

Divergent Mechanisms of Glucocorticoid Resistance in Experimental Models of Pediatric Acute Lymphoblastic Leukemia

Petra S. Bachmann,^{1,2} Rosemary Gorman,¹ Rachael A. Papa,¹ Jane E. Bardell,¹ Jette Ford,⁴ Ursula R. Kees,⁴ Glenn M. Marshall,^{1,3} and Richard B. Lock^{1,2}

¹Children's Cancer Institute Australia for Medical Research, ²The University of New South Wales, and ³The Centre for Children's Cancer and Blood Disorders, Sydney Children's Hospital, Sydney, Australia; and ⁴The Telethon Institute for Child Health Research, and Centre for Child Health Research, The University of Western Australia, Perth, Australia

Abstract

Cell line models of glucocorticoid resistance in childhood acute lymphoblastic leukemia (ALL) almost invariably exhibit altered glucocorticoid receptor (GR) function. However, these findings are incongruous with those using specimens derived directly from leukemia patients, in which GR alterations are rarely found. Consequently, mechanisms of glucocorticoid resistance in the clinical setting remain largely unresolved. We present a novel paradigm of glucocorticoid resistance in childhood ALL, in which patient biopsies have been directly established as continuous xenografts in immune-deficient mice, without prior *in vitro* culture. We show that the GRs from six highly dexamethasone-resistant xenografts (*in vitro* IC₅₀ >10 μmol/L) exhibit no defects in ligand-induced nuclear translocation and binding to a consensus glucocorticoid response element (GRE). This finding contrasts with five commonly used leukemia cell lines, all of which exhibited defective GRE binding. Moreover, whereas the GRs of dexamethasone-resistant xenografts were transcriptionally active, as assessed by the ability to induce the *glucocorticoid-induced leucine zipper* (*GILZ*) gene, resistance was associated with failure to induce the *bim* gene, which encodes a proapoptotic BH3-only protein. Furthermore, the receptor tyrosine kinase inhibitor, SU11657, completely reversed dexamethasone resistance in a xenograft expressing functional GR, indicating that pharmacologic reversal of glucocorticoid resistance in childhood ALL is achievable. [Cancer Res 2007;67(9):4482–90]

Introduction

Glucocorticoids are among the most effective agents used in the treatment of lymphoid malignancies, including childhood acute lymphoblastic leukemia (ALL; ref. 1) due to their ability to induce apoptosis of lymphoid cells (2). The glucocorticoid-induced apoptotic response is mediated through the glucocorticoid receptor (GR), a member of the nuclear receptor family of ligand-dependent transcription factors, which is localized in the cytoplasm of unstimulated cells (3).

Ligand binding to the GR results in its dissociation from chaperone proteins that tether the receptor in the cytoplasm, causing a conformational change in the protein that unmask domains for nuclear translocation, receptor dimerization, DNA binding, and transactivation (2). The activated receptor-ligand complex translocates to the nucleus where it binds as a homodimer to specific glucocorticoid response elements (GRE) found in the promoter and enhancer regions of glucocorticoid-responsive genes. Changes in gene expression ultimately result in proteolytic activation of caspases that cleave critical cellular substrates and are responsible for the hallmark changes associated with apoptosis (4).

A number of recent studies have attempted to identify critical glucocorticoid-regulated genes, with the emerging view that an interactive network of genes undergoes altered expression before the onset of apoptosis (5–7). Interestingly, the only proapoptotic protein identified to date to be up-regulated by glucocorticoids in several models of glucocorticoid-induced apoptosis is the BH3-only Bcl-2 family member, Bim (6, 8–12). A critical role for Bim in glucocorticoid-induced apoptosis of human precursor B (pre-B) ALL cells was recently identified (10). BH3-only Bcl-2 family members (including Bim, Bad, Bid, Bik, Bmf, Puma, Noxa, and Hrk) are related to other Bcl-2 proteins through only a single region of homology, the BH3 domain, and are potent inducers of apoptosis through the activation of Bax (13). The formation of pores in the outer mitochondrial membrane by Bax/Bak oligomers leads to loss of mitochondrial transmembrane potential and the subsequent release of proapoptotic factors such as cytochrome *c* and SMAC/DIABLO into the cytoplasm that promote the activation of initiator caspases (2).

Despite the central role of glucocorticoids in all protocols for the treatment of ALL, mechanisms by which they act on their target cells, and lesions underlying resistance, remain poorly understood (2). This is due, in part, to lack of suitable experimental systems to study the disease. Due to the limited proliferative capacity of primary leukemia cells *in vitro*, studies investigating glucocorticoid resistance have focused to a greater extent on laboratory-derived ALL cell lines. Resistance in such cell lines is almost invariably associated with defects in receptor-ligand interactions, such as mutations in the ligand binding domain of the GR (14–16). However, similar studies using primary patient samples have detected only isolated cases of somatic mutations in the GR, leading to poor glucocorticoid response (17, 18). Although existing data are conflicting, the majority of studies have failed to associate glucocorticoid resistance in patient samples with either relative expression of the various isoforms of the receptor (19, 20), polymorphisms within the coding region of the *GR* gene (18), or expression levels of cochaperone proteins associated with the GR (21).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

P.S. Bachmann and R. Gorman contributed equally to this work.

Children's Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children's Hospital.

Requests for reprints: Richard Lock, Children's Cancer Institute Australia, P.O. Box 81, High Street, Randwick, NSW 2031, Australia. Phone: 61-2-9382-1846; Fax: 61-2-9382-1850; E-mail: rlock@ccia.unsw.edu.au.

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We have previously shown that the *in vivo* and *in vitro* dexamethasone responses of a panel of childhood ALL biopsies established as xenografts in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice reflected the clinical outcome of the patients from whom the xenografts were derived (9, 22). In the current study, we report that all highly resistant xenografts exhibited ligand-dependent GR DNA binding and transcriptional induction of *glucocorticoid-induced leucine zipper* (*GILZ*), a direct transcriptional target of the GR. However, there was a clear disparity between sensitive and resistant xenografts in their ability to induce transcription of *bim*, a gene that lacks GREs in its promoter and seems not to be a direct transcriptional target of the GR. These results are in direct contrast with glucocorticoid-resistant cell lines, which exhibited defects upstream of nuclear translocation of the GR and/or DNA binding. Our finding of transcriptionally functional GR in all childhood ALL xenografts derived directly from biopsy specimens indicates that reversal of glucocorticoid resistance in the clinic is achievable. Hence, we also report the complete reversal of high-level glucocorticoid resistance and restitution of *Bim* induction in childhood ALL xenograft cells cotreated with the receptor tyrosine kinase (RTK) inhibitor, SU11657.

Materials and Methods

NOD/SCID mouse model of childhood ALL. Procedures by which we previously established continuous xenografts from childhood ALL biopsies in NOD/SCID mice and assessed their *in vivo* sensitivity to dexamethasone are described in detail elsewhere (22, 23). Xenograft characteristics are presented in Table 1. Leukemia progression in dexamethasone- or vehicle control-treated mice was monitored by quantifying human CD45 (pan leukocyte)-positive (huCD45⁺) cells versus total murine CD45⁺ and huCD45⁺

cells (% huCD45⁺) in the peripheral blood (PB). ALL-10, ALL-4CL, and ALL-7CL (see below) were monitored using anti-human CD19 antibodies due to low expression of cell surface CD45. Mouse event-free survival (EFS) was calculated by the number of days from treatment initiation until the proportion of human cells in the PB reached 25%. For comparisons between xenografts, the median EFS for control mice was subtracted from the median EFS for drug-treated mice to generate a tumor growth delay (TGD). All experimental studies had approval from the Human Research Ethics Committee and the Animal Care and Ethics Committee of the University of New South Wales.

Development and characterization of xenograft-derived strains. Cell lines were established *in vitro* from ALL-4 (ALL-4CL) and ALL-7 (ALL-7CL) xenograft cells according to our previously published method (24). Once established as continuously growing lines, the cells were adapted to RPMI 1640 supplemented with 20% fetal bovine serum (FBS), 1 mmol/L pyruvate, nonessential amino acids, 10 μmol/L 2-mercaptoethanol, and 2 mmol/L L-glutamine. Because these cultured cells require high cell density and exhibit prolonged population doubling times, they will collectively be referred to as xenograft-derived strains (XDS) to distinguish them from the established leukemia cell lines also used in this study (see below).

Expression of a range of lineage-specific and differentiation markers on the surface of XDS and on cells harvested from the spleens of engrafted mice was analyzed using standard procedures (22, 23). Cytogenetic analysis was done at SydneyGenetics, Sydney IVF (Sydney, NSW, Australia) using standard procedures (25). Chromosomal spreads were prepared from cultures following colcemid-induced metaphase arrest (0.2 μg/mL for 30 min at 37°C). Cells were resuspended in 0.075 mol/L KCl hypotonic solution for 20 min at room temperature, then fixed with methanol/acetic acid (3:1), dropped onto microscope slides, and GTG banded according to standard techniques (25). Finally, cells were examined at a chromosome resolution ranging from 200 to 400 bands per haploid set. Chromosomal abnormalities were identified and described according to International System for Human Cytogenetic Nomenclature (ISCN; 1995).

Table 1. Description of experimental model systems and *in vivo/in vitro* responses to dexamethasone

Designation	Model type	ALL subtype	<i>In vitro</i> IC ₅₀	<i>In vivo</i> TGD (days)	<i>In vitro</i> stratification	<i>In vivo</i> stratification
ALL-2	Xenograft	Common	Refer to ref. (9)	30.3	Resistant	Intermediate
ALL-3	Xenograft	Pre-B	Refer to ref. (9)	66.3	Sensitive	Sensitive
ALL-4	Xenograft	Ph ⁺ -ALL	Refer to ref. (9)	2.3	Resistant	Resistant
ALL-7	Xenograft	Biphen	Refer to ref. (9)	34.4	Resistant	Intermediate
ALL-8	Xenograft	T-ALL	Refer to ref. (9)	17.2	Sensitive	Resistant
ALL-10	Xenograft	Common	Refer to ref. (9)	37.3	Resistant	Intermediate
ALL-11	Xenograft	Common	Refer to ref. (9)	62.2	Sensitive	Sensitive
ALL-16	Xenograft	T-ALL	Refer to ref. (9)	52.8	Sensitive	Sensitive
ALL-17	Xenograft	Common	Refer to ref. (9)	31.3	Sensitive	Intermediate
ALL-18	Xenograft	Common	Refer to ref. (9)	nd	Resistant	nd
ALL-19	Xenograft	Common	Refer to ref. (9)	5.8	Resistant	Resistant
ALL-4CL	XDS	Ph ⁺ -ALL	>10 μmol/L	2.4	Resistant	Resistant
ALL-7CL	XDS	Biphen	>10 μmol/L	23.2	Resistant	Resistant
CEM WT	Cell line	T-ALL	94.2 nmol/L	nd	Sensitive	nd
CEM MTX-R3	Cell line	T-ALL	>10 μmol/L	nd	Resistant	nd
Jurkat	Cell line	T-ALL	>10 μmol/L	nd	Resistant	nd
HAL-01	Cell line	Pro-B	>10 μmol/L	nd	Resistant	nd
Nalm-6	Cell line	Pre-B	52.7 nmol/L	nd	Sensitive	nd
UOC-B1	Cell line	Pro-B	>10 μmol/L	nd	Resistant	nd
REH	Cell line	Pre-B	>10 μmol/L	nd	Resistant	nd

Abbreviations: Biphen, biphenotypic ALL; Common, CD10⁺ pre-B ALL; Intermediate, *in vivo* TGD 25–50 d; nd, not done; Ph⁺-ALL, Philadelphia chromosome-positive ALL; Pre-B, B-cell precursor ALL; Resistant, *in vitro* IC₅₀ > 10 μmol/L, *in vivo* TGD <25 d; Sensitive, *in vitro* IC₅₀ < 100 nmol/L, *in vivo* TGD, >50 d; T-ALL, T-lineage ALL.

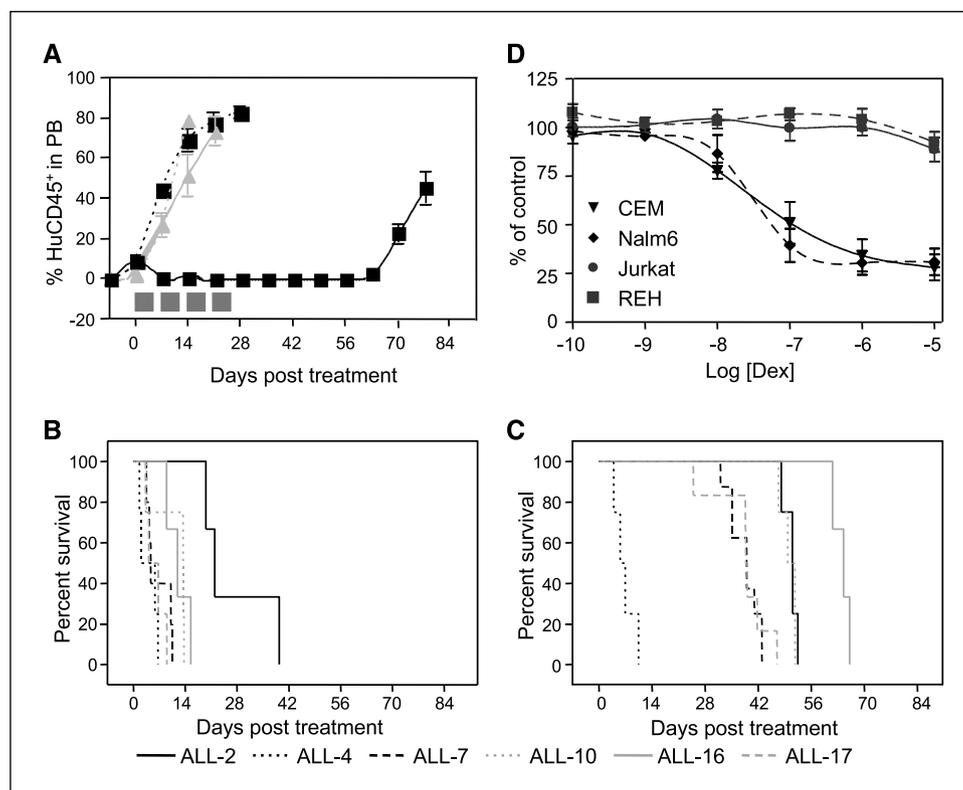


Figure 1. *In vivo* and *in vitro* responses of childhood ALL xenografts and cell lines to dexamethasone. **A**, *in vivo* responses of representative xenografts, ALL-3 and ALL-19, to dexamethasone. The % huCD45⁺ cells in the PB of mice engrafted with cells from ALL-3 (black squares) or ALL-19 (gray triangles) is shown at weekly intervals in mice treated with dexamethasone (15 mg/kg i.p. Monday–Friday \times 4 wks, solid lines) or saline control (dotted lines). Boxes, treatment periods. Points, the mean of groups of four mice; bars, SD. **B** and **C**, Kaplan-Meier plots of mouse EFS from the start of treatment in mice engrafted with six continuous xenografts, for saline-treated controls (**B**) or dexamethasone-treated animals (**C**). Numbers below lines, xenografts. **D**, MTT assay of four ALL cell lines exposed to various concentrations of dexamethasone for 48 h.

***In vitro* cell culture.** Jurkat and REH cell lines were obtained from the American Type Culture Collection, and UOC-B1 and Hal-01 cell lines were kindly provided by Dr. A. Thomas Look (Dana-Farber Cancer Institute, Boston, MA). CEM WT, CEM MTX-R3, and Nalm-6 cells used in the study were laboratory stock cell lines (9). These cell lines were maintained in suspension culture in RPMI 1640 supplemented with 10% FBS and 2 mmol/L L-glutamine.

For all experiments described in this study, xenograft cells were retrieved from cryostorage and resuspended in QBSF-60 medium (Quality Biological) supplemented with Flt-3 ligand (20 ng/mL; kindly provided by Amgen), penicillin (100 units/mL), streptomycin (100 μ g/mL), and L-glutamine (2 mmol/L; QBSF-60/F). Viability was determined by exclusion of 0.2% trypan blue. For drug treatments, cells were equilibrated in QBSF-60/F in a humidified atmosphere overnight at 37°C, 5% CO₂ before addition of dexamethasone or SU11657 (kindly provided by Pfizer Inc., Groton, CT). An equivalent volume of media only was added to control cells. Cells were harvested at the appropriate time points by centrifugation at 490 \times g for 10 min, aspirating media, and washing cells twice with sterile PBS.

In vitro dexamethasone sensitivity was assessed using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, exactly as described previously (9). Cell viability was calculated as a percentage of untreated controls. Results presented are the mean \pm SE of at least three independent experiments. IC₅₀ (inhibitory concentration, 50%) values were calculated from cumulative survival curves.

Analysis of protein expression. The preparation of whole-cell extracts, separation of cytoplasmic and nuclear fractions using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce), determination of protein concentration, and analysis of cellular proteins by immunoblotting have been described in detail elsewhere (9). Polyclonal antibodies specific for the following proteins were used: GR (Santa Cruz Biotechnology), actin and Bim (Sigma), and DNA topoisomerase I (topo I) and Bax (BD Biosciences Pharmingen). Secondary antibodies used were horseradish peroxidase (HRP) conjugates of either anti-mouse or anti-rabbit immunoglobulin G (Amersham Biosciences, Buckinghamshire, England).

GR-DNA Binding ELISA. GR-DNA binding affinity in nuclear extracts was quantified by TransAM Transcription Factor ELISA (Active Motif). Nuclear extracts were prepared as above, and 10- μ g aliquots of protein were assayed for GR DNA-binding activity according to the manufacturer's instructions. GR-DNA binding activity was expressed relative to an equivalent amount of positive control nuclear extract prepared from HeLa cells treated with dexamethasone (100 nmol/L, 1 h).

Real-time reverse transcription-PCR. Real-time quantitative reverse transcription-PCR (RT-PCR) was carried out using standard techniques. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized in a 20- μ L reaction volume using 2 μ g of total RNA, 500 ng of random primers (Roche), and 200 units of M-MLV Reverse Transcriptase (Invitrogen). The reaction mixture was ultimately diluted to 50 μ L with nuclease-free water.

Primers and probes for an early response gene (*GILZ*) possessing GREs in its promoter region and a late response gene (*bim*) that does not were purchased from Assays-on-Demand (Applied Biosystems). Quantitative real-time RT-PCR analysis was carried out in triplicate under the following cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, as per the manufacturer's instructions. Reactions without template and/or enzyme were used as negative controls. Elongation factor-1 α (EF-1 α) was used as an internal standard in each reaction, with primer and probe concentrations optimized to not interfere with target gene amplification (primers EF-1 α F, 5'-CTGAACCATCCAGGCCAAAT-3'; EF-1 α R, 5'-GCCGTGTGGCAATCCAAT-3'; Probe, 5'-VIC-AGCGCCGCTATGCCCTG-TAMRA-3'). RNA quantity was normalized to the corresponding EF-1 α control by the Δ C_t method. Fold differences were calculated by comparison with the vehicle-treated control at each time point.

Results

Experimental model of dexamethasone resistance in pediatric ALL. To investigate mechanisms of glucocorticoid resistance in childhood ALL, our experimental model system included

Table 2. Karyotype and immunophenotype of XDS compared with original xenograft

Xenograft/XDS	Karyotype (% of cells)	Immunophenotype
ALL-4	45, XY, der (9)t (9;13) (p13;q11.2), t (9;22) (q34;q11.2), -13 (80%) 45, XY, der (9)t (9;13) (p13;q11.2), t (9;22) (q34;q11.2), -13, t (4;8) (q21;q24.3) (20%)	HLA-DR+/CD45 ⁺ /19 ⁺ /22 ⁺ /10 ⁺ /38 ⁺ /11b ⁻ /33 ⁻
ALL-4CL	45, XY, der (9)t (9;13) (p13;q11.2), t (9;22) (q34;q11.2), -13 (100%)	HLA-DR+/CD45 ⁺ /19 ⁺ /22 ⁺ /10 ⁺ /38 ⁺ /11b ⁻ /33 ⁻
ALL-7	45, XY, dic (8;9) (p11.2;p13), t (17;19) (q22;p13.3) (71%) 46, XY, del (13) (q14?q34),der (16)?t (7;16) (p15;q24), t (17;19) (q22;p13.3) (29%)	HLA-DR+/CD45 ⁺ /19 ⁺ /22 ⁺ /10 ⁺ /38 ⁺ /11b ⁺ /33 ⁺
ALL-7CL	46, XY, del (13) (q14?q34),der (16)?t (7;16) (p15;q24), t (17;19) (q22;p13.3) (100%)	HLA-DR+/CD45 ⁻ /19 ⁺ /22 ⁺ /10 ⁺ /38 ⁺ /11b ⁻ /33 ⁺

continuous xenografts, XDS, and ALL cell lines, the details of which are shown in Table 1. We have previously described the development of a panel of continuous ALL xenografts in NOD/SCID mice, showed their relevance to the clinical disease, and documented their *in vivo* and *in vitro* sensitivity to dexamethasone (9, 22, 23). Figure 1A illustrates the range of *in vivo* responses of representative xenografts to dexamethasone, showing the proportion of huCD45⁺ cells in PB over time of mice engrafted with ALL-3 or ALL-19, then treated with either dexamethasone or saline control. The progression of ALL-19 was not affected by treatment with dexamethasone, showing no obvious difference from saline control-treated mice. In contrast, the progression of ALL-3 was delayed by dexamethasone treatment for ~60 days.

Kaplan-Meier plots showing EFS of mice engrafted with six continuous xenografts and treated with saline or dexamethasone (Fig. 1B and C) show the range of responses to treatment. ALL-18 was excluded due to its slow rate of engraftment (23). *In vivo* TGD values and *in vitro* dexamethasone sensitivity, determined by MTT assay, are indicated in Table 1, with significant concordance between the two assays (relative cell survival by MTT assay at 10 nmol/L dexamethasone versus *in vivo* TGD, *r* = 0.77; *P* = 0.0098). A notable exception was ALL-8, which was previously shown to have down-regulated GR expression (9) and was sensitive *in vitro* but resistant *in vivo*. We have previously shown that the stratification of xenografts according to patient outcome reveals a significant difference in TGD values (22). Furthermore, of the three most sensitive xenografts (TGD, 52.8–66.3 days, ALL-3, ALL-11, and ALL-16), only one patient experienced a relapse, and all remained alive >10 years from diagnosis. In contrast, the three most resistant (TGD, 2.3–17.2 days, ALL-4, ALL-8, and ALL-19) all relapsed and died within 18 months of diagnosis; and of the four exhibiting intermediate sensitivity (TGD, 30.3–37.3 days, ALL-2, ALL-7, ALL-10, and ALL-17), three relapsed, and two died of disease (22). The results indicate that this panel of xenografts is a clinically relevant model. Similar *in vivo* dexamethasone sensitivity data for all xenografts except ALL-4 and ALL-10, and the *in vitro* data for all continuous xenografts, was previously published (9, 22).

Xenograft cells are principally suitable only for short-term *in vitro* culture (22), although we have successfully established XDS from two xenografts, ALL-4CL and ALL-7CL, by passaging at high density until a cell line was established. The conditions required to develop these XDS were identical to those necessary to establish cell lines from primary patient specimens (24). Comparison of the karyotypes of these XDS with their respective parental xenografts provides evidence that a clonal subpopulation (dominant in the case of ALL-4, but minor for ALL-7) emerged *in vitro* from the xenograft mixed populations (Table 2). Moreover, whereas the immunophenotype of ALL-4CL was identical to ALL-4, the

emergence of ALL-7CL resulted in the loss of cell surface CD45 and CD11b expression (Table 2). Despite these changes, ALL-4CL and ALL-7CL exhibited responses to dexamethasone both *in vivo* and *in vitro* that were comparable to their respective parental xenografts (Table 1). In addition, the XDS showed similar patterns of organ infiltration to the parental xenografts when inoculated into NOD/SCID mice (data not shown). The population doubling times for ALL-4CL and ALL-7CL were 33 and 55 h, respectively.

Cell lines have routinely been used to define mechanisms of glucocorticoid resistance in ALL (14, 26). Several human ALL cell lines were also investigated in this study, which exhibited sensitivity (CEM WT, Nalm-6) or resistance (CEM MTX-R3, Jurkat, HAL-01, UOC-B1, REH) to dexamethasone (Table 1). Representative MTT cytotoxicity assays for CEM WT, Nalm-6, Jurkat, and REH cells are shown in Fig. 1D.

GR expression and function. Glucocorticoid resistance mechanisms identified in ALL cell lines almost invariably involve defects in the GR. Consequently, GR expression and function were rigorously tested in our experimental model of dexamethasone resistance. Previous studies have shown that 10/11 continuous

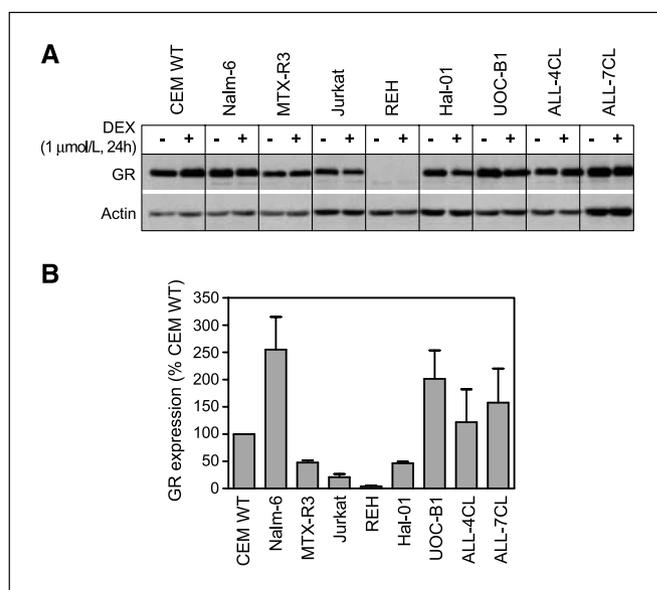


Figure 2. GR protein expression in leukemia cell lines. A, leukemia cell lines were treated with 1 μmol/L dexamethasone or solvent control for 24 h, and whole cell lysates were prepared and subjected to immunoblot analysis for GR and actin. B, basal GR expression was quantified relative to actin and expressed as a percentage of CEM WT. Columns, mean of three independent experiments; bars, SE.

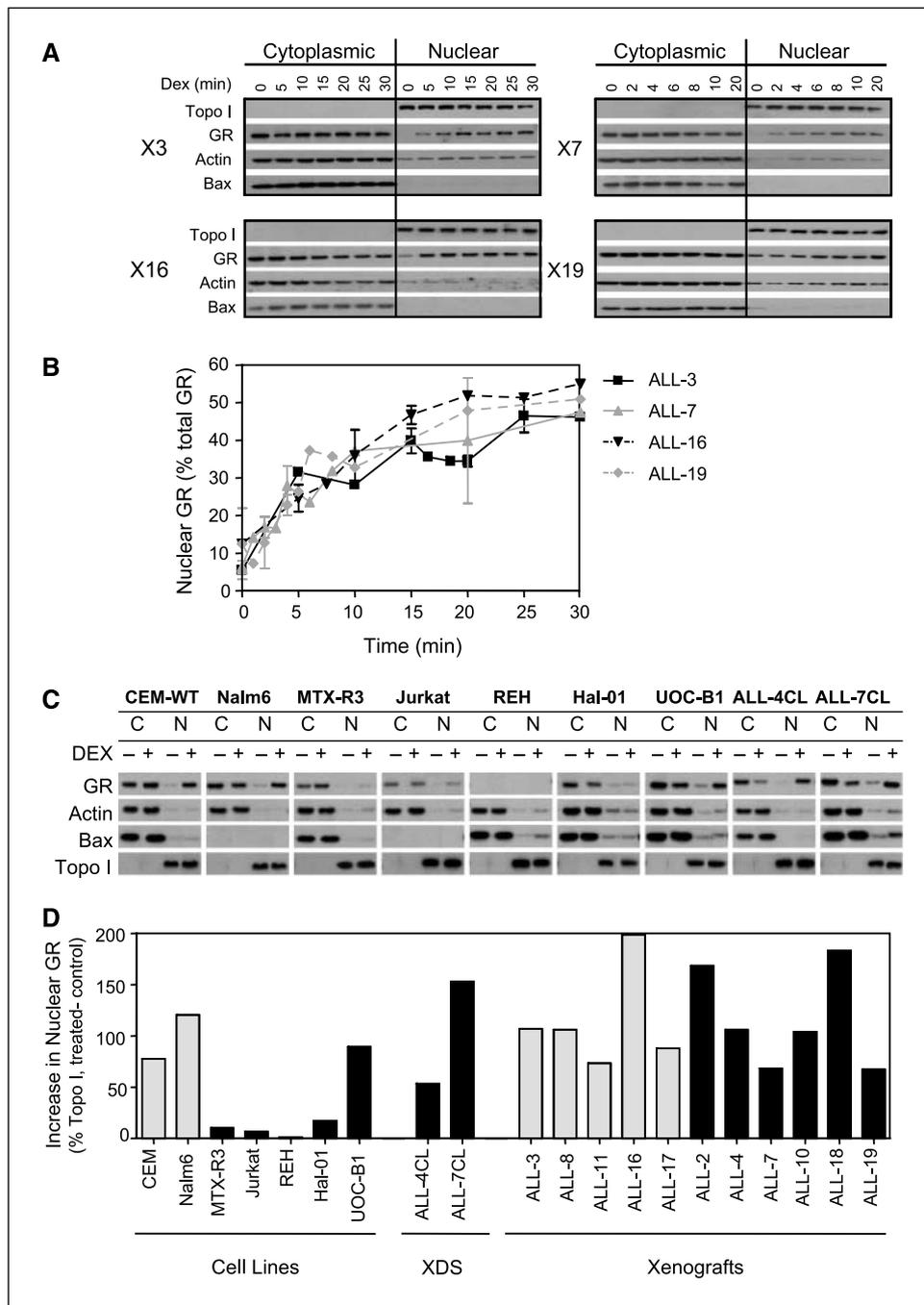


Figure 3. Ligand-induced GR nuclear translocation in experimental models of glucocorticoid resistance. *A* and *B*, time course of GR nuclear translocation in ALL-3 (X3), ALL-7 (X7), ALL-16 (X16), and ALL-19 (X19). Cells were treated with dexamethasone (1 $\mu\text{mol/L}$) and harvested at various time points up to 30 min for the separation into nuclear and cytoplasmic fractions. Equal amounts of protein (30 μg) from each fraction were immunoblotted for GR, topo I (nuclear), actin (loading control), and Bax (cytoplasmic; *A*). In (*B*), the proportion of nuclear GR is expressed as a percentage of total GR in cytoplasmic and nuclear fractions. *Points*, mean of three independent experiments plotted over time; *bars*, SE. *C* and *D*, nuclear translocation of GR in ALL cell lines. Cells were exposed to dexamethasone (1 $\mu\text{mol/L}$, 5 h), separated into cytoplasmic (*C*) and nuclear (*N*) fractions, and processed for immunoblotting as described in (*A*). In (*D*), the ligand-induced increase in nuclear GR is compared between all model types used. The GR signal in nuclear extracts was expressed as a percentage of the control Topo I signal, and the increase in nuclear GR was expressed as treated minus control. *Gray and black bars*, samples stratified as sensitive or resistant, respectively, to dexamethasone *in vitro* (see Table 1).

xenografts tested exhibited no defects in GR expression, with only the T-cell ALL (T-ALL), ALL-8, expressing significantly lower levels of receptor (9). In addition, unlike cell lines, none of the xenografts tested showed induction of the GR after exposure to dexamethasone, regardless of glucocorticoid sensitivity. In contrast, Fig. 2*A* and *B* shows that the pre-B ALL cell line REH expresses no detectable GR, whereas dexamethasone-resistant CEM MTX-R3, Jurkat, and HAL-01 all express levels of GR <50% than those of dexamethasone-sensitive CEM WT cells. Dexamethasone-resistant UOC-B1 cells, as well as the ALL-4CL and ALL-7CL XDS, expressed levels of GR comparable to or greater than CEM WT cells. The CEM WT cell line also showed autoinduction of the GR following exposure to dexamethasone, consistent with previous reports (27),

although no other cell line showed a similar change in GR expression.

We have previously shown nuclear translocation of the GR following exposure of 11 continuous xenografts to dexamethasone for 5 h (9), and Fig. 3*A* and *B* extend these findings to confirm no notable differences in the kinetics of ligand-induced GR nuclear translocation between two sensitive (ALL-3 and ALL-16) and two resistant (ALL-7 and ALL-19) xenografts. In contrast, Fig. 3*C* shows that 4/5 highly dexamethasone-resistant ALL cell lines (CEM MTX-R3, Jurkat, REH, and Hal-01) exhibited defective GR nuclear translocation in the presence of ligand, with only UOC-B1 cells showing GR translocation. The XDS ALL-4CL and ALL-7CL also exhibited ligand-induced GR nuclear translocation (Fig. 3*C*). These

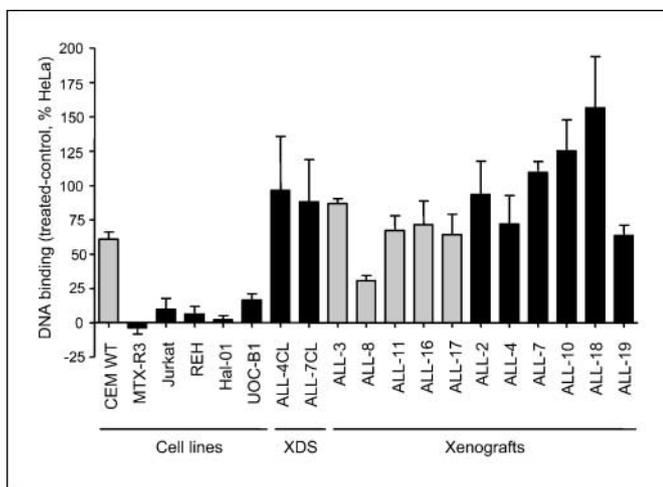


Figure 4. GR:GRE binding in leukemia cell lines, XDS, and xenografts. Ligand-induced binding of GR to a GRE was assessed using a DNA-binding ELISA. The OD_{450 nm} of each sample was normalized to the HeLa positive control, and DNA binding expressed as the bound GR following treatment with dexamethasone (1 μmol/L, 1 h) minus that of a solvent-treated control. Gray and black bars, samples stratified as sensitive or resistant, respectively, to dexamethasone *in vitro* (see Table 1). Columns, mean of at least three independent experiments; bars, SE.

results, summarized in Fig. 3D, imply divergent mechanisms of glucocorticoid resistance within this ALL experimental model system.

To further explore differential mechanisms of glucocorticoid resistance, the ability of the GR in nuclear extracts of dexamethasone-treated cells to bind a GRE was assessed using a DNA-binding ELISA assay (Fig. 4). Although all xenografts and XDS, regardless of whether they were resistant to dexamethasone, exhibited ligand-induced DNA binding to varying degrees, all of the resistant cell lines (including UOC-B1, which exhibited GR nuclear translocation) were defective in DNA binding activity. The decreased DNA binding exhibited by ALL-8 is consistent with the reduced GR expression we have previously documented in this

xenograft (9). These data confirm fundamental differences in mechanisms of glucocorticoid resistance in long-term *in vitro* cultured ALL cell lines compared with continuous xenografts established *in vivo*, as well as XDS.

Several recent large-scale gene expression studies have identified glucocorticoid-induced genes in ALL model systems (5, 6, 28–30). We have previously shown defective induction of one of these genes, *Bim*, at the protein level, in dexamethasone-resistant xenografts (9). The *bim* gene promoter does not contain a consensus GRE (6, 31), and therefore, we compared the induction of *bim* and a gene that does contain a GRE, *GILZ*, using quantitative real-time RT-PCR. Figure 5A clearly shows that xenograft cells, regardless of whether they were resistant to dexamethasone, induced *GILZ* in response to ligand treatment. *GILZ* expression reached a maximum within 1 to 2 h of dexamethasone exposure. In agreement with our previous study (9), the disparity between sensitive and resistant xenografts seemed to emerge in induction of *bim*, with resistant xenografts (ALL-2 and ALL-19) clearly defective (Fig. 5B). In contrast, all glucocorticoid-resistant cell lines were defective in both *GILZ* (Fig. 5C) and *bim* (Fig. 5D) induction in response to dexamethasone treatment, in agreement with their deficiencies in GR nuclear translocation (Fig. 3C) and DNA binding (Fig. 4). Both XDS (ALL-4CL and ALL-7CL) exhibited induction of *GILZ* but not *bim* (Fig. 5C and D), consistent with dexamethasone-resistant xenografts but not cell lines. The real-time quantitative RT-PCR data shown in Table 3 confirm that the *GILZ* gene was induced in all xenografts in response to dexamethasone treatment, regardless of their sensitivity to dexamethasone, but only in the sensitive CEM cell line and not the five resistant cell lines. These data again highlight the differences in glucocorticoid resistance mechanisms within this ALL experimental model system.

Pharmacologic reversal of glucocorticoid resistance in ALL xenograft cells. The above observations of dexamethasone-induced GR DNA binding and induction of gene transcription in resistant ALL xenograft models, along with observations that GR defects are rare in primary patient biopsies (17–20, 32), suggest that pharmacologic reversal of glucocorticoid resistance is achievable

Figure 5. Effects of dexamethasone on *GILZ* and *bim* expression in xenograft cells and leukemia cell lines. Xenograft cells cultured *in vitro* (A and B) or leukemia cell lines and XDS (C and D) were exposed to dexamethasone (1 μmol/L), harvested at times up to 16 h, and processed for real-time RT-PCR quantitation of *GILZ* (A and C) and *bim* (B and D) mRNA expression. Data are expressed relative (fold) to solvent-treated controls at each time point. Points, mean of three independent experiments; bars, SE.

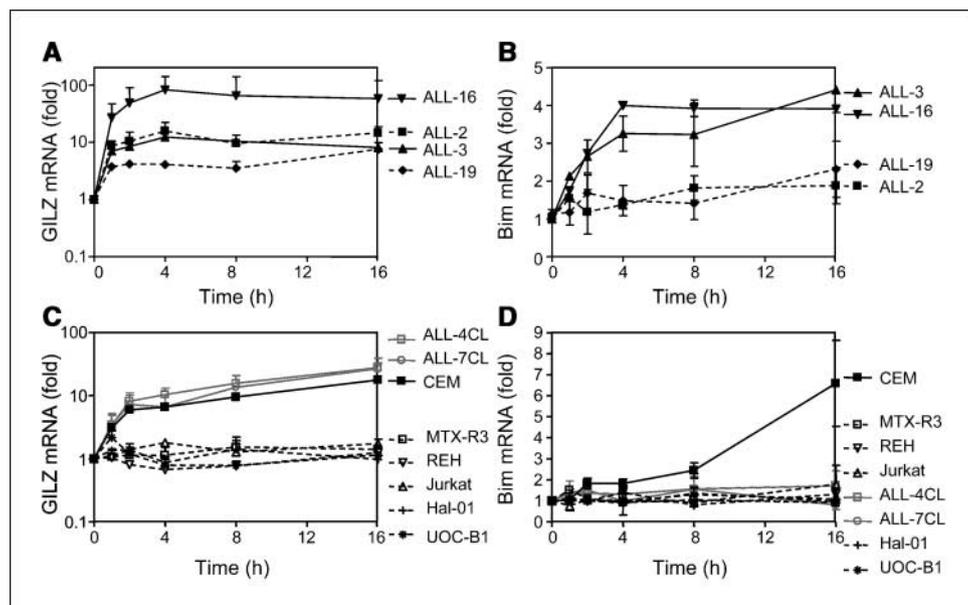


Table 3. Induction of *GILZ* mRNA by dexamethasone in xenografts and cell lines

Xenograft/cell line	<i>GILZ</i> mRNA expression*
ALL-2	15.7 ± 3.7
ALL-3	12.3 ± 1.1
ALL-4	5.5 ± 1.2
ALL-7	13.2 ± 2.8
ALL-8	5.8 ± 1.6
ALL-10	2.2 ± 0.5
ALL-16	82.1 ± 33.4
ALL-17	6.3 ± 0.4
ALL-19	4.1 ± 0.2
CEM	6.6 ± 0.1
MTX-R3	1.1 ± 0.4
Jurkat	1.6 ± 0.1
REH	0.8 ± 0.1
Hal-01	0.9 ± 0.2
UOC-B1	0.8 ± 0.2

*Fold increase induced by dexamethasone (1 μmol/L, 4 h) relative to solvent-treated control, mean ± SE of three separate experiments.

in the clinical setting. With this aim, we have been able to completely reverse dexamethasone resistance in ALL-7 using the RTK inhibitor SU11657 (Fig. 6A and B). At concentrations of up to 10 μmol/L, SU11657 alone was not cytotoxic toward ALL-7 cells (Fig. 6B). However, coincubation of 10 nmol/L SU11657 with 100 nmol/L dexamethasone caused an approximate 20% decrease in ALL-7 cell viability compared with controls, with a concomitant decrease in cell viability as the SU11657 concentration was increased. Coincubation of 10 μmol/L SU11657 with 100 nmol/L dexamethasone caused a >95% loss of cell viability. Moreover, the dexamethasone IC₅₀ value of ALL-7 cells coincubated with 1 μmol/L SU11657 was <10 nmol/L (Fig. 6A), consistent with the three most sensitive xenografts, ALL-3, ALL-11, and ALL-16 (Table 1 and ref. 9). SU11657 was unable to reverse dexamethasone resistance in any of the five cell lines expressing defective GR (data not shown). Figure 6C and D shows that the combination of SU11657 and dexamethasone also caused a greater than additive induction of Bim protein isoforms in ALL-7 cells, consistent with the reversal of dexamethasone resistance.

Discussion

Despite the central role of glucocorticoids in all protocols for the treatment of childhood ALL, the mechanisms underlying resistance remain poorly understood. The data described in this report present a new paradigm for elucidating glucocorticoid resistance mechanisms in pediatric ALL and for bridging the gap in understanding between studies using laboratory cell lines and those using primary biopsy specimens. In contrast with leukemia cell line studies, our findings present clear evidence that glucocorticoid resistance in xenografts established directly from biopsy material is associated with functional GR signaling pathways and transcriptional induction of a primary target of the GR, *GILZ*. However, glucocorticoid resistance manifested in the failure of the resistant xenografts to induce mRNA expression of the proapoptotic Bcl-2 family member, *bim*. Furthermore, we provide

proof of principle for the ability to pharmacologically modulate glucocorticoid resistance with RTK inhibitors in a highly resistant xenograft, resulting in restored Bim induction and apoptosis in response to dexamethasone.

In all leukemia cell lines analyzed, glucocorticoid resistance manifested itself at the level of the receptor, as evidenced by defects in their ability to bind DNA and induce expression of the early-induced gene, *GILZ*. The GR mutations underlying the CEM WT subline, CEM MTX-R3, and the Jurkat cell line have previously been described (14, 33). However, before the current report, the lesions underlying resistance in the REH, Hal-01, and UOC-B1 cell lines were unclear. In this study, we show that glucocorticoid resistance in the REH cell line can be attributed to a lack of detectable GR. Although the Hal-01 cell line has detectable GR protein, our results show an impaired response to glucocorticoid treatment evidenced by a lack of ligand-induced nuclear translocation, presumably due to a mutation in the receptor resulting in either impaired ligand binding or defective nuclear localization signals. Only one of the

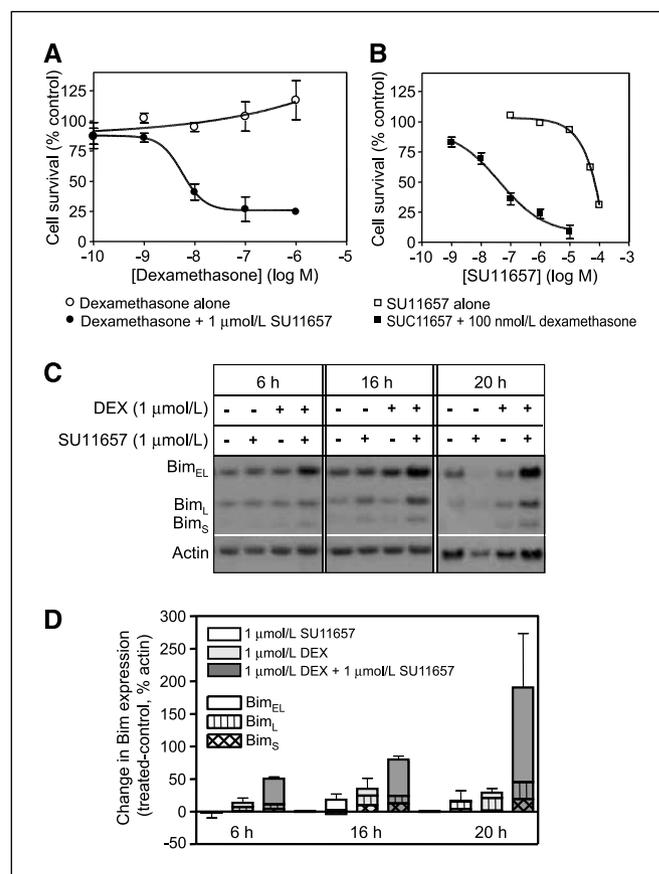


Figure 6. Pharmacologic reversal of glucocorticoid resistance in ALL-7. A, MTT assay of ALL-7 cells exposed to a range of dexamethasone concentrations either alone (○) or in the presence of 1 μmol/L SU11657 (●). B, MTT assay of ALL-7 cells exposed to a range of SU11657 concentrations either alone (□) or in the presence of 100 nmol/L dexamethasone (■). C, whole cell lysates were prepared from ALL-7 cells treated with 1 μmol/L dexamethasone, or the combination of up to 20 h and immunoblotted for Bim isoforms and actin. D, quantified results of changes in Bim expression following treatment with 1 μmol/L SU11657, 1 μmol/L dexamethasone, or both for up to 20 h. The expression of each Bim isoform detected was normalized to actin and summed. The relative contribution of each isoform to total Bim expression is indicated by shading (solid, Bim_{EL}; vertical lines, Bim_L; hatched, Bim_S). Each treatment is expressed with the basal Bim levels subtracted. Columns, mean of three independent experiments; bars, SE.

resistant cell lines included in this study (UOC-B1) underwent ligand-induced nuclear translocation, but failed to show DNA binding activity and *GILZ* induction.

Although the reasons for the disparity between the incidence of mutations in cell line models versus clinical samples remains unclear, the results from xenografts more closely represent the clinical situation, where GR mutations are infrequently encountered and cannot account for the prevalence of glucocorticoid resistance (17, 18). In support of this, sequencing of the entire coding region of the GR in one sensitive (ALL-3) and four resistant xenografts (ALL-2, ALL-4, ALL-7, and ALL-19) failed to reveal any mutations affecting the GR protein.⁵

In contrast to the findings in cell lines, all xenografts, independent of sensitivity to dexamethasone, showed an ability to activate GR signaling pathways and induce *GILZ*, a primary target of the GR containing at least three functional GREs in its promoter (34). Global changes in gene expression have been further evaluated in two sensitive (ALL-3 and ALL-16) and two resistant (ALL-7 and ALL-19) xenografts using chemiluminescence-based gene arrays.⁶ Following treatment with dexamethasone, all four xenografts induced primary GR targets, including *GILZ* and *FKBP5* (34), as well as other genes identified as glucocorticoid-regulated genes in cell line models, *CD53/MOX44* and *BTG1* (6, 7). In addition to the cell line models, these genes have also been identified as being regulated by glucocorticoid treatment *in vivo* by microarray analysis of primary patient samples (30). Although the individual contributions of these genes to glucocorticoid-induced apoptosis remain unclear, these findings indicate that the resistant xenografts are able to activate glucocorticoid signaling pathways, and that the factor(s) preventing the induction of *bim* are downstream of the transactivating functions of the receptor.

Bim was first identified as a mediator of glucocorticoid-induced apoptosis in microarray studies of murine and human leukemia cell lines (6). These results were confirmed at the protein level in leukemia cell lines and in our xenograft model of ALL (6, 9, 11, 12). The functional role of Bim was subsequently shown by small interfering RNA and short hairpin RNA inhibition of Bim expression in a highly sensitive pre-B leukemia cell line conferring resistance to glucocorticoid-induced apoptosis (10). Studies of knock-out mice of various BH3-only proteins provided evidence that only knock-out of Bim and Puma confer any degree of resistance to glucocorticoid treatment in murine thymocytes (8, 35). Although the role of Bim has been established in glucocorticoid-induced apoptosis of ALL cells, no studies to date have identified a similar role for Puma (12), although increases in *puma* mRNA have been reported in primary murine thymocytes exposed to dexamethasone (36).

Glucocorticoid-induced Bim up-regulation does not seem to be the direct result of transcriptional activity of the GR because the human *bim* promoter does not contain any GREs, and apoptosis is dependent on *de novo* protein synthesis (6, 12). However, the details of the signaling pathways upstream of *bim* induction remain unclear. Regulation of the various isoforms of Bim is complex and involves both transcriptional and post-translational mechanisms, dependent on cellular context (37). Increases in Bim expression may be the result of (a) activation of the *bim* gene by transcription factors, including Foxo3a/FKHRL1, RUNX3, and E2F1

(38–40); (b) release of the two largest isoforms of Bim, Bim_{EL} and Bim_L, from the cytoskeleton, where they are sequestered in healthy cells (41); (c) relief of extracellular signal-regulated kinase-1/2-mediated phosphorylation of Bim_{EL}, which targets it for proteasomal degradation in healthy cells (42); and (d) demethylation of the *bim* promoter in leukemia cell lines (43).

The SU series of compounds, originally developed as antiangiogenic agents, are multitargeted small-molecule RTK inhibitors displaying differential selectivity for class III and V RTKs, including platelet-derived growth factor receptors, vascular endothelial growth factor receptors, Flt-3, and Kit (44). Preclinical and phase I/II studies have established the efficacy of this family of RTK inhibitors against a variety of malignancies that are resistant to conventional chemotherapy (45, 46). In this study, we showed that the RTK inhibitor SU11657 (as well as SU11248, SU5416, and SU6668)⁷ acts synergistically with dexamethasone to modulate signaling through Bim and induce apoptosis in a highly glucocorticoid-resistant xenograft, ALL-7, conferring dexamethasone IC₅₀ values comparable with the most sensitive xenografts. These findings provide proof-of-principle evidence for the ability to therapeutically modulate glucocorticoid resistance in patients.

It is currently unclear how inhibition of RTK activity is able to reverse glucocorticoid resistance in ALL-7. Further studies are under way to delineate the critical signaling pathways affected by RTK inhibition in this xenograft and whether inhibition of a single pathway or simultaneous inhibition of multiple pathways restores glucocorticoid sensitivity. In this panel of xenografts, there seems to be divergent mechanisms of resistance upstream of Bim because the RTK inhibitors tested were unable to circumvent resistance in other highly resistant xenografts. However, because all xenografts contain GRs that seem to be functional, it is likely that glucocorticoid sensitivity could be restored by the interruption of the appropriate signaling pathway. Recently, the mTOR inhibitor rapamycin has been identified as a modulator of glucocorticoid resistance in leukemia cell lines by a mechanism involving the down-regulation of the antiapoptotic protein Mcl-1 (47). Similar studies of multiple myeloma cell lines and primary cells also identified additional compounds that sensitize resistant cells to dexamethasone, including rapamycin and *R*-etodolac, that may also be of benefit in leukemia cells (48, 49). In a clinical setting, individual patients may have to be molecularly profiled to identify those who may benefit from combination treatment with glucocorticoids and RTK inhibitors.

In conclusion, our findings show that mechanisms underlying glucocorticoid resistance in xenograft models of childhood ALL, which have been shown to recapitulate the primary disease state, are the result of defective signaling pathways that abrogate induction of the proapoptotic protein Bim. This finding is in contrast with those using glucocorticoid-resistant cell lines, which exhibit a lack of GR binding to GREs, indicative of defects at the level of the GR. Importantly, this study shows for the first time that high-level glucocorticoid resistance can be completely reversed by the coadministration of concentrations of RTK inhibitors that are achievable in the plasma of patients (50) and indicates that pharmacologic modulation of glucocorticoid resistance in the clinical setting is feasible. This novel paradigm of glucocorticoid resistance (Supplementary Fig. S1) in childhood ALL may assist in

⁵ Y.W. Phua and R.B. Lock, unpublished observations.

⁶ P.S. Bachmann and R.B. Lock, unpublished observations.

⁷ J.E. Bardell and R.B. Lock, unpublished observations.

the identification of clinically relevant mechanisms of resistance and the optimization of resistance reversal strategies before their testing in patients.

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Divergent Mechanisms of Glucocorticoid Resistance in Experimental Models of Pediatric Acute Lymphoblastic Leukemia

Petra S. Bachmann, Rosemary Gorman, Rachael A. Papa, et al.

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