Title

Wnt inhibition leads to improved chemosensitivity in paediatric acute lymphoblastic leukaemia

Journal

British Journal of Haematology, 167(1)

ISSN

0007-1048

Authors

Dandekar, Smita
Romanos-Sirakis, Eleny
Pais, Faye
et al.

Publication Date

2014-10-01

DOI

10.1111/bjh.13011

Peer reviewed

Published in final edited form as:
Br J Haematol. 2014 October ; 167(1): 87–99. doi:10.1111/bjh.13011.

Wnt Inhibition Leads to Improved Chemosensitivity in Pediatric Acute Lymphoblastic Leukemia

Smita Dandekar[#1], **Eleny Romanos-Sirakis**[#1,7], **Faye Pais**[2], **Teena Bhatla**[1], **Courtney Jones**[1], **Wallace Bourgeois**[3], **Stephen P. Hunger**[4], **Elizabeth A. Raetz**[1], **Michelle L. Hermiston**[2], **Ramanuj Dasgupta**[1,5], **Debra J. Morrison**[1], and **William L. Carroll**[1,6,#]

[1]NYU Cancer Institute, NYU Langone Medical Center, New York, NY

[2]Department of Pediatrics, University of California School of Medicine, San Francisco, California

[3]NYU School of Medicine

[4] University of Colorado School of Medicine, Aurora, Colorado

[5]Department of Biochemistry and Molecular Pharmacology, NYU Langone Medical Center, New York, NY

[6]Department of Pathology, NYU Langone Medical Center, New York, NY

[7]Department of Pediatrics, Staten Island University Hospital

[#] These authors contributed equally to this work.

Summary

While childhood acute lymphoblastic leukemia (ALL) is now highly curable, the dismal prognosis for children who relapse warrants novel therapeutic approaches. Previously, using an integrated genomic analysis of matched diagnosis - relapse paired samples, we identified overactivation of the Wnt pathway as a possible mechanism of recurrence. To validate these findings and document whether Wnt inhibition may sensitize cells to chemotherapy, we analyzed the expression of Activated β-catenin (and its downstream target *BIRC5*) using multiparameter phosphoflow cytometry and tested the efficacy of a recently developed Wnt inhibitor, iCRT14, in ALL cell lines and patient samples. We observed increased activation of β-catenin at relapse in 6 /10 patients. Furthermore, treatment of leukemic cell lines with iCRT14 led to significant downregulation of Wnt target genes and combination with traditional chemotherapeutic drugs resulted in a synergistic decrease in viability as well as a significant increase in apoptotic cell death. Finally, pre-treatment of purified blasts from patients with relapsed leukemia with the Wnt inhibitor followed by exposure to prednisolone, restored chemosensitivity in these cells. Our results

[#]Address for Correspondence William L. Carroll MD NYU Cancer Institute Smilow 1201 522 First Avenue New York NY 10016 Phone: 212-263-327 Fax: 212-263-9190 william.carroll@nyumc.org.

Authorship Contributions

SD, ER, DM, TB, CJ, MH and WLC planned experiments. SD, ER, FP, MH, DM and WB performed experiments and analyzed data. SD, DM, RD and W.L.C. wrote the manuscript. R.D. contributed the iCRT reagents and consulted with the research. W.L.C. directed the research. All authors discussed the results and reviewed the manuscript.

Disclosure of Conflicts of Interest

There are no conflicts to declare.

demonstrate that overactivation of the Wnt pathway may contribute to chemoresistance in relapsed childhood ALL and that Wnt-inhibition may be a promising therapeutic approach.

Keywords

acute lymphoblastic leukemia; phosphoflow cytometry; Wnt inhibition; chemosensitivity; relapse

The prognosis for children with ALL has improved dramatically over the last five decades (Hunger, Lu et al. 2012). However up to 20% of children will experience relapse, and the prognosis for these children is poor, with documented 5-year event free survival rates (EFS) rates of 12% and 35% for early (< 36 months from diagnosis) and late relapse (≥ 36 months), respectively (Nguyen, Devidas et al. 2008, van den Berg, de Groot-Kruseman et al. 2011). Intensification of treatment has not impacted the prognosis for the majority of patients highlighting the critical need for novel approaches for the prevention and treatment of relapsed and refractory disease. Intrinsic drug resistance is a key factor in the poor response to retrieval therapy as evidenced by lower remission rates, early second relapse and by ex vivo analysis of drug sensitivity at relapse vs. diagnosis (Klumper, Pieters et al. 1995, Raetz, Borowitz et al. 2008).

Aberrant Wnt signaling has been linked to cancers of the liver, colon, breast, skin (Polakis 2000, Miyoshi, Rosner et al. 2002, Miyoshi and Hennighausen 2003, Moon, Kohn et al. 2004) and more recently hematologic malignancies including acute myeloid leukemia (AML) and ALL (Reya and Clevers 2005, Gang, Hsieh et al. 2013). The transcriptional co-activator β-catenin is the key mediator of canonical Wnt signaling. Wnt activation results in increased accumulation of cytosolic β-catenin, which then translocates to the nucleus and interacts with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of high-mobility group (HMG) transcription factors to activate transcription of target genes (Nusse 1999, Staal and Clevers 2000). Also, many of the known target genes of the signaling pathway (*BIRC5, CCND1 and MYC)* are involved in cellular differentiation, proliferation and survival and have been implicated in carcinogenesis (Gehrke, Gandhirajan et al. 2009), making them attractive targets for therapy.

Previously, we performed an integrated genomic analysis of relapsed ALL to discover the underlying biological pathways responsible for drug resistance (Hogan, Meyer et al. 2011). We observed that many of the known negative regulators of the Wnt pathway were deleted and/or hypermethylated and/or downregulated at relapse in many patients, including the direct Wnt inhibitors: *WIF1, PTPRO, SFRP2, SFRP4, SFRP5, FZD10, DKK2* and *DKK3* (Nelson and Nusse 2004, Takahashi-Yanaga and Kahn 2010). Inhibitors of the β-catenin/TCF/LEF activity, including *APC, WT1* and many cadherin (*CDH1, CDH11* and *CD13*) and SOX genes (*SOX2, SOX3, SOX8, SOX9, SOX11, SOX14* and *SOX21*), were also negatively regulated. *BIRC5*, a downstream target of the Wnt pathway, which encodes the anti-apoptosis protein Survivin, was upregulated by gene expression array and validated by quantitative RT-PCR. Interestingly, *PTPRO*, a negative feedback inhibitor of the Wnt pathway, that binds to Wnt and blocks its association with other receptors, was hypermethylated and downregulated (Kim, Kim et al. 2010).

We hypothesized that overactivation of the Wnt pathway may have a critical function in mediating therapy resistance in some patients. To address this issue directly, we compared the alteration of Wnt signaling network activity (activated β-catenin) and the expression of downstream targets in 10 diagnosis-relapse paired patient samples, as well as the impact of a recently developed Wnt inhibitor, iCRT14 (Gonsalves, Klein et al. 2011) in ALL cell lines and primary patient samples. *BIRC5* was chosen as readout for β-catenin/TCF transcriptional activity because we have shown it to be upregulated at relapse and because overexpression has been associated with poor outcomes in certain cancers (Adida, Haioun et al. 2000, Paik, Shak et al. 2004, Fangusaro, Caldas et al. 2006). Collectively, our results indicate that overactivation of the Wnt pathway is often observed at relapse and that inhibition of Wnt signaling restores chemosensitivity in resistant disease.

Methods

Cell Lines, Patient Samples and Tissue Culture

B and T-lineage acute lymphoblastic leukemia cell lines (Reh, Nalm6, UOCB1 and Molt-4 cells), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 1% Penicillin/Streptomycin under 5% CO_2 at 37°C. Reh and Molt-4 cell lines were purchased from ATCC (American Type Culture Collection) (Manassas, VA, USA) and Nalm6 from DSMZ (Leibnitz Institut-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) (Braunschweig, Germany). UOCB1 cell line was received as a gift. Stock solutions of iCRT14 (ChemDiv, San Diego, CA) and etoposide were prepared in dimethyl sulfoxide, doxorubicin, cytarabine (Sigma-Aldrich, St. Louis, MO) and lithium chloride in ddH_20, prednisolone (Pharmacia, St Paul, MN) in 0.9% NaCl and 6-thioguanine (6TG) in 0.1N NaOH. Drugs were serially diluted in RPMI and added to the culture media at the indicated concentrations.

Phosphoflow cytometry was performed on a total of 10 matched pairs of diagnosis and relapse samples from individual patients that were obtained from the Children's Oncology Group (COG) tissue bank as cryopreserved, diagnostic bone marrow aspirates or peripheral blood samples.

An additional 8 samples (4 diagnosis, 4 relapse) were collected from patients treated or enrolled on correlative biology studies at the NYU Langone Medical Center. All primary samples were enriched from bone marrow or peripheral blood collections by Ficoll-Hypaque centrifugation and maintained in fresh culture medium (RPMI1640) containing 20% fetal bovine serum, 0.01% ITS (insulin, transferrin and sodium selenide- Sigma-Aldrich, St. Louis, MO) solution and 1% penicillin-streptomycin, before being treated as outlined below. All patients (or guardians) provided informed consent for the use of these samples for research studies in accordance with the Declaration of Helinski.

Small Molecule Compounds

Novel small molecule inhibitors of the Wnt pathway, iCRTs (inhibitor of catenin responsive transcription), were previously identified by a screen for compounds (Gonsalves, Klein et al. 2011) that interfere with the β-catenin-TCF protein-protein interaction, leading to inhibition

of target gene transcription without indiscriminate degradation of β-catenin or inhibition of the β-catenin interaction with E-cadherin and other junction proteins key to the structural integrity of cells. iCRT14 has also been shown to interfere with the binding of TCF to DNA (Gonsalves, Klein et al. 2011),(Lee, Madar et al. 2013). iCRTs 3, 5 and 14 (ChemDiv, San Diego, CA), were prepared in DMSO. They were tested in 3 B-lineage ALL cell lines (UOCB1, Nalm6 and Reh) by measuring the effect of varying dose concentrations of the compounds on cell viability after 48 hours of treatment and iCRT14 was selected for further experiments as it was the most potent compound across all cell lines (Figure S1). IC_{50} of the candidate compounds were shown to be in the nM to μM range (Gonsalves, Klein et al. 2011)

Phosphoflow Cytometry

10 paired diagnosis-relapse patient samples obtained from the COG cell bank were thawed, washed, viability determined on a ViaCell, and adjusted to 2×10^6 viable cells/mL in serum free media containing 0.5% BSA. Reh cells were used as a positive control. After resting cells for one hour at 37°, cells were fixed with 4% PFA (paraformaldehyde), permeabilized with 95% methanol and stained simultaneously for Caspase 3 V450 (C92-605, BD-560627) (San Jose, CA, USA), CD10 PE-Cy7 (BD-341092) (San Jose, CA, USA), Active β-catenin FITC (Clone 8E7, Millipore-05-665) (Billerica, MA, USA), and Survivin AlexaFluor647 (71G4B7, Cell Signaling 2866S) (Danvers, MA, USA) as previously described (Zhang, Ding et al. 2012). Samples were processed on a BD FACS Verse (San Jose, CA, USA) and analyzed using FLOWJO (Ver.9.6), Microsoft Excel (Ver12.2.5) (Redmond, WA, USA), and Prism (Ver. 5 software) (La Jolla, CA, USA). After eliminating doublets, forward and side scatter were used to eliminate cellular debris. Viable leukemic blasts were identified as CD10 positive, caspase 3 negative cells. This subgroup was gated upon for further analysis.

Quantitative Reverse Transcription PCR Analysis

To determine relative expression of each gene of interest, total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and q-reverse transcription -PCR was performed using the I-Script II complementary DNA Synthesis kit (Biorad, Hercules, CA) and the PerfeCTA SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD). Synthesis of PCR products was monitored by Agilent Technology's Mx3005P QPCR system (Santa Clara, CA, USA) and the results were normalized to *β2M* levels. Expression data were plotted relative to no treatment control samples using the ΔΔCt method. RT-PCR primers purchased from Qiagen: *BIRC5* QT01679664, *AXIN2* QT00037639 and *MYC* QT00035406. β*2M* primer sequences: Forward 5'-ATGTGTCTGGGTTTCATCCATCC-3', Reverse 5'-AGTCACATGGTTCACACGGCA-3'

Western Blotting

UOCB1 and Molt-4 cells were plated at 1 million cells/mL, treated with 20μM iCRT14 for 24 and 48 hours, lysed in RIPA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, and 0.5% NaDeoxycholate) containing protease inhibitors (Roche, Indianapolis, IN) and assayed for MYC, AXIN2, total caspase 3, cleaved caspase 3 and cleaved PARP while lysate for BIRC5 was prepared as previously described (Morrison, Hogan et al. 2012). Actin was used as a loading control. The signal was visualized using the ECL System (Amersham,

Piscataway, NJ, USA). Antibodies to the following proteins were used: BIRC5 1:100 (Santa Cruz Biotechnology, Dallas, TX, USA), MYC 1:2000 (Santa Cruz Biotechnology, Dallas, TX, USA), AXIN2 1: 200 (Abgent, San Diego, CA, USA), cleaved PARP 1:1000 (Cell Signaling Technology, Danvers, MA, USA), total caspase 3 1:500 (Cell Signaling Technology, Danvers, MA, USA), cleaved caspase 3 1:500 (Cell Signaling Technology, Danvers, MA, USA) and Actin 1:10,000 (Abcam) (Cambridge, MA, USA). HRP-conjugated anti-mouse IgG, 1:10,000 (GE Healthcare) (Piscataway, NJ, USA) and HRP-conjugated anti-rabbit IgG, 1:10,000 (GE Healthcare) (Piscataway, NJ, USA) were used as the secondary antibodies.

Cell Viability Assays

CellTiter-Glo Luminescent Cell Viability assays (Promega, Madison, WI) were performed on patient samples and cell lines grown in the conditions described above. Cell lines were seeded in 96-well plates at a concentration of 0.4 million cells/mL and treated with iCRT14 (0-40 μM). A panel of chemotherapeutic agents used in the therapy of ALL was added concurrently at hour 0 for the concurrent assays (etoposide, prednisolone and doxorubicin) and after 48 hours of iCRT pretreatment for the pretreatment assays (etoposide, prednisolone, doxorubicin, cytarabine and 6TG). iCRT14 doses were added daily to the cells in the pretreatment assays. CellTiter-Glo reagent was added and luminescence was recorded using a Flexstation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and the SoftMax Pro Data Acquisition and Analysis Software (Molecular Devices, Sunnyvale, CA, USA) at hour 24 for the concurrent assays and hour 72 for the pretreatment assays. Drug combination effects were analyzed by the Calcusyn software (Biosoft, Cambridge, GB, UK) according to the combination index (CI) equation of Chou-Talalay (Chou 2010). This analysis describes the drug interaction as: CI >1.1 antagonistic, 0.9-1.1 additive, <0.9 synergistic. Patient samples were pretreated with iCRT14 for only 24 hours because of their known decreased viability.

Apoptosis Assays

Cell lines were seeded in 96-well plates (2 million cells/mL) and treated with iCRT14 (0-40uM) for 24, 48 or 72 hours. Chemotherapy was added either concurrently (etoposide 0.4 μM) or after 48 hours of pretreatment with iCRT14 (etoposide [0-4 μM], prednisolone [0-500 μg/ml], and doxorubicin [0-500 nM]). iCRT14 doses were refreshed daily. 24 hours after the addition of the chemotherapy, the cells were stained with Annexin-V-PE and 7AAD (Annexin V-PE Apoptosis Detection Kit, BD Pharmingen) (Piscataway, NJ, USA) and evaluated for apoptosis by flow cytometry using the FACScan (Becton-Dickinson, Franklin lakes, NJ, USA). Data was analyzed using the FlowJo software (version 7.6.5, TreeSTAr Inc. Ashland, OR, USA).

Luciferase Reporter Assays

Reh cells were infected with the 7 TFP (7xTcf-FFluc//SV40-PuroR) reporter lentivirus to make a stable Wnt reporter cell line.(Fuerer and Nusse 2010) The cells were treated with 10 mM concentration of lithium chloride (LiCl) for 24 hours to induce Wnt activation, and then plated in a 96-well plate at a concentration of 0.3 million cells/mL. 20 μM of iCRT14 was added to the cells at the time of plating and the assay was performed 24 hours after addition

of iCRT14. Presto Blue Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was used to normalize the data. Presto blue was added to each well and the fluorescence intensity in each well was measured after 1 hour of incubation, using a Flexstation 3 Microplate Reader (Molecular Devices) and the SoftMax Pro Data Acquisition and Analysis Software (Molecular Devices). Luciferase levels were measured using the Dual Glo Luciferase system (Promega, WI, USA). Results are reported as Relative Luciferase Units (RLU) and normalized to the Presto blue fluorescent signal.

Results

Multiparameter Phosphoflow Cytometry Reveals Overactivation of the Wnt/β-catenin Pathway at Relapse

To test our hypothesis that hyperactivation of the WNT/β-catenin pathway mediates chemotherapy resistance and contributes to relapse in a substantial number of patients, we used multiparameter phosphoflow cytometry to evaluate the level of Wnt/β-catenin pathway activation with antibodies validated for phosphoflow on 10 paired diagnosis-relapse primary patient samples. We selected phospho-Activated β-catenin and BIRC5 (one of the downstream targets of the Wnt pathway) as markers of Wnt/β-catenin pathway activation. The pairs were stained simultaneously for caspase 3 (used as a marker of cell viability), CD10, activated β-catenin and BIRC5 and the expression of activated β-catenin and BIRC5 was compared in diagnosis and relapsed samples from the same patient by gating on the CD10 positive, caspase 3 negative viable leukemic cell population (Figure 1A, Figure S2-A). Comparison of the mean fluorescent intensity (MFI) of activated β-catenin and BIRC5 indicated that activated β-catenin was upregulated in 6 of the 10 patients at relapse (Patients 2, 3, 4, 6, 9 and 10) (Figure 1B), with a statistically significant difference between diagnosis and relapse for activated β-catenin (P=0.0232) (Figure S2-B). BIRC5 was increased at relapse in 6 patients (Patients 2, 6, 7, 8, 9 and 10). In 4 pairs, the upregulation of BIRC5 coincided with activated β-catenin (Patients 2, 6, 9 and 10) (Figure 1C) and in 1 pair (Patient 3, increased activated β-catenin was associated with the presence of a subpopulation of BIRC5 high cells. Interestingly, 1 pair showed up-regulation of activated β-catenin without significant activation of BIRC5 (Patient 4) and another patient showed the reverse (Patient 7) (Figure 1B and S2-A). Half of the patients showed a bimodal distribution of BIRC5 both at diagnosis as well as relapse with patients 2, 6 and 10 showing a shift towards a greater percentage of cells demonstrating high BIRC5 expression from diagnosis to relapse suggesting preferential selection of this subclone at relapse (Figure S2-A).

Treatment of B and T Lineage Leukemic Cells With iCRT14 Shows Decreased Expression of Wnt Target Genes

Using phospho flowcytometry, upregulation of the Wnt signaling network activity was observed in ALL cell lines (Figure S3). To test the effect of iCRT14 on Wnt target genes in leukemic cell lines, mRNA expression of *BIRC5, AXIN2 and MYC* was evaluated. We have previously shown that *BIRC5* is upregulated at relapse in pediatric ALL and preclinical models support it as a target (Morrison, Hogan et al. 2012). *AXIN2* was selected as it is regarded to be a general indicator of Wnt pathway activity and *MYC* was selected as it is a known proto-oncogene widely implicated in cancer (He, Sparks et al. 1998). Initially, Reh,

UOCB1 and Molt-4 cell lines were treated with varying concentrations of iCRT14 [0, 10, 20 and 30 μM] for 24 hours and a dose responsive decrease in *BIRC5* mRNA expression was observed (Figure S4). To test the expression of target genes at two time points (24 and 48 hours), the 20 μM dose concentration was selected as it showed a 40-60% decrease in *BIRC5* mRNA expression across all cell lines at hour 24. *BIRC5* mRNA was decreased at both time points for all cell lines tested (UOCB1, Reh, Nalm6 and Molt-4), with 70-80% decrease in expression by hour 48 (Figure 2A). Protein lysates prepared from iCRT14 treated and vehicle treated cells showed a corresponding decrease in the protein levels of BIRC5 and MYC in UOCB1 and Molt-4 cells at both 24 and 48 hours and a decrease in AXIN2 in UOCB1 cells at both time points (Figure 2B).

To further examine the inhibitory role of iCRT14, a Wnt reporter stable cell line was pretreated with the GSK3β inhibitor, lithium chloride (LiCl), which induced Wnt activation, and then treated with iCRT14 for 24 hours. A 50% decrease in reporter activity was observed after treatment with iCRT14 (Figure S5). These findings provide support for the inhibitory role of iCRT14 in these cell lines, in agreement with published data.(Gonsalves, Klein et al. 2011)

iCRT14 Sensitizes Leukemic Cells to Traditional Chemotherapy

To determine if Wnt inhibition can sensitize leukemic cells to chemotherapy, B-lineage cell lines (UOCB1, Nalm6 and Reh) were pretreated with the Wnt-inhibitor for 48 hours followed by the addition of traditional chemotherapeutic agents. Since we observed a significant decrease in the expression of Wnt target genes in UOCB1 and Molt-4 cells as well as increased apoptosis in UOCB1 cells at the 48 hour time point (Figure S6-A); this interval was selected as the optimal interval for priming the leukemic cell lines with the inhibitor before treatment with chemotherapy. Effects on cell viability were assessed at hour 72 (i.e. 24 hours after adding chemotherapy).

All 3 cell lines showed a dose dependent decrease in cell viability after treatment with iCRT14 alone for 72 hours, with a decrease of at least 80% by hour 72 with the maximum dose (40μM). In addition, combination of iCRT14 with the chosen traditional chemotherapeutic agents demonstrated additive to synergistic effects as determined by the Calcusyn program (Figure 3F and S7). The UOCB1 cells showed great synergism with all 5 chemotherapeutic agents tested (CI=0.1-0.88) (Figure 3A-E). The Nalm6 cells were most sensitive to iCRT14 showing a 50% or greater decrease in cell viability with the first dose of 10μM alone, hence were treated with doses of 0-30μM only. Since the Wnt inhibitor alone caused significant decrease in cell viability in these cells, the combination of chemotherapy and iCRT showed additive to synergistic effects (CI=0.05-1.1). In the Reh cells, all agents other than cytarabine showed robust synergism in combination with iCRT14 (CI=0.03-0.55). These data suggest that pretreatment with the Wnt inhibitor sensitizes leukemic cell lines to the effects of chemotherapy. We also tested concurrent administration of iCRT14 and chemotherapy (etoposide [0-10 μM], prednisolone [0-500 μg/mL] and doxorubicin [0-125 nM]) at 24 and 48 hours which resulted in additive and synergistic effects only in UOCB1 cell line at the 24 hour time point (Figure S8), and did not show induction of apoptosis

(Figure S6 B-C); further establishing that iCRT pretreatment is essential for sensitizing leukemic cells to the effects of chemotherapy.

To demonstrate that this effect was not limited to iCRT14, experiments were replicated with iCRT3, in UOCB1 cells. A similar dose dependent decrease in cell viability with the inhibitor alone and excellent synergism with chemotherapy was observed, suggesting that interruption of the β-catenin-TCF interaction is detrimental to the viability of the leukemic cells (Figure S9).

iCRT14 Synergizes With Traditional Chemotherapy to Potentiate Apoptosis and Reverse Chemoresistance

Since pretreatment with iCRT14 synergizes with chemotherapy to markedly decrease cell viability, we examined if the combination could further enhance apoptotic cell death. UOCB1 and Reh cells were pretreated with 20 and 40 μM of iCRT14 for 48 hours, followed by the addition of chemotherapeutic agents in 2 dose concentrations (etoposide [0.8 and 4 μM], prednisolone [300 and 500 μg/ml] and doxorubicin [200 and 500 nm]) for another 24 hours. Nalm6 cells were very sensitive to the Wnt inhibitor as seen previously with the cell viability assays and were treated with lower dose concentrations of iCRT14 (5 and 10 μM) to detect the synchronous effects of chemotherapy. Cells were stained with Annexin V-PE and 7AAD to identify the percentage of apoptotic cells. iCRT14 alone contributed significantly to apoptosis with a 10-30% increase in the percentage of apoptotic cells at the lowest dose which increased further to 15-50 % with the second dose (Figure 4 A-C). The combination of iCRT14 and chemotherapy demonstrated an increased amount of cell death in all cell lines with > 80 % apoptosis across all cell lines by hour 72 with the maximal dose of chemotherapy.

Change in the protein levels of apoptotic markers were examined as a corollary to Annexin-V staining. Treatment of UOCB1 cells with 20μM of iCRT14 showed cleavage of caspase 3 starting at hour 24 that was very pronounced by hour 48. As the cleaved caspase 3 signal increased, there was a concomitant decrease in the total caspase 3 signal. Cleaved PARP was readily detected by 24 hours and increased further at hour 48 (Figure 4D).

Ex vivo Treatment of Patient Samples With iCRT14 and Prednisolone Confirms Enhanced Chemosensitivity with Wnt Inhibition.

To validate our findings in primary samples, leukemic blasts from 4 newly diagnosed (1-4) and 4 relapsed patient samples (5-8) were examined for changes in chemosensitivity to prednisolone after the addition of iCRT14. Prednisolone was chosen as previous work by Klumpers et al demonstrated that leukemic blasts from patients with relapsed ALL express the highest level of resistance to glucocorticoids at relapse.(Klumper, Pieters et al. 1995) All 4 diagnosis samples, showed a significant decrease in viability with prednisolone alone and 3 out of 4 patients, showed a further decrease in viability with addition of iCRT14 (Figure 5A). However, as chemotherapy alone decreased viability markedly in this chemotherapy naive group, synergism could not be established with iCRT14. As expected all relapse patient samples were much less sensitive to prednisolone alone as compared to diagnosis samples (40% decrease in viability as compared to 80%). Interestingly, all the relapsed

patients demonstrated a decrease in viability with the addition of the inhibitor alone at both doses and a 50% or greater decline further in viability in combination with prednisolone, showing increased chemosensitivity in response to Wnt inhibition (Figure 5B). Patient 7 was very sensitive to the Wnt inhibitor, with a 90% decrease in viability with iCRT14 alone. The combination of prednisolone and iCRT14 showed additive to synergistic effects (CI=0.7 and 1) in one of the patients (patient 6) while 3 patients showed strong synergism with both doses of the inhibitor (CI=0.03-0.6).

Discussion

The Wnt pathway is an evolutionarily conserved signaling network, that plays a critical role in the development of various organ systems and has been implicated in hematopoietic stem cell (HSC) maintenance and cell fate determination(Kleber and Sommer 2004). Stimulation of normal hematopoietic progenitor cells with Wnt proteins or overexpression of activated β-catenin increases self- renewal and multilineage reconstitution in mouse models (Murdoch, Chadwick et al. 2003), (Reya, Duncan et al. 2003).

In addition to playing a vital role in the self-renewal of HSCs, Wnt signaling also contributes significantly to the survival and expansion of lymphocyte progenitors. *LEF1* deficient mice have been shown to have defects in the pro-B cell proliferation, both in vivo and in vitro. Aberrations in the Wnt pathway have been implicated in the induction of many hematologic malignancies(Staal and Clevers 2005) including acute myeloid leukemia (AML)(Muller-Tidow, Steffen et al. 2004, Wang, Krivtsov et al. 2010), chronic lymphoid leukemia (CLL)(Lu, Zhao et al. 2004), blast crisis of chronic myeloid leukemia (CML) (Jamieson, Ailles et al. 2004), multiple myeloma(Derksen, Tjin et al. 2004) and more recently ALL (Khan, Bradstock et al. 2007).

In pre B ALL, the *E2A-PBX1* fusion protein resulting from the t (1;19) chromosomal translocation, has been shown to upregulate the expression of *WNT 16B* suggesting that Wnt 16B mediated autocrine growth mechanisms may contribute to the biology of the disease and targeted inhibition of *WNT 16B* has been shown to induce apoptosis (McWhirter, Neuteboom et al. 1999, Mazieres, You et al. 2005). Moreover, the activation of β-catenin has also been proposed as a mechanism for Notch independent induction of T-ALL (Guo, Dose et al. 2007).

Our previous work, implicated overactivation of the Wnt pathway at relapse in childhood ALL.(Hogan, Meyer et al. 2011) Through our multiplatform analysis, we demonstrated that several negative regulators of the Wnt pathway were selectively deleted hypermethylated, and/or downregulated at relapse. *BIRC5,* a downstream target of the Wnt pathway, was upregulated in our analysis both by gene expression and by RT-PCR. Others have also explored the role of epigenetic regulation of Wnt inhibitors in the biology of ALL. Roman-Gomez et al observed down regulation of the expression of the Wnt inhibitors *SFRP1, SFRP2, SFRP4, SFRP5, WIF1, DKK3* and *HDPR1* by promoter hypermethylation, in ALL cell lines and lymphoblasts from both children and adults with newly diagnosed ALL, along with a concordant upregulation of Wnt target genes *TCF1, LEF1, CCND1, WNT16 and FZ3* (Roman-Gomez, Cordeu et al. 2007). Abnormal methylation of the Wnt inhibitors was

associated with a decreased 10 year disease free survival (25% vs. 66%, $p < 0.001$) and overall survival (28% vs. 61%, p=0.001). Importantly, treatment with a demethylating agent 5-aza-2'-deoxycytidine downregulated the expression of Wnt target genes. Likewise we have recently demonstrated that treatment with the DNA methyltransferase inhibitor (DNMTi) decitabine can lead to re-expression of (2-31 fold) induction of expression of *WT1* and *PTPRO* in cell lines (Bhatla, Wang et al. 2012).

We have validated our hypothesis by demonstrating enhanced activation of the Wnt pathway at relapse in patients by demonstrating upregulation of activated β-catenin in 6 of 10 patients from diagnosis to relapse, with concordant increase in activated β-catenin and BIRC5 in 4 of the 6 pairs. The fact that Wnt/β-catenin was activated in a significant number but not all patients is consistent with our previous data demonstrating that multiple pathways mediate disease recurrence (Hogan, Meyer et al. 2011). The high expression of BIRC5 at relapse is also consistent with previous work (Hogan, Meyer et al. 2011). In 1 patient (Patient 8) the changes in activated β-catenin and BIRC5 were discordant suggesting the complex heterogeneity of cell signaling networks.

Wnt signaling leads to the activation of several downstream targets of the pathway, many of which are known proto-oncogenes such as *MYC*, CCND1, and *BIRC5,* thus providing a possible explanation for the association with chemoresistance.(He, Sparks et al. 1998, Tetsu and McCormick 1999, Zhang, Otevrel et al. 2001) Our experiments show consistent downregulation of the Wnt target genes *BIRC5, MYC and AXIN2* with Wnt inhibition that is associated with increased chemosensitivity. *BIRC5*, a member of the inhibitor of apoptosis (IAP) family, regulates cell cycle progression and inhibits caspase function (Pennati, Folini et al. 2007), thus mediating chemoresistance. We have previously shown near consistent upregulation of *BIRC5* at relapse and that downregulation of *BIRC5* by targeting it with both short-hairpin RNA and locked antisense oligonucelotide, induces apoptosis and leads to increased chemo sensitivity (Morrison, Hogan et al. 2012).

Likewise, Khan et al have shown that exogenous Wnt proteins can increase cell survival and proliferation in the pre-B ALL cell lines Nalm6, Reh and LK63 (Khan, Bradstock et al. 2007). The bone marrow microenvironment and direct contact between leukemic cells and marrow stromal cells (MSCs) are essential for leukemic cell survival and have a protective effect against chemotherapy (Mudry, Fortney et al. 2000). Yang et al have demonstrated that the Wnt pathway contributes to the protection of ALL cells by bone marrow stromal support(Yang, Mallampati et al. 2013). Consistent with our findings that Wnt inhibition is a novel therapeutic approach for relapsed ALL, Gang et al very recently have shown that ICG-001, a CREB-binding protein (CBP) inhibitor(Emami, Nguyen et al. 2004) that disrupts the CBP/β- and γ-catenin interactions, leads to decreased self-renewal capacity of the ALL cells, downregulates BIRC5 and helps eradicate the drug resistant clones in ALL (Gang, Hsieh et al. 2013).

Collectively this work indicates that disruption of the Wnt pathways may be an attractive therapeutic strategy for relapsed ALL. Significant research efforts are currently underway at targeting the Wnt pathway with various small molecules such as XAV939, a Tankyrase inhibitor that destabilizes β-catenin by increasing the levels of axin (Huang, Mishina et al.

2009); Pyrvinium, a CK1 inhibitor (Thorne, Hanson et al. 2010), ICG-001, and most recently, a small molecule inhibitor that blocks Porcupine, the enzyme promoting palmitoylation, and thus secretion of Wnt ligands (Lu, Ma et al. 2009). Phase I clinical trials are ongoing with LGK974, an orally bioavailable porcupine inhibitor, in melanoma and breast cancer; with OMP-18R5, a monoclonal antibody targeting the frizzled receptors and blocking their associated with Wnt ligands and with PRI-724, a small molecule inhibitor of the CBP/β-catenin interaction, in advanced solid tumors and in myeloid leukemias. Evaluation of these agents in children with relapsed or refractory leukemia is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by US NIH grants R01 CA140729 and R21 CA152838-02 to WLC; R01 CA155125-01 to R.D; NYU Langone Cancer Center Support Grant 5 P30 CA16087-30; St. Baldrick's Foundation, Campini Foundation, and the Pepp Family Foundation grants to MH and the Pediatric Cancer Foundation to SD. Additional support was provided by grants from the National Cancer Institute to COG, including U10 CA98543 (COG Chair's grant), U10 CA98413 (COG Statistical Center), and U24 CA114766 (COG Specimen Banking)

References

Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, Dombret H, Reyes F, Diebold J, Gisselbrecht C, Salles G, Altieri DC, Molina TJ. Prognostic significance of survivin expression in diffuse large B-cell lymphomas. Blood. 2000; 96(5):1921–1925. [PubMed: 10961895]

Bhatla T, Wang J, Morrison DJ, Raetz EA, Burke MJ, Brown P, Carroll WL. Epigenetic reprogramming reverses the relapse-specific gene expression signature and restores chemosensitivity in childhood B-lymphoblastic leukemia. Blood. 2012; 119(22):5201–5210. [PubMed: 22496163]

Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010; 70(2):440–446. [PubMed: 20068163]

Derksen PW, Tjin E, Meijer HP, Klok MD, MacGillavry HD, van Oers MH, Lokhorst HM, Bloem AC, Clevers H, Nusse R, van der Neut R, Spaargaren M, Pals ST. Illegitimate WNT signaling promotes proliferation of multiple myeloma cells. Proc Natl Acad Sci U S A. 2004; 101(16):6122–6127. [PubMed: 15067127]

Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Kim HY, Moon SH, Ha JR, Kahn M. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci U S A. 2004; 101(34):12682–12687. [PubMed: 15314234]

Fangusaro JR, Caldas H, Jiang Y, Altura RA. Survivin: an inhibitor of apoptosis in pediatric cancer. Pediatr Blood Cancer. 2006; 47(1):4–13. [PubMed: 16534789]

Fuerer C, Nusse R. Lentiviral vectors to probe and manipulate the Wnt signaling pathway. PLoS One. 2010; 5(2):e9370. [PubMed: 20186325]

Gang EJ, Hsieh YT, Pham J, Zhao Y, Nguyen C, Huantes S, Park E, Naing K, Klemm L, Swaminathan S, Conway EM, Pelus LM, Crispino J, Mullighan CG, McMillan M, Muschen M, Kahn M, Kim YM. Small-molecule inhibition of CBP/catenin interactions eliminates drug-resistant clones in acute lymphoblastic leukemia. Oncogene. 2013

Gehrke I, Gandhirajan RK, Kreuzer KA. Targeting the WNT/beta-catenin/TCF/LEF1 axis in solid and haematological cancers: Multiplicity of therapeutic options. Eur J Cancer. 2009; 45(16):2759–2767. [PubMed: 19729298]

Gonsalves FC, Klein K, Carson BB, Katz S, Ekas LA, Evans S, Nagourney R, Cardozo T, Brown AM, DasGupta R. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of

the Wnt/wingless signaling pathway. Proc Natl Acad Sci U S A. 2011; 108(15):5954–5963. [PubMed: 21393571]

Guo Z, Dose M, Kovalovsky D, Chang R, O'Neil J, Look AT, von Boehmer H, Khazaie K, Gounari F. Beta-catenin stabilization stalls the transition from double-positive to single-positive stage and predisposes thymocytes to malignant transformation. Blood. 2007; 109(12):5463–5472. [PubMed: 17317856]

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science. 1998; 281(5382):1509–1512. [PubMed: 9727977]

Hogan LE, Meyer JA, Yang J, Wang J, Wong N, Yang W, Condos G, Hunger SP, Raetz E, Saffery R, Relling MV, Bhojwani D, Morrison DJ, Carroll WL. Integrated genomic analysis of relapsed childhood acute lymphoblastic leukemia reveals therapeutic strategies. Blood. 2011; 118(19): 5218–5226. [PubMed: 21921043]

Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Zhang Y, Wiessner S, Hild M, Shi X, Wilson CJ, Mickanin C, Myer V, Fazal A, Tomlinson R, Serluca F, Shao W, Cheng H, Shultz M, Rau C, Schirle M, Schlegl J, Ghidelli S, Fawell S, Lu C, Curtis D, Kirschner MW, Lengauer C, Finan PM, Tallarico JA, Bouwmeester T, Porter JA, Bauer A, Cong F. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature. 2009; 461(7264): 614–620. [PubMed: 19759537]

Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, Reaman GH, Carroll WL. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. J Clin Oncol. 2012; 30(14):1663–1669. [PubMed: 22412151]

Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, Sawyers CL, Weissman IL. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med. 2004; 351(7):657–667. [PubMed: 15306667]

Khan NI, Bradstock KF, Bendall LJ. Activation of Wnt/beta-catenin pathway mediates growth and survival in B-cell progenitor acute lymphoblastic leukaemia. Br J Haematol. 2007; 138(3):338–348. [PubMed: 17614820]

Kim M, Kim H, Jho EH. Identification of ptpro as a novel target gene of Wnt signaling and its potential role as a receptor for Wnt. FEBS Lett. 2010; 584(18):3923–3928. [PubMed: 20804755]

Kleber M, Sommer L. Wnt signaling and the regulation of stem cell function. Curr Opin Cell Biol. 2004; 16(6):681–687. [PubMed: 15530781]

Klumper E, Pieters R, Veerman AJ, Huismans DR, Loonen AH, Hahlen K, Kaspers GJ, van Wering ER, Hartmann R, Henze G. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. Blood. 1995; 86(10):3861–3868. [PubMed: 7579354]

Lee E, Madar A, David G, Garabedian MJ, Dasgupta R, Logan SK. Inhibition of androgen receptor and beta-catenin activity in prostate cancer. Proc Natl Acad Sci U S A. 2013; 110(39):15710–15715. [PubMed: 24019458]

Lu D, Zhao Y, Tawatao R, Cottam HB, Sen M, Leoni LM, Kipps TJ, Corr M, Carson DA. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2004; 101(9):3118–3123. [PubMed: 14973184]

Lu J, Ma Z, Hsieh JC, Fan CW, Chen B, Longgood JC, Williams NS, Amatruda JF, Lum L, Chen C. Structure-activity relationship studies of small-molecule inhibitors of Wnt response. Bioorg Med Chem Lett. 2009; 19(14):3825–3827. [PubMed: 19410457]

Mazieres J, You L, He B, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM. Inhibition of Wnt16 in human acute lymphoblastoid leukemia cells containing the t(1;19) translocation induces apoptosis. Oncogene. 2005; 24(34):5396–5400. [PubMed: 16007226]

McWhirter JR, Neuteboom ST, Wancewicz EV, Monia BP, Downing JR, Murre C. Oncogenic homeodomain transcription factor E2A-Pbx1 activates a novel WNT gene in pre-B acute lymphoblastoid leukemia. Proc Natl Acad Sci U S A. 1999; 96(20):11464–11469. [PubMed: 10500199]

Miyoshi K, Hennighausen L. Beta-catenin: a transforming actor on many stages. Breast Cancer Res. 2003; 5(2):63–68. [PubMed: 12631383]

Miyoshi K, Rosner A, Nozawa M, Byrd C, Morgan F, Landesman-Bollag E, Xu X, Seldin DC, Schmidt EV, Taketo MM, Robinson GW, Cardiff RD, Hennighausen L. Activation of different Wnt/beta-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors. Oncogene. 2002; 21(36):5548–5556. [PubMed: 12165853]

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet. 2004; 5(9):691–701. [PubMed: 15372092]

Morrison DJ, Hogan LE, Condos G, Bhatla T, Germino N, Moskowitz NP, Lee L, Bhojwani D, Horton TM, Belitskaya-Levy I, Greenberger LM, Horak ID, Grupp SA, Teachey DT, Raetz EA, Carroll WL. Endogenous knockdown of survivin improves chemotherapeutic response in ALL models. Leukemia. 2012; 26(2):271–279. [PubMed: 21844871]

Mudry RE, Fortney JE, York T, Hall BM, Gibson LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. Blood. 2000; 96(5):1926–1932. [PubMed: 10961896]

Muller-Tidow C, Steffen B, Cauvet T, Tickenbrock L, Ji P, Diederichs S, Sargin B, Kohler G, Stelljes M, Puccetti E, Ruthardt M, deVos S, Hiebert SW, Koeffler HP, Berdel WE, Serve H. Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. Mol Cell Biol. 2004; 24(7):2890–2904. [PubMed: 15024077]

Murdoch B, Chadwick K, Martin M, Shojaei F, Shah KV, Gallacher L, Moon RT, Bhatia M. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. Proc Natl Acad Sci U S A. 2003; 100(6):3422–3427. [PubMed: 12626754]

Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science. 2004; 303(5663):1483–1487. [PubMed: 15001769]

Nguyen K, Devidas M, Cheng SC, La M, Raetz EA, Carroll WL, Winick NJ, Hunger SP, Gaynon PS, Loh ML. Factors influencing survival after relapse from acute lymphoblastic leukemia: a Children's Oncology Group study. Leukemia. 2008; 22(12):2142–2150. [PubMed: 18818707]

Nusse R. WNT targets. Repression and activation. Trends Genet. 1999; 15(1):1–3. [PubMed: 10087922]

Paik S, Shak S, Tang G, Kim C, Baker J, Cronin M, Baehner FL, Walker MG, Watson D, Park T, Hiller W, Fisher ER, Wickerham DL, Bryant J, Wolmark N. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004; 351(27):2817–2826. [PubMed: 15591335]

Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy: fulfilled promises and open questions. Carcinogenesis. 2007; 28(6):1133–1139. [PubMed: 17341657]

Polakis P. Wnt signaling and cancer. Genes Dev. 2000; 14(15):1837–1851. [PubMed: 10921899]

Raetz EA, Borowitz MJ, Devidas M, Linda SB, Hunger SP, Winick NJ, Camitta BM, Gaynon PS, Carroll WL. Reinduction platform for children with first marrow relapse of acute lymphoblastic Leukemia: A Children's Oncology Group Study[corrected]. J Clin Oncol. 2008; 26(24):3971–3978. [PubMed: 18711187]

Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005; 434(7035):843–850. [PubMed: 15829953]

Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 2003; 423(6938): 409–414. [PubMed: 12717450]

Roman-Gomez J, Cordeu L, Agirre X, Jimenez-Velasco A, San Jose-Eneriz E, Garate L, Calasanz MJ, Heiniger A, Torres A, Prosper F. Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. Blood. 2007; 109(8):3462–3469. [PubMed: 17148581]

Staal FJ, Clevers H. Tcf/Lef transcription factors during T-cell development: unique and overlapping functions. Hematol J. 2000; 1(1):3–6. [PubMed: 11920163]

Staal FJ, Clevers HC. WNT signalling and haematopoiesis: a WNT-WNT situation. Nat Rev Immunol. 2005; 5(1):21–30. [PubMed: 15630426]

Takahashi-Yanaga F, Kahn M. Targeting Wnt signaling: can we safely eradicate cancer stem cells? Clin Cancer Res. 2010; 16(12):3153–3162. [PubMed: 20530697]

Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature. 1999; 398(6726):422–426. [PubMed: 10201372]

Thorne CA, Hanson AJ, Schneider J, Tahinci E, Orton D, Cselenyi CS, Jernigan KK, Meyers KC, Hang BI, Waterson AG, Kim K, Melancon B, Ghidu VP, Sulikowski GA, LaFleur B, Salic A, Lee LA, Miller DM 3rd, Lee E. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1alpha. Nat Chem Biol. 2010; 6(11):829–836. [PubMed: 20890287]

van den Berg H, de Groot-Kruseman HA, Damen-Korbijn CM, de Bont ES, Schouten-van Meeteren AY, Hoogerbrugge PM. Outcome after first relapse in children with acute lymphoblastic leukemia: a report based on the Dutch Childhood Oncology Group (DCOG) relapse all 98 protocol. Pediatr Blood Cancer. 2011; 57(2):210–216. [PubMed: 21337680]

Wang Y, Krivtsov AV, Sinha AU, North TE, Goessling W, Feng Z, Zon LI, Armstrong SA. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science. 2010; 327(5973):1650–1653. [PubMed: 20339075]

Yang Y, Mallampati S, Sun B, Zhang J, Kim SB, Lee JS, Gong Y, Cai Z, Sun X. Wnt pathway contributes to the protection by bone marrow stromal cells of acute lymphoblastic leukemia cells and is a potential therapeutic target. Cancer Lett. 2013; 333(1):9–17. [PubMed: 23333798]

Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, Easton J, Chen X, Wang J, Rusch M, Lu C, Chen SC, Wei L, Collins-Underwood JR, Ma J, Roberts KG, Pounds SB, Ulyanov A, Becksfort J, Gupta P, Huether R, Kriwacki RW, Parker M, McGoldrick DJ, Zhao D, Alford D, Espy S, Bobba KC, Song G, Pei D, Cheng C, Roberts S, Barbato MI, Campana D, Coustan-Smith E, Shurtleff SA, Raimondi SC, Kleppe M, Cools J, Shimano KA, Hermiston ML, Doulatov S, Eppert K, Laurenti E, Notta F, Dick JE, Basso G, Hunger SP, Loh ML, Devidas M, Wood B, Winter S, Dunsmore KP, Fulton RS, Fulton LL, Hong X, Harris CC, Dooling DJ, Ochoa K, Johnson KJ, Obenauer JC, Evans WE, Pui CH, Naeve CW, Ley TJ, Mardis ER, Wilson RK, Downing JR, Mullighan CG. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012; 481(7380):157–163. [PubMed: 22237106]

Zhang T, Otevrel T, Gao Z, Ehrlich SM, Fields JZ, Boman BM. Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res. 2001; 61(24):8664–8667. [PubMed: 11751382]

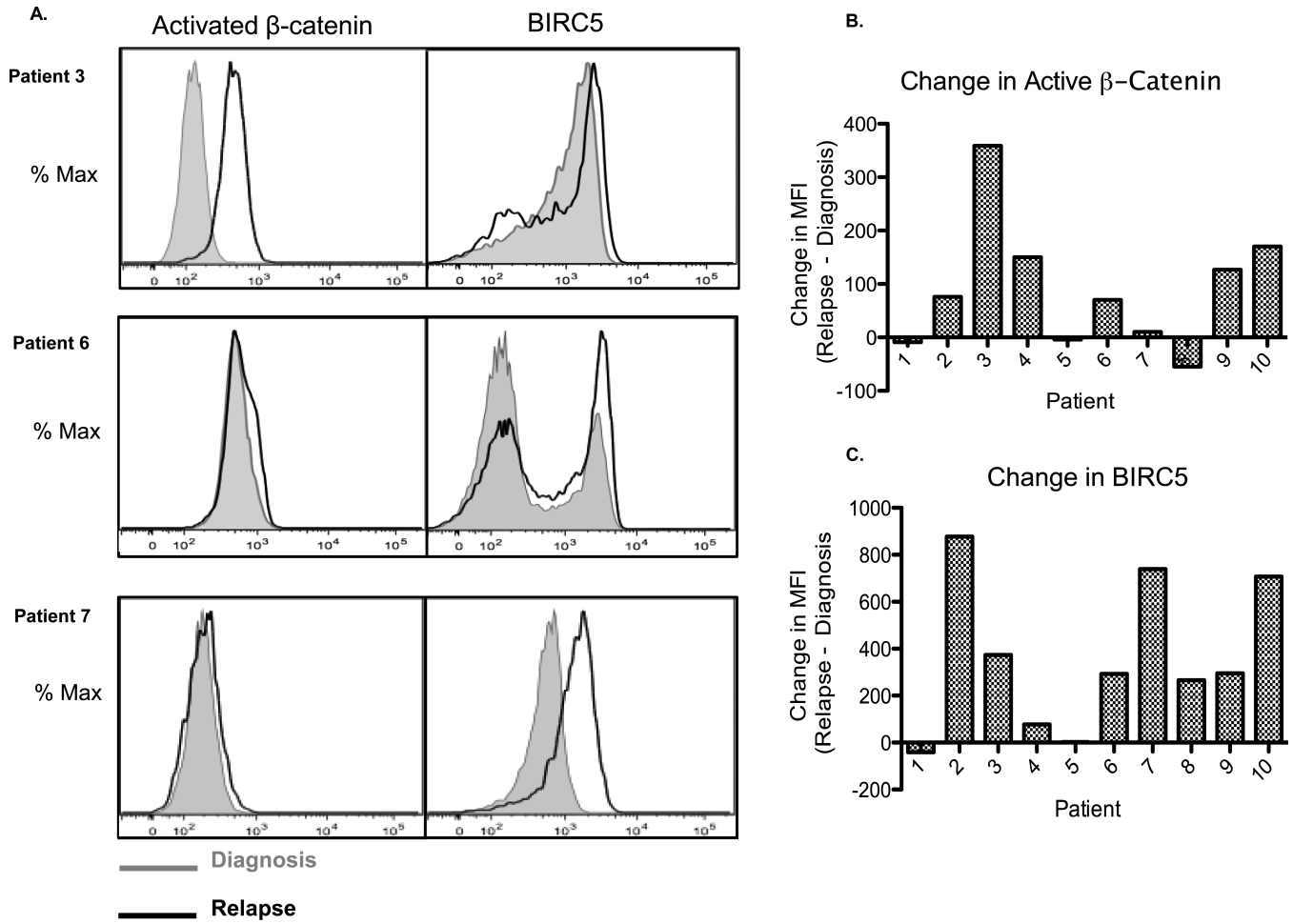
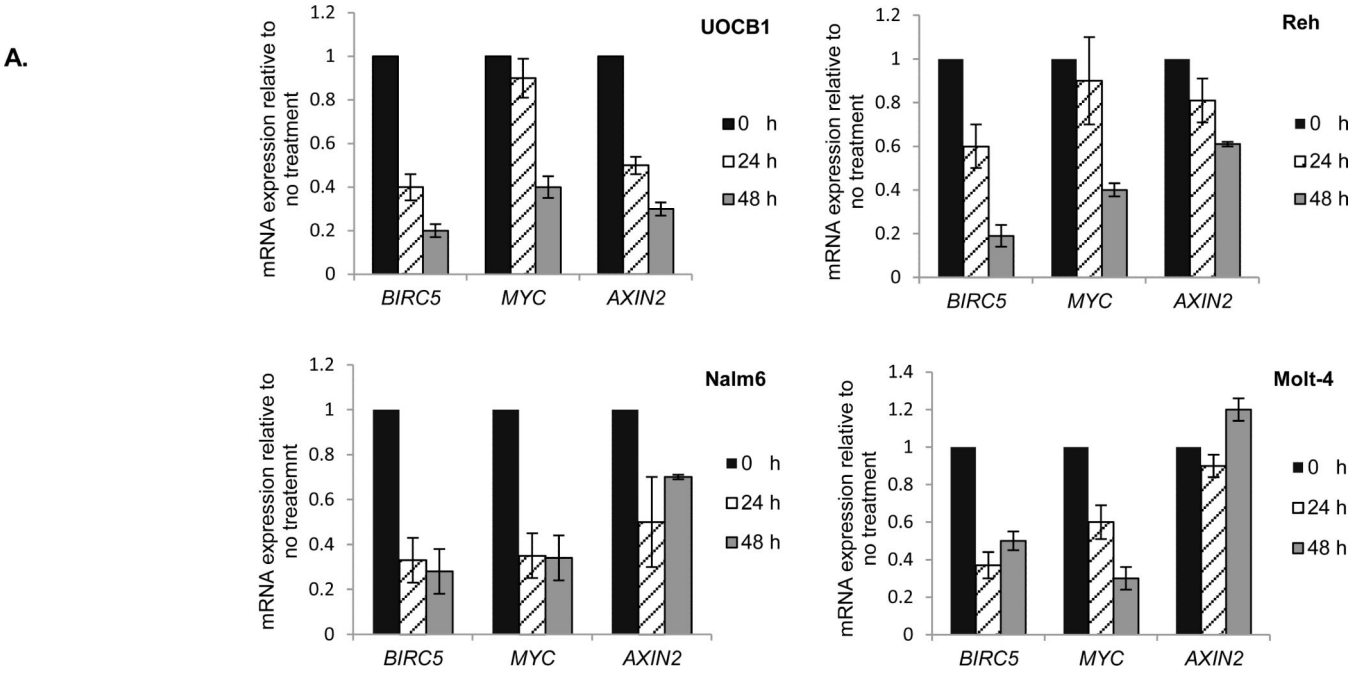


Figure 1. Wnt signaling network activity in paired diagnosis-relapse patient samples

10 matched paired Diagnosis- Relapse patient samples were fixed with 4 % PFA, permeabilized with 95% methanol and then stained simultaneously with antibodies for caspase 3, CD10, activated β-catenin and BIRC5. CD10 positive, caspase 3 negative live leukemic blast population was gated upon and shifts in the expression of activated β-catenin and BIRC5 examined from diagnosis to relapse. **(A)** Representative histograms of 3 matched diagnosis-relapse patient pairs demonstrating upregulation of activated β-catenin (Patient 3) and BIRC5 (Patient 7) at relapse and selection of a BIRC5 high subclone (Patient 6) at relapse compared to diagnosis. Results are plotted for all 10 patient pairs as change in the Mean Fluorescence Intensity (MFI) from diagnosis to relapse for **(B)** Activated β-Catenin and **(C)** BIRC5



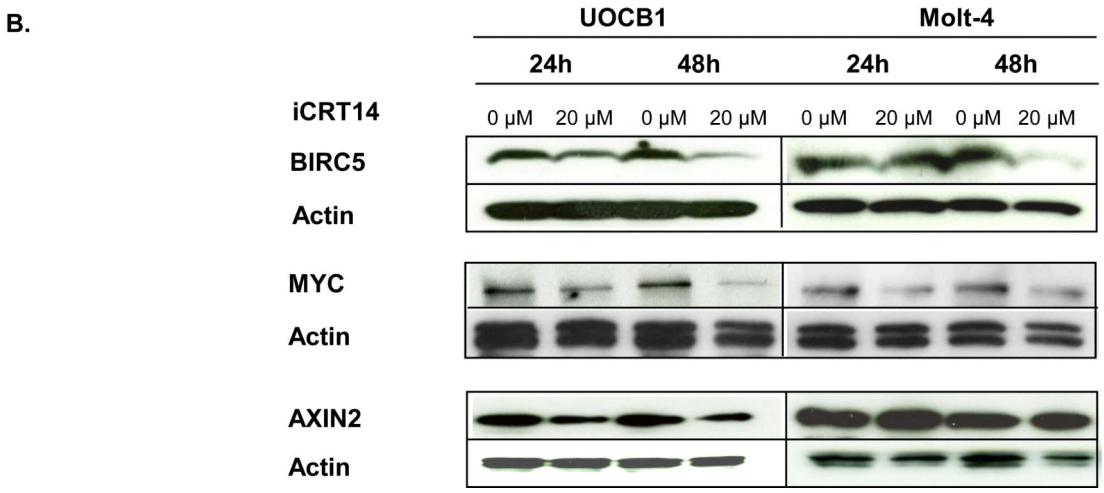
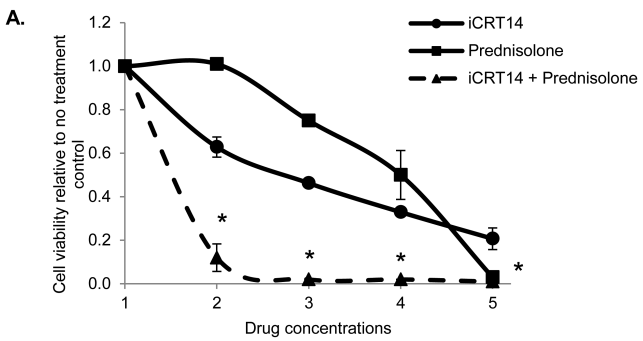
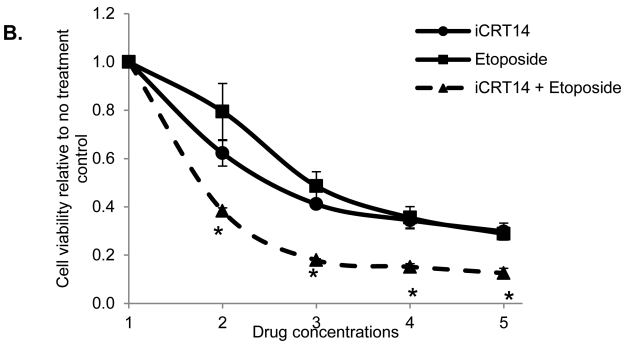
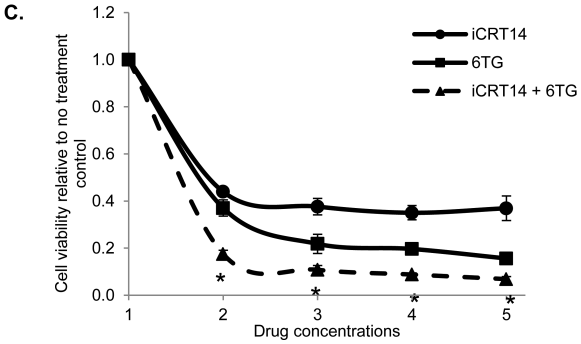


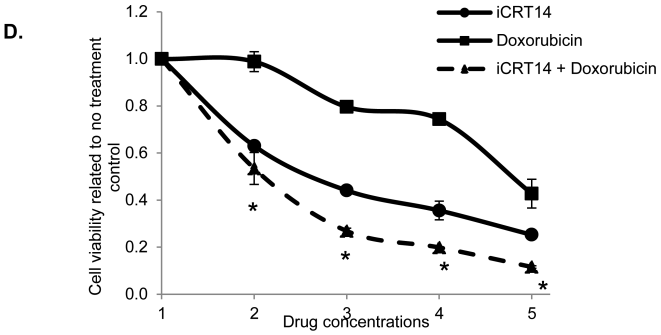
Figure 2. Wnt target genes are down regulated following incubation with Wnt inhibitor in ALL cell lines

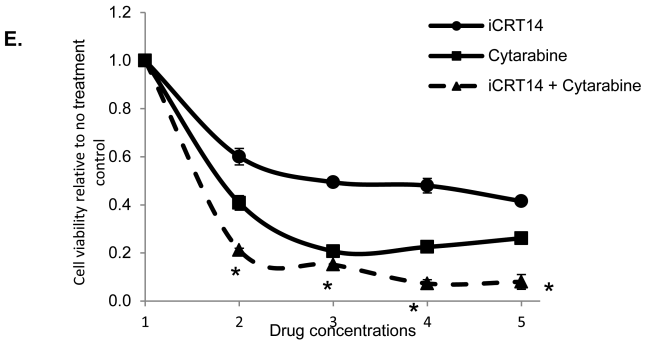
UOCB1 and Molt-4 cell lines were treated with 20 μM concentration of iCRT14 for 24 and 48 hours. **(A)** Quantitative real time PCR determining mRNA expression of 3 Wnt pathway target genes. Results are expressed relative to the levels observed at baseline without exposure to the inhibitor. Each experiment was performed in triplicate. Error bars represent standard deviation of the mean**. (B)** Western blot analysis of BIRC5, MYC and AXIN2 protein levels with and without treatment with the inhibitor. Actin was used as the loading control











F.

UOCB1

Dose	Etoposide	Prednisolone	Doxorubicin	6TG	Cytarabine
2	0.67	0.73	0.87	0.12	0.17
3	0.47	0.62	0.78	0.13	0.17
4	0.67	0.73	0.88	0.13	0.1
5	0.73	0.81	0.85	0.12	0.25

Nalm6

Dose	Etoposide	Prednisolone	Doxorubicin	6TG	Cytarabine
2	1.1	2	1.1	0.6	1.02
3	0.9	0.09	1.06	0.5	1.09
4	0.8	0.05	0.9	0.5	0.9
5	1.05	0.05	0.6	0.3	0.7

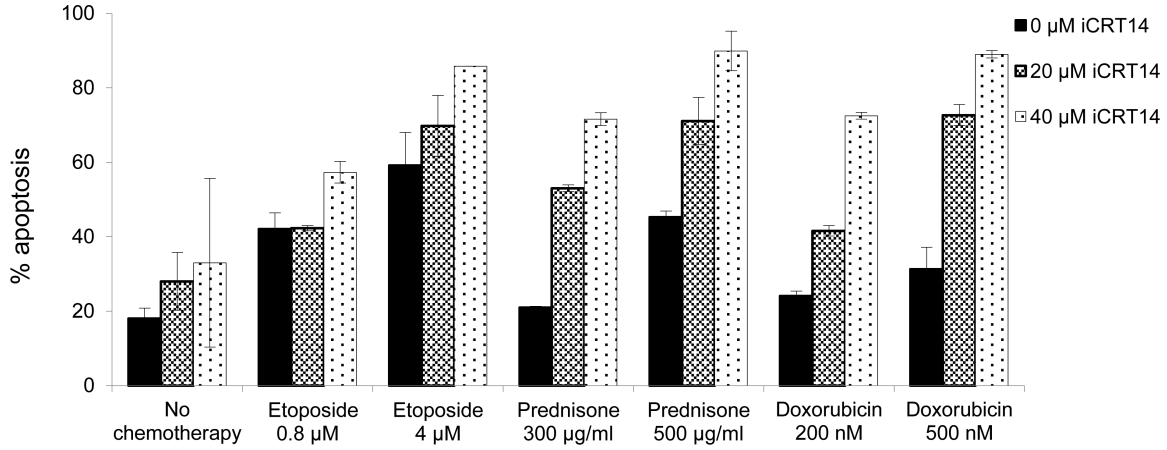
Reh

Dose	Etoposide	Prednisolone	Doxorubicin	6TG	Cytarabine
2	0.4	0.45	0.55	0.48	1.3
3	0.17	0.16	0.29	0.47	1.3
4	0.1	0.04	0.29	0.44	1.9
5	0.1	0.03	0.18	0.43	3

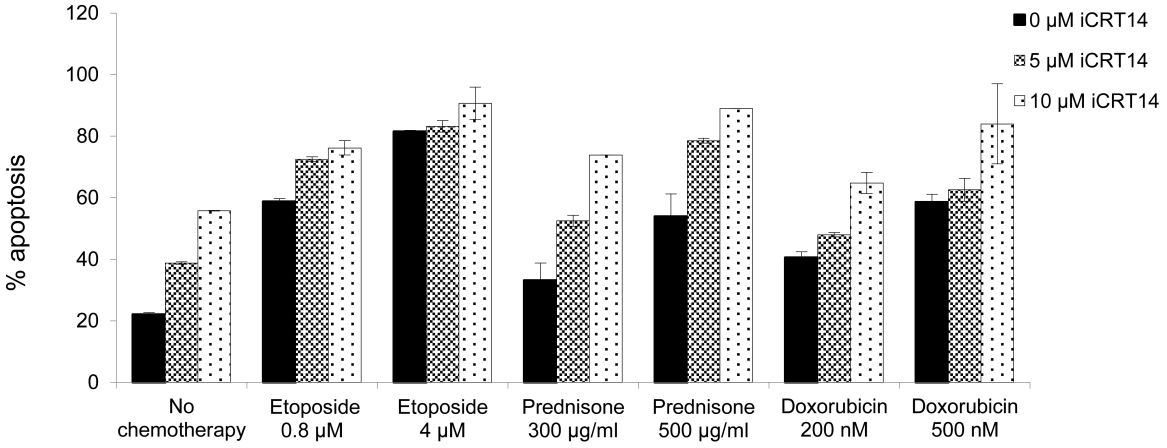
Figure 3. iCRT14 pretreatment synergizes with chemotherapy to reduce the cell viability of UOCB1 cells

UOCB1 cells were treated with iCRT14 for 48 hours before adding **(A)** Prednisolone (0-500 μg/mL) **(B)** Etoposide (0-1 μM) **(C)** 6TG (0-20 μg/mL) **(D)** Doxorubicin (0-125 nM) and **(E)** Cytarabine (0-5 μg/mL). Cell viability was assessed at 72 hours. * indicates synergistic drug combination (CI<0.9). Results graphed show the effect of chemotherapy alone, iCRT14 alone and various dose combinations of the inhibitor and chemotherapy. Error bars represent standard deviation of the mean**. (F)** Table of combination index (CI) for all cell lines tested at all drug combinations.

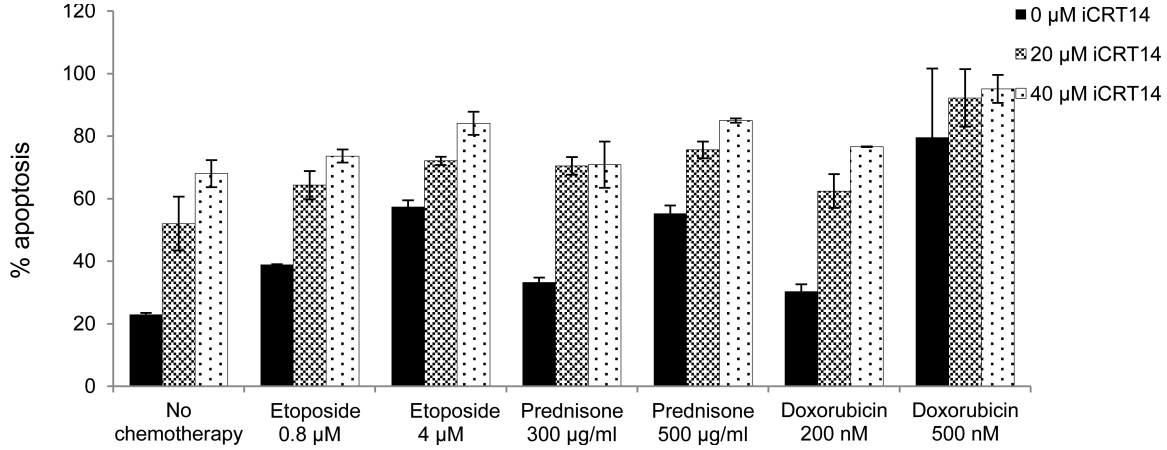
A. UOCB1



B. Nalm6



C. Reh



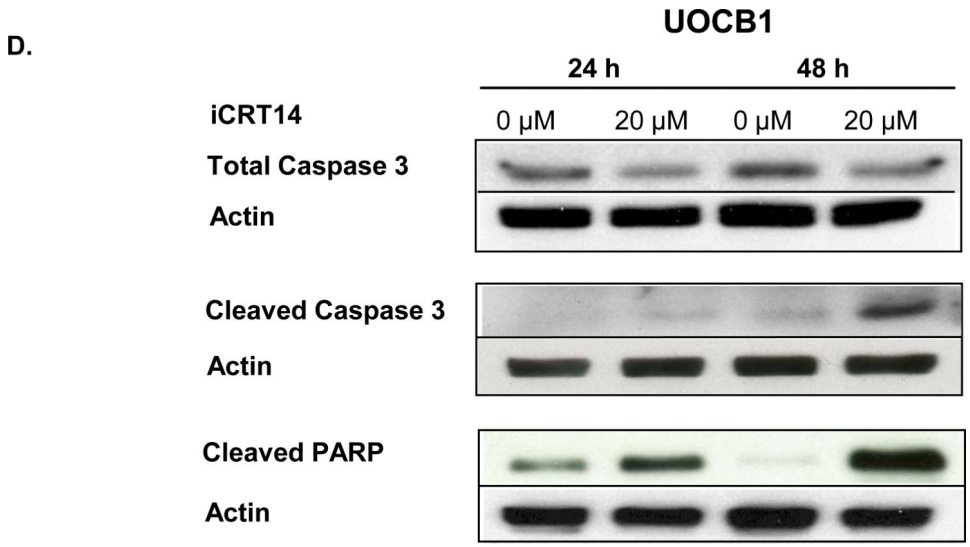


Figure 4. Combination of iCRT14 and chemotherapy induces apoptosis in ALL cell lines

B-All lines, **(A)** UOCB1, **(B)** Nalm6, and **(C)** Reh, were treated with or without iCRT14 (2 doses) in the presence or absence of etoposide (0-4 μM), prednisolone (0-500 μg/mL) and doxorubicin (0-500 nM) and stained with Annexin V-PE and 7AAD and assayed for apoptosis (% apoptosis ± SD). **(D)** Western blots showing cleavage of caspase 3 and PARP in UOCB1 cells after treatment with 20 μM of iCRT14 in comparison to the untreated cells. Actin was used as the loading control.

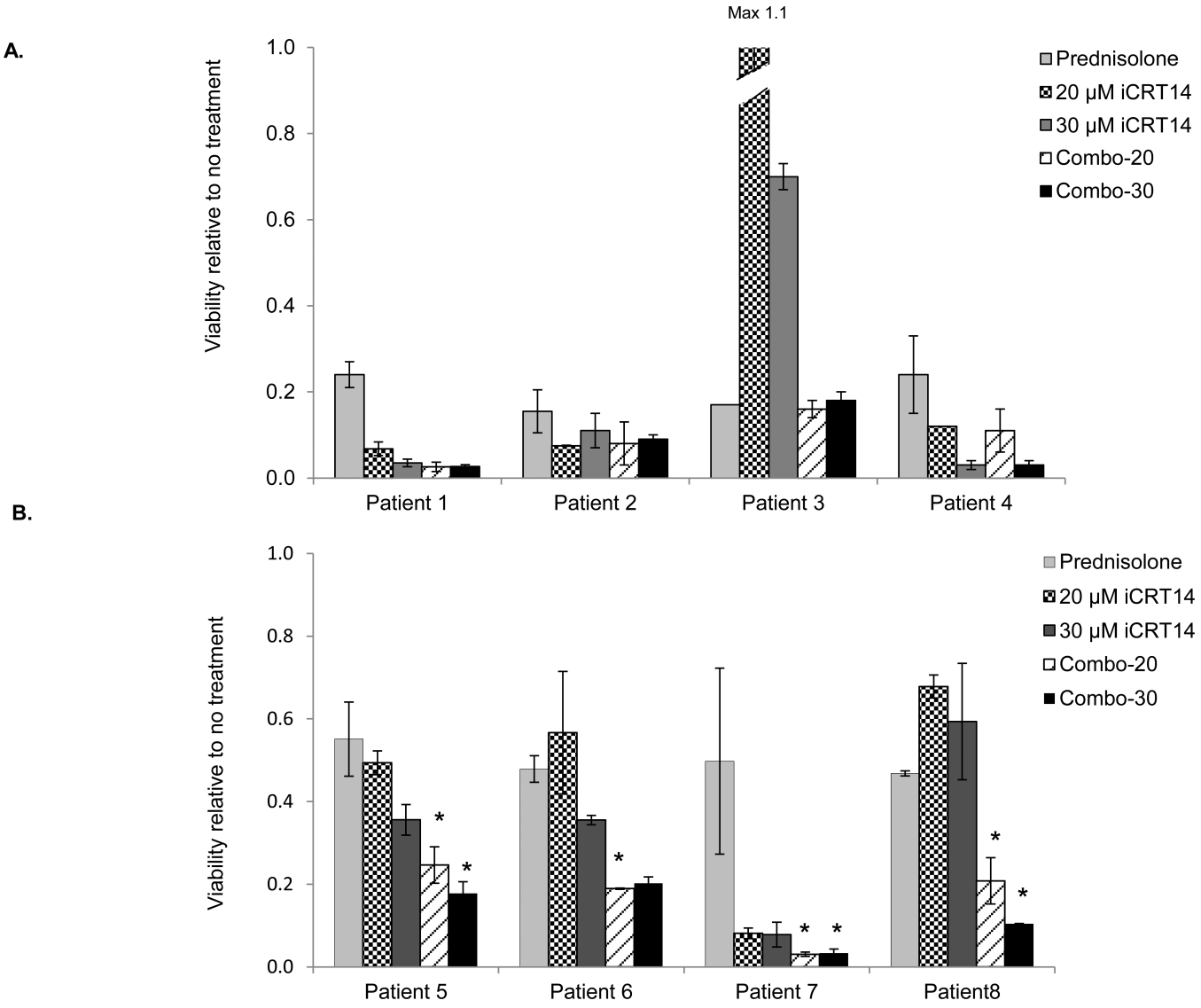


Figure 5. Viability of patient samples significantly decreased following ex vivo treatment with a combination of prednisolone and iCRT14 as compared to either agent alone

Cell titerglo cell viability assays were performed on patient-derived blast cells, from 4 newly diagnosed and 4 relapsed patients, pretreated with iCRT14 for 24 hrs (20 and 30 μM) with or without addition of 24hrs of prednisolone (200 μg/mL) at hour 24. Results are graphed for **(A)** Diagnosis samples (Patients 1-4) and **(B)** Relapsed patient samples (Patients 5-8). For each patient, the first bar represents the % of cells viable after treatment with prednisolone; the next 2 bars show the effect of 20 and 30 μM of iCRT14 by itself and the last 2 bars show the % viable cells after treatment with prednisolone in combination with the 2 doses of the Wnt inhibitor. * indicates synergistic combinations as determined by Calcusyn program (CI<0.9). Error bars represent standard deviation of the mean.