \* $p < 0.05$  using paired *t*-test. B: CALU-6 cells ( $5 \times 10^6$ /animal) were injected *s.c.* into 8 to 10-week-old Swiss nu/nu mice. Animals were treated *s.c.*, as indicated and described in the Materials and Methods, on days 0-4, 7-11 and 14-18 with dexamethasone as 50  $\mu\text{g}/\text{kg}/\text{day}$ . Tumor sizes were measured with calipers. Mean data  $\pm$  standard error of one representative of two independent experiments are shown; \* $p < 0.01$  using two-sample equal variance *t*-test. Co: Control.

GC-induced apoptosis and we further studied these 65 GC-resistant primary leukemia samples. Dexamethasone and prednisolone reduced spontaneous apoptosis in 21/65 (32%) GC-induced apoptosis-resistant primary leukemia cells, suggesting an anti-apoptotic, pro-survival effect of GCs on at least a fraction of primary acute leukemia cells. The *in vitro* effects of dexamethasone and prednisolone correlated strongly (Figure 5B). The effect on samples obtained at initial diagnosis did not differ from those of samples obtained at relapse (data not shown). Taken together, these data show also GCs enhanced tumor cell survival also in primary, patient-derived leukemia cells. These data describe increased survival as a completely new action of GCs on primary leukemia cells.

Out of the 65 GC-resistant samples, a subset of 21 primary samples exhibited spontaneous proliferation *in vitro* and was analyzed further. A single sample exhibited an extraordinary phenotype upon stimulation with dexamethasone: dexamethasone-induced proliferation. The sample was obtained from a 4.5-year-old girl at first diagnosis of a common acute lymphoblastoid leukemia (pre-B-ALL) who received dexamethasone within induction therapy. Measurement of apoptosis by forward side scatter analysis in FACScan showed 14% of cells surviving under control conditions, due to high spontaneous apoptosis *in vitro*, while 40% and 41% of cells survived on treatment with

dexamethasone and prednisolone, respectively, revealing a significant anti-apoptotic effect of GCs on primary tumor cells (Figure 5C). While primary leukemia cells exhibited almost no *in vitro* proliferation under control conditions, treatment with dexamethasone led to the formation of numerous huge colonies *in vitro* (Figure 5D, left). Colony formation was accompanied by markedly increased ATP consumption (Figure 5D, right). Thus in this primary ALL tumor sample, dexamethasone induced both survival and proliferation.

Taken together, our data show GC-induced proliferation of numerous solid tumor cell lines and GC-induced survival of primary leukemia cells. Thus, anti-apoptotic off-target actions represent a widely spread phenotype induced by GCs across different tumor cell entities.

## Discussion

Our data represent the first evaluation of GC-induced survival and/or proliferation across a significant number of different tumor cell types. We show an unexpectedly high frequency of this tumor-directed adverse effect of GCs by using (i) various different and highly precise techniques to measure cell proliferation *in vitro*; (ii) both *in vitro* and *in vivo* experiments; (iii) cell lines as well as primary tumor cells from patients; and (iv) tumor cells of different origin, including both hematopoietic and cells from solid tumors. As

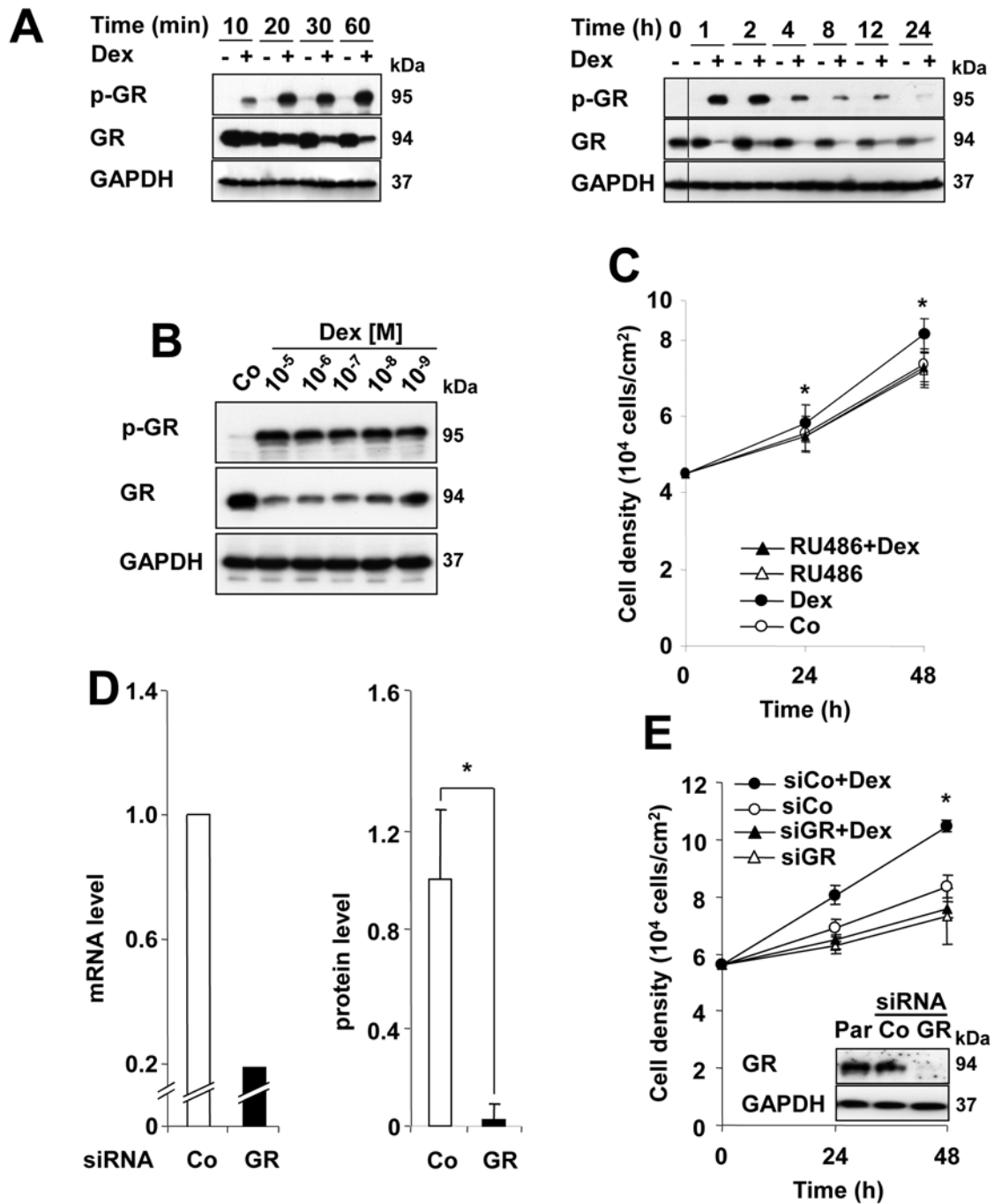
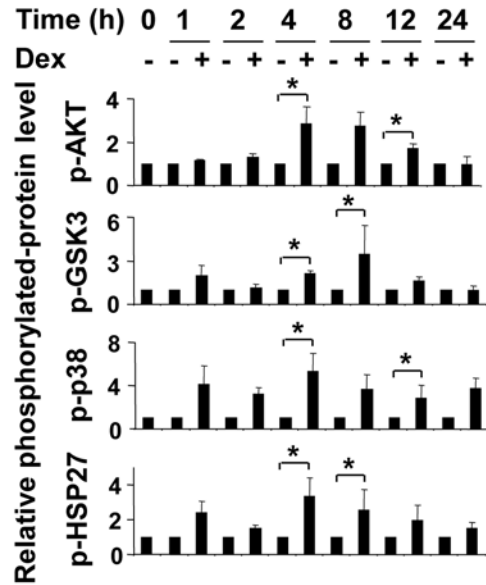
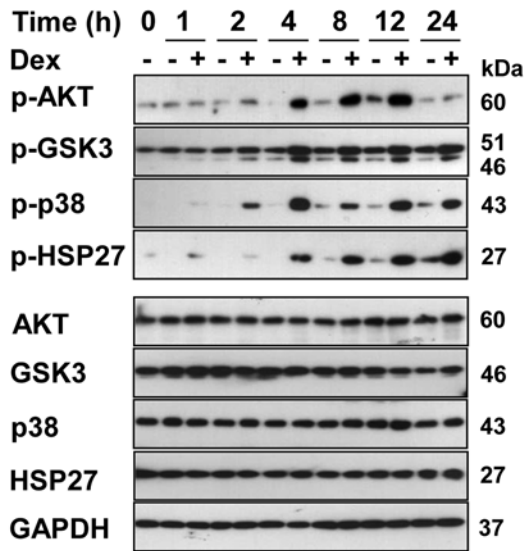


Figure 3. Dexamethasone (Dex)-induced growth is mediated by the glucocorticoid receptor (N3RC1). A: MCF-7 cells were treated with Dex ( $10^{-5}$  M) for the time periods indicated and western blot was performed of total cellular protein. B: MCF-7 cells were treated with Dex at the indicated concentrations for 4 hours and western blot of total cellular protein was performed. C: MCF-7 cells were treated with or without the N3RC1 antagonist RU486 ( $10^{-6}$  M) for 1 hour followed by Dex ( $10^{-6}$  M) for another 48 hours. Tumor cell growth was measured using time-lapse microscopy. The means of three independent experiments  $\pm$  standard error are shown; \* $p < 0.05$  using paired t-test. D: MCF-7 cells were treated with control or N3RC1-specific siRNA as described in the Materials and Methods. Knock-down efficiency was determined either by quantitative real time polymerase chain reaction (qRT-PCR) after 12 hours using HRPT1 for normalization of relative quantification (left;  $n=1$ ) or by western blotting followed by quantification after 24 hours. The means of three independent experiments  $\pm$  standard error are shown for the western blot analysis; \* $p < 0.05$  using paired t-test (right). E: MCF-7 cells were treated with control or N3RC1-specific siRNA as described in the Materials and Methods and treated with Dex ( $10^{-5}$  M) 12 hours after transient transfection for the time periods indicated. Tumor cell growth was measured using time-lapse microscopy together with western blot performed after 24 hours. The means of three independent experiments  $\pm$  standard error are shown; \* $p < 0.05$  using paired t-test. Co: Control; Par: Parental.

**A**



**B**

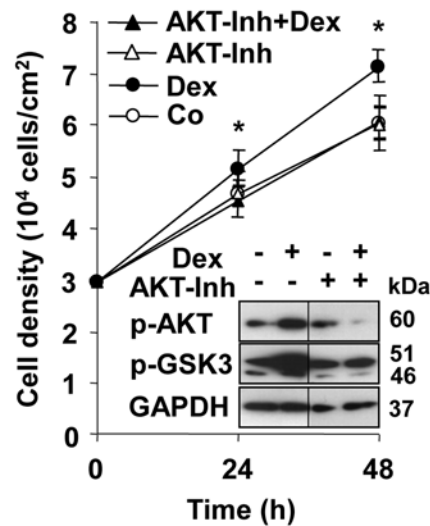
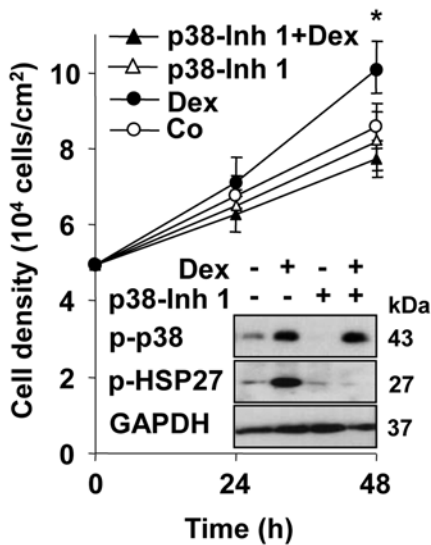


Figure 4. Inhibitors of protein kinase B (AKT) and p38-mitogen-activated protein kinase (MAPK) inhibit dexamethasone (Dex)-induced cell proliferation. A: MCF-7 cells were treated with Dex ( $10^{-5}$  M) for the time periods indicated and western blot of total cellular protein was performed (left). Blots were quantified using AIDA Image Analyzer software; normalization was performed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative amount of phosphorylated protein was calculated versus untreated cells which were set to 1 for each time point. The means of three independent experiments  $\pm$  standard error are shown;  $*p < 0.05$  using paired *t*-test (right). B: MCF-7 cells were pre-treated for 1 hour with p38-MAPK inhibitor 1, namely SB202190 ( $10^{-5}$  M, left) or AKT Inhibitor VIII ( $5 \times 10^{-8}$  M, right) followed by Dex ( $10^{-5}$  M) for another 48 hours. Tumor cell growth was measured using time lapse microscopy. In parallel, western blots were performed of identically treated cells, except that cells were harvested 4 hours after Dex treatment. The means of five independent experiments  $\pm$  standard error are shown;  $*p < 0.05$  using paired *t*-test. For clearness of presentation, the order of samples was rearranged in the right western blots without any further manipulation. The separating lines mark the reassembly. Co: Control; Inh: inhibitor.



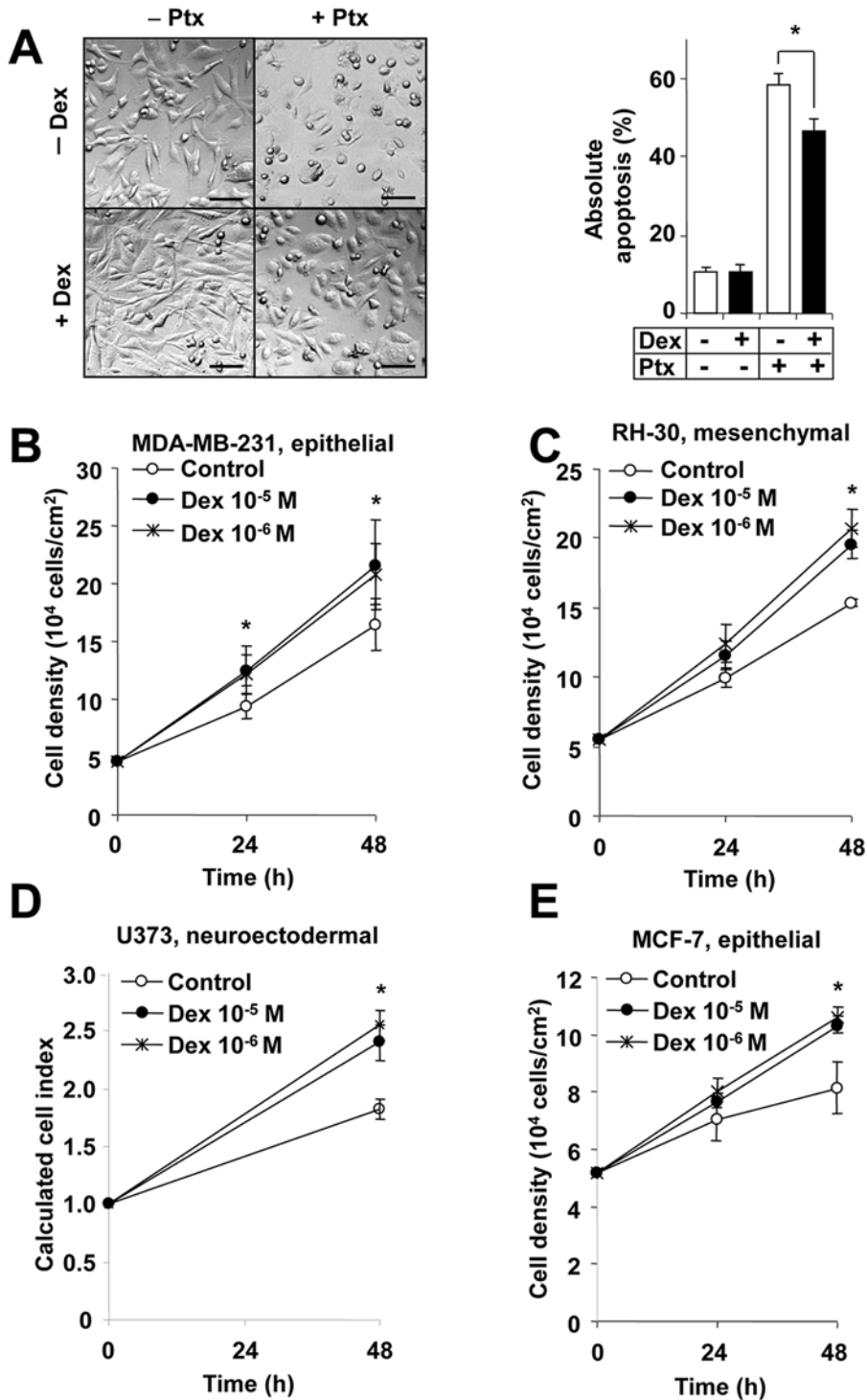


Figure 5. Dexamethasone enhances survival and growth of primary, patient-derived leukemia cells. A: A total of 139 samples of fresh patient-derived tumor cells were treated with Dex ( $10^{-5}$  M) and apoptosis was measured by forward side scatter analysis in FACScan as described in the Materials and Methods. Samples were classified as exhibiting apoptosis or survival, if the drug induced more than 10% specific apoptosis or survival, respectively; otherwise samples were classified as exhibiting no effect. Specific survival was calculated as specific apoptosis in % subtracted from 100%. B: Scatter diagram of specific survival induced by Dex ( $10^{-5}$  M) against specific survival induced by prednisolone (Pred) ( $10^{-4}$  M). A positive linear relationship with correlation coefficient  $r=0.9$ ;  $*p<0.001$  using Pearson product moment correlation. C, D: The most outstanding sample data are shown. The sample was from a child with leukemia. C: The original FACScan data incorporated into A, 48 hours after stimulation. D: Light microscopy images (left) and ATP consumption (right) of the cultures after 24 hours. Arrows indicate colonies and at the lower left, numbers of colonies in a well with 105 seeded cells are indicated as detected by a blinded operator. Scale bars 0.1 mm. Co: Control.

a signaling mechanism, the glucocorticoid receptor was found to mediate dexamethasone induced tumor cell proliferation.

Of overall clinical significance in oncology, our data indicate that the non-use of GCs might actually improve the prognosis of patients with cancer. Non-use of GCs represents a straightforward procedure which is realized more easily and at lower costs compared to the evaluation of new therapeutic options. Clinical studies are necessary to show whether cancer patients might benefit from restricted application of GCs.

The main message of our data is that GCs increase the number of living tumor cells in a wide range of different tumor entities. In general, GCs use two mechanisms to do so: an anti-apoptotic, pro-survival action combined with a pro-proliferative action. The pro-proliferative action of GCs was known from rare studies on single solid tumor cell lines (13, 15, 28). Our data show that this tumor-directed adverse effect of GCs is widespread in numerous cell lines from solid tumors. The anti-apoptotic, pro-survival action of GCs was well known for cell lines of solid tumors (2). Here we show for the first time that GC-induced survival also involves hematopoietic tumor cells, namely acute leukemia.

Since the early 1950s, tumor-directed adverse effects of GCs have been discussed in the literature. So why has GC-induced tumor proliferation not been studied more intensively so far? One reason might lie in the technical difficulties of measuring tumor cell proliferation *in vitro*. The proliferation rate (100% in controls) was augmented to 140% on average by dexamethasone; since frequently used conventional tests such as the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, ATP consumption and BrdU incorporation suffer from high intra- and interassay variances, these tests might not detect these differences. The use of modern, more sensitive and reliable techniques, such as time-lapse microscopy, followed by automated analysis or electrical impedance measurement now facilitate studies on GC-induced tumor proliferation.

For our studies, we used GCs exclusively at concentrations at or below those measured in the plasma of patients during anticancer therapy (16, 29). As very low concentrations of dexamethasone induce proliferation *in vitro* (Figure 2A) and as dexamethasone is metabolized with a long half life in humans ( $9 \pm 3$  hours) (30), our data suggest that unfavorable plasma concentrations of dexamethasone might persist for prolonged periods of time in patients with cancer.

GCs are not the first drugs which have been shown to be able to induce either apoptosis or proliferation of tumor cells. We have recently shown that TNF-related apoptosis-inducing ligand (TRAIL) induces either do so, depending on the intracellular signaling network regulating both effects. For TRAIL, we delineated the signaling mechanism responsible for the life and death decision (18, 31) and were consequently able to convert TRAIL-induced proliferation

into TRAIL-induced apoptosis (17). GCs are likely another category of adjuvants/cytotoxic drugs which can induce non-target side-effects by the promotion of pro-survival cell functions. Furthermore, our data clearly underline the anti-apoptotic effect of GCs on the efficacy of cytotoxic drugs, which is not surprising when our recently published data are taken into account which clearly demonstrate that one class of anticancer agents can also reduce the activity of the second during combination therapy (32).

Our data show that the addition of inhibitors of proliferation pathways, such as of p38-MAPK or AKT disable dexamethasone induced tumor cell proliferation *in vitro* (Figure 4B, data not shown). p38-MAPK and AKT inhibitors in current clinical testing might therefore represent promising candidates for a putative co-application with dexamethasone to prevent dexamethasone induced survival and proliferation of tumor cells.

In certain clinical trials on solid tumors, the use of GCs was associated with significantly lower therapy response rates and increased metastatic potential (7, 8). Our data might help to understand the underlying phenotypes and signaling mechanisms responsible for these clinical findings. In sum, these clinical and preclinical studies, although limited, might argue towards a restricted use of GCs during anticancer therapy of solid tumors.

GCs play an important beneficial role for therapy of hematopoietic malignancies and non-response to GCs is associated with impaired prognosis, *e.g.* in ALL (27). In our study, GCs increased the number of living tumor cells only if the tumor cells display resistance to apoptosis induction by GCs. Our data strengthen the widespread desire to individualize therapy. In hematological malignancies, predictive parameters and biomarkers are required which discriminate patient tumor cells prone towards GC-induced cell death from those that are not.

In summary, our data show that GCs induce pro-survival and pro-proliferative effects *in vitro* in several different tumor types. Our data encourage a detailed preclinical and clinical re-evaluation aiming to define how GCs can be safely applied in patients with cancer.

## Acknowledgements

The skilled technical work of U. Borgmeier and L. Mura from Helmholtz Zentrum München and R. Meilbeck from Dr. von Haunersches Kinderspital is kindly appreciated. This article contains data from the Ph.D. thesis of EB.

This work was supported by Bettina Bräu-Stiftung (to IJ).

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Received July 2, 2012

Revised August 6, 2012

Accepted August 7, 2012