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Targeting Wnt/β-catenin and PI3K/Akt/mTOR pathways in T-cell acute lymphoblastic leukemia

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disorder that results from clonal transformation of T-cell precursors. Phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) and canonical Wnt/β-catenin signaling pathways play a crucial role in T-cell development and in self-renewal of healthy and leukemic stem cells. Notably, β-catenin is a transcriptional regulator of several genes involved in cancer cell proliferation and survival. In this way, aberrations of components belonging to the aforementioned networks contribute to T-ALL pathogenesis. For this reason, inhibition of both pathways could represent an innovative strategy in this hematological malignancy. Here, we show that combined targeting of Wnt/β-catenin pathway through ICG-001, a CBP/β-catenin transcription inhibitor, and of the PI3K/Akt/mTOR axis through ZSTK-474, a PI3K inhibitor, downregulated proliferation, survival, and clonogenic activity of T-ALL cells. ICG-001 and ZSTK-474 displayed cytotoxic effects, and, when combined together, induced a significant increase in apoptotic cells. This induction of apoptosis was associated with downregulation of Wnt/β-catenin and PI3K/Akt/mTOR pathways. All these findings were confirmed under hypoxic conditions that mimic the bone marrow niche where leukemic stem cells are believed to reside. Taken together, our findings highlight potentially promising treatment consisting of co-targeting Wnt/β-catenin and PI3K/Akt/mTOR pathways in T-ALL settings.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological disorder that results from clonal transformation of T-cell precursors and represents 10-15% of ALL cases in children and up to 25% in adults (Vadillo et al., 2018). Despite significant progress over the past decades, drug-resistance remains a major problem in the treatment of T-ALL, especially in adults. Dose escalation of current chemotherapeutics is limited by acute and chronic toxicities. Therefore, new treatment options are required (Evangelisti et al., 2014; Martelli et al., 2019).

Phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) and canonical Wnt/β-catenin signaling pathways are implicated in healthy T-cell development, whereas aberrations such as mutations, deletions or overexpression of components belonging to the aforementioned networks contribute to T-ALL pathogenesis (Bongiovanni et al., 2017; Evangelisti et al., 2018b; Martelli et al., 2019; McCubrey et al., 2014).

The PI3K/Akt/mTOR signaling pathway is involved in many cellular processes, such as cell growth, survival, transcription, translation, apoptosis, metabolism, motility, and autophagy (Laplante and Sabatini, 2012). Moreover, it controls different steps in the development, differentiation, and activation of normal T-cells. Hence, dysregulation of this signaling network may lead to T-ALL onset and progression and is associated with a worse patient outcome (Evangelisti et al., 2014; Martelli et al., 2019).

The Wnt/β-catenin pathway plays an important role both in embryogenic development and during the entire life of mammals. It has been shown that this signaling axis regulates skin and hair growth, maintains intestinal homeostasis, contributes to hematopoiesis (Malhotra and Kincade, 2009), influences cell migration (Webster and Weeraratna, 2013), genomic stability (Hoffmeyer et al., 2012), and apoptosis (Pecina-Slaus, 2010). Importantly, it is involved in self-renewal of healthy stem cells (Majidinia et al., 2018; Uribe-Etxebarria et al., 2019) as well as of both solid cancer (Najafi et al., 2019) and leukemia stem cells (LSCs) (Lento et al., 2013). An aberrant increase in the levels of β-catenin exerts oncogenic effects via the activation of downstream gene expression programs in colorectal cancer (Morin et al., 1997; Rahmani et al., 2018; Vermeulen et al., 2010), endometrial cancer (Giannakis et al., 2014; Liu et al., 2019), breast cancer (Gao et al., 2019; Khramtsov et al., 2010; Mohammadi-Yeganeh et al., 2019; Rahmani et al., 2019), prostate cancer (Pashirzad et al., 2019), bladder cancer (Xie et al., 2019), lung cancer (Wang et al., 2018b), glioblastoma (He et al., 2019) as well as in blood malignancies (Staal and Sen, 2008; Zhao et al., 2007). More specifically, ≥85% of pediatric T-ALL patients display increased β-catenin expression and upregulation of the Wnt target genes *AXIN2, C-MYC, TCFL* and *LEF* (Arensman et al., 2014; Fernandes et al., 2017). Furthermore, about 30% of adult T-ALL patients displayed high expression of lymphoid enhancer-

binding factor 1 (LEF1) mRNA (Guo et al., 2015). LEF1 exerts its role in regulating cell proliferation and survival by activation of the Wnt target genes through the recruitment of β-catenin (Petropoulos et al., 2008).

The canonical Wnt/β-catenin pathway is triggered by the binding of Wnt ligands to low-density lipoprotein receptor-related protein (LRP) 5/6 and frizzled receptors (Niehrs, 2012). This binding leads to Dishevelled (Dvl) activation and sequestering of glycogen synthase kinase (GSK) 3β that is unable to phosphorylate β-catenin at Ser33/Ser37/Thr41, thereby preventing the ubiquitination and degradation of β-catenin via the proteasome. Therefore, inactivation of GSK3β (through Akt-dependent Ser9 phosphorylation, for example) leads to stabilization, accumulation, and translocation of β-catenin to the nucleus where it associates with transcription factors, notably T cell factor (TCF) and LEF, thereby activating target genes involved in cell growth and survival, including *C-MYC*, *CCND1*, *BIRC5*, and *CDKN1a* (Brantjes et al., 2002). It is well known that high levels of the aforementioned genes are found in several types of cancer (Velculescu et al., 1999). Of note, c-myc is a direct target of the Wnt/β-catenin pathway, as well as of Notch1 signaling, the most frequently mutated signaling network in T-ALL (Evangelisti et al., 2018b). c-myc activation is involved in tumor initiation, progression, and maintenance (Dang, 2012). *BIRC5* is another gene activated by the Wnt/β-catenin pathway that involved in oncogenesis and its product, survivin, is downstream of c-myc (Ma et al., 2005; Park et al., 2011). However, survivin expression is also regulated via PI3K/Akt/mTOR signaling (Glienke et al., 2012). It has been demonstrated that survinin is overexpressed in ALL primary cells (Gang et al., 2014) where its targeting exerts marked cytotoxic effects.

A Wnt/β-catenin inhibitor, LGK974, is currently undergoing clinical trials for the treatment of pancreatic adenocarcinoma and colorectal cancers (NCT01351103). CWP232291 is another β-catenin inhibitor currently tested for acute myeloid leukemia (NCT01398462). Moreover, the ICG-001-derived compound, PRI-724, entered early-phase clinical trials for advanced solid tumors (NCT01302405) and hematological malignancies (NCT01606579). ICG-001 is a small molecule that inhibits Wnt/β-catenin-mediated transcription, downregulating the expression of a subset of β-catenin/TCF-responsive genes (Emami et al., 2004). It acts specifically by binding the cAMP-responsive element binding (CREB)-binding protein (CBP), thereby disrupting CBP interactions with β-catenin.

Evidence suggests that both PI3K/Akt/mTOR and Wnt/β-catenin pathways contribute to carcinogenesis and sustain neoplastic cell proliferation (Deming et al., 2014; Jefferies et al., 2017; Yang et al., 2015; Zhang et al., 2019). On the bases of these premises, inhibition of Wnt/β-catenin

and PI3K/Akt/mTOR pathways is regarded as a possible innovative therapeutic strategy for cancer treatment (Arques et al., 2016; Park et al., 2019).

Surprisingly, however, the efficacy of simultaneous targeting of PI3K/Akt/mTOR and Wnt/β-catenin pathways has never been explored in T-ALL preclinical models.

Here, we demonstrate that ICG-001 and ZSTK-474 (a PI3K inhibitor) displayed a marked cytotoxic activity against T-ALL cell lines and, more importantly, we show that the combined use of these compounds was synergistic. We also demonstrate that ICG-001/ZSTK-474 combined treatment resulted in apoptotic cell death characterized by a concomitant downregulation of c-myc and survivin and dampened the colony-forming activity of T-ALL cells. Importantly, ICG-001/ZSTK-474 combined treatment was cytotoxic to T-ALL cells cultured under hypoxic conditions, an observation that appears particularly relevant when considering that LSCs reside in the hypoxic bone marrow (BM) microenvironment (Hira et al., 2017).

Materials and methods

Cell lines and reagents

T-ALL cell lines were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). All T-ALL cells were cultured in RPMI-1640 medium (Life Technologies Italia, Monza, Italy) supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) at 37˚C in a humidified atmosphere of 5% CO_2. ICG-001 and ZSTK-474 were from Selleck Chemicals (Houston, TX, USA). Primary antibodies were from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assay

T-ALL cell lines were cultured in the presence of the vehicle (DMSO 0.1%) or increasing drug concentrations, and cell viability was determined using the MTT [3-(4,5-Dimethylthythiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation kit (Roche Diagnostic, Basel, Switzerland), according to manufacturer's instructions. The combination effect and potential synergism were evaluated from quantitative analysis of dose-effect relationship, based on the Chou and Talalay method (Evangelisti et al., 2018a). For each experiment, a combination index (CI) was calculated using the CalcuSyn software (Biosoft, Cambridge, UK). This method of analysis generally defines CI values of 0.9 to 1.1 as additive, 0.3 to 0.9 as synergistic, and <0.3 as strongly synergistic, whereas values >1.1 are considered antagonistic.

Annexin V-FITC/PI staining and cell cycle analysis

Apoptosis and cell cycle analysis were performed as previously described (Evangelisti et al., 2018a), using an FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) with the appropriate software (CXP, Beckman Coulter).

Western blot analysis

Western blot analysis was performed as previously described (Evangelisti et al., 2018a). Cells were lysed using M-PER Mammalian Protein Extraction Reagent supplemented with the Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Rockford, IL, USA). Unless otherwise specified in the figure legends, 30 μg of protein was blotted to each lane. Antibody to β-actin served as loading control. Band density measurement was performed using a BioRad (Hercules, CA, USA) densitometer (GS 800) equipped with Quantity One Software.

Quantitative real time-PCR (qRT-PCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions, and 1 μg of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). Gene expression was assessed using the TaqMan® Gene Expression Master Mix, using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Results were normalized to the level of the ubiquitously expressed RNA 18S ribosomal 1 gene, while the Universal Human Reference RNA (Agilent) was used as control. Results were expressed as $2^{-\Delta Ct}$ (ΔCt = [(CTgene of interest − CT internal control) to compare the relative gene expression among samples, and as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct$ = [(CT gene of interest − CT internal control) sample − (CT gene of interest − CT internal control) universal]) to compare gene expression of the treated cell lines with that of untreated control (Lonetti et al., 2016).

Colony-forming assays

T-ALL cells were cultured in methylcellulose-based medium (MethoCult SF^{BIT} H4236, StemCell Technologies, Vancouver, BC, Canada) at 1.6×10^2 cells/ml. Colony forming units (CFUs) were scored after 12 days of incubation at 37°C in a fully humidified 5% CO_2 atmosphere. Results are shown as the fold change of the number of CFUs compared with untreated conditions.

Subcellular fractionation

Cells were collected, centrifuged, and washed in PBS. Pellets were resuspended in 1 ml of TM2 buffer (10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$) for 1 minute. Then, Triton 0.2% was added and the cell suspension was passed twice through a syringe fitted with a 22 ½ gauge needle. Next, $MgCl_2$ 3mM was added to the suspension, which was centrifuged for 10 minutes at 800 rpm. Nuclear pellets were washed twice in TM5 buffer (10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$), while the supernatant was transferred into a new vial and used as the cytoplasmic fraction (Poli et al., 2014).

Immunofluorescence microscopy

To determine the subcellular localization of β-catenin, T-ALL cells were cytocentrifuged (Thermo Electron Corporation, Pittsburgh, PA, USA) at low acceleration and 200 rpm for 5 minutes or seeded on coverslips and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized with 0.2% Triton X-100/PBS for 10 minutes and were blocked with 3% BSA/PBS for 1 hour. Anti-β-Catenin antibody diluted (1:100) in 3% BSA-containing PBS was added to the cells on the slide and incubated overnight at 4 °C. Slides were washed with PBS, then incubated with FITC-conjugated anti-rabbit immunoglobulin G (Dako Denmark A/S, Glostrup Denmark) antibody (1: 50) for 1 hour at room temperature. Cytoplasm was stained with Alexa Fluor 555 Phalloidin (ThermoFisher Scientific, Waltham, MA, USA). Slides were washed 3 times for 10 minutes at room temperature with PBS/Tween and mounted with an anti-fade reagent in glycerol containing DAPI (Molecular Probes, Eugene, OR, USA). Confocal imaging was performed using a Nikon A1 confocal laser scanning microscope, equipped with a 60×, 1.4 NA objective and with 405, 488, and 561 nm laser lines. Z-stacks were collected at optical resolution of 100 nm/pixel with pinhole diameter set to 1 Airy unit and z-step size to 200 nm. All image analyses were performed using NIS-Elements software (Nikon). The degree of cytoplasmic translocation of β-catenin can be assessed in a qualitative manner by measures, in single optical sections, the β-catenin mean fluorescence intensity ratio (N:C ratio) between representative region of interests of nucleus and cytoplasm. The statistical significance of the differences between the experimental points was evaluated by Student's t-test.

Gene silencing by siRNA

Jurkat and RPMI-8402 cells were transfected with Nucleofector Kit V from Lonza (Walkersville, MD, USA) with 750 nmol/L of ON-TARGET plus CTNNB1 siRNA (Dharmacon, Lafayette, CO, USA) using an Amaxa apparatus, following the manufacturer's instructions. Scramble siRNA was also from Dharmacon. β-catenin silencing was confirmed by western blot analysis.

Statistical analysis

Statistical analyses were performed using Student's *t* test or one-way ANOVA (Dunnett's test) at a significance level of $p<0.05$ (Prism software, GraphPad Software, San Diego, CA, USA).

Results

ICG-001 and ZSTK-474 induce cytotoxic effects and are synergistic in T-ALL cell lines

To evaluate the status of the Wnt/β-catenin and PI3K/Akt/mTOR pathways in T-ALL, we first analyzed by western blot the expression of β-catenin, Akt and their phosphorylated forms in a panel of T-ALL cell lines (Figure 1A). Protein levels varied among the different cell lines, showing a high expression of total β-catenin in HPB-ALL, Molt-4, Jurkat, CEM-S, and RPMI-8402 cells. Remarkably, β-catenin was unphosphorylated (i.e. active) in almost all the cell lines. As to Ser473 Akt, its levels were particularly high in the above outlined five cell lines. The analysis of the ratio between p-β-catenin/β-catenin documented that the ratio was particularly low in HPB-ALL, Molt-4, Jurkat, CEM-S, and RPMI-8402 cells (Figure 1B).

Then, we analyzed the potential anticancer effects of 2 drugs: ICG-001, a CBP/β-catenin-dependent transcription inhibitor, and ZSTK-474, a PI3K inhibitor, administered either alone and in combination. ICG-001 and ZSTK-474 were tested on five different T-ALL cell lines by incubating cells for 48 hours with increasing concentrations of the drugs. ICG-001 significantly reduced T-ALL cells viability, displaying IC_{50} ranging from 1.5 μM for Molt-4 cells to 17.2 μM for Jurkat cells, while ZSTK-474 IC_{50} varied from 1.6 μM for Molt-4 cells to 3.6 μM for RPMI-8402 cells. We also investigated whether ICG-001 and ZSTK-474 could synergize in T-ALL cells. Combined treatment was more effective in inhibiting cell viability that single treatments, as synergisms were observed in all cell lines, as documented by the CI values (Figure 1C and 1D).

Based on these findings, we focused on CEM-S, Jurkat, and RPMI-8402 cell lines that all display PI3K/Akt/mTOR and Wnt/β-catenin activated networks as well as the most effective drug synergism. Moreover, in the subsequent experiments we used fixed drug concentrations: 10 μM for ICG-001 and 2 μM for ZSTK-474 for CEM-S and Jurkat cells, while for RPMI-8402 cells we used 5 μM ICG-001 and 1 μM for ZSTK-474.

Flow cytometric analysis of PI-stained Jurkat cells treated with ICG-001 or ZSTK-474 alone for 24 hours documented an accumulation of cells in the G_0/G_1 phase of the cell cycle, accompanied by a decrease of cells in the S or G_2/M phases. In contrast, with combined treatment, we detected a strong increase in the sub-G_1 cell fraction that corresponds to death cells (Figure 2A). To evaluate whether the cytotoxic effects of the combined treatment could be related to apoptosis, flow cytometric analysis of Annexin V/PI-stained samples was performed. As shown in Figure 2B, we observed an increase

in the percentage of both early apoptotic (positive for Annexin V) and/or late apoptotic (positive for both Annexin V and PI) cells upon 24 and 48 hours of ICG-001 and ZSTK-474 treatment. Consistently with this, western blot analysis documented an increased cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP), upon combined treatment, in the three cell lines (Figure 2C).
Taken together, our findings demonstrated that ICG-001 and ZSTK-474 have cytostatic effects when administrated separately while they induce apoptosis when administrated together.

Wnt/β-catenin and PI3K/Akt/mTOR signaling pathway are modulated by ICG-001 and ZSTK-474

To understand how ICG-001 and ZSTK-474 act at molecular level, we analyzed the expression of some components of Wnt/β-catenin (Figure 3A and 3B) and PI3K/Akt signaling at the transcript and/or protein level.

Firstly, we analyzed the expression of 2 canonical Wnt/β-catenin target genes, involved in cancer cell proliferation and survival that are activated by nuclear translocation of β-catenin: *C-MYC* and *BIRC5*. *BIRC5* encodes the protein survivin (Di Giacomo et al., 2017; Verdecia et al., 2000). We treated T-ALL cell lines for 24 hours with ICG-001, ZSTK-474 or the combination of the 2 drugs and we evaluated the expression of the 2 genes by qRT-PCR. ICG-001 and, to some extent, ZSTK-474, were able to decrease mRNA levels of both *C-MYC* and *BIRC5*. However, the combined treatment was much more effective than single treatments (Figure 3A).

We then incubated T-ALL cells with the 2 inhibitors at different time points, and we performed western blot analysis of protein expression (Figure 3B). After 24 hours of treatment, we observed that ICG-001 and ZSTK-474 negatively affected Wnt/β-catenin signaling, as indicated by a marked decrease in c-myc, survivin, and total β-catenin levels. The decrease was exacerbated in samples treated with both the drugs for 48 hours (Figure 3B). We also observed an increase in p-β-catenin (Ser33/Ser37/Thr41) and a decrease in p-GSK3 (phosphorylated at Ser 9) levels is response to combined treatment, especially after 48 hours (Figure 3B). Taken together, these results indicated that the combination of the 2 drugs dampened Wnt/β-catenin signaling pathway, most likely through activation of GSK3β and proteasomal degradation of β-catenin. Furthermore, the PI3K/Akt/mTOR signaling status was also examined by western blot analysis, which demonstrated a dephosphorylation of Ser473 Akt and S6 ribosomal protein (S6RP), whereas total Akt and S6RP levels were unaffected (Figure 3C).

ICG-001 and ZSTK-474 negatively affect clonogenic activity of T-ALL cells

We next investigated whether ICG-001 combined with ZSTK-474 could affect the self-renewal capacity of T-ALL cells, through a CFU assay in a semi-solid medium (Figure 4). Clonogenic activity was assessed in CEM-S, Jurkat, and RPMI-8402 cells. Interestingly, we observed that combined treatment, compared to each single treatment, caused a marked inhibition of T-ALL cell clonogenic activity (Figure 4).

ICG-001 and ZSTK-474 induce translocation of β-catenin from nucleus to cytoplasm.

To be active, β-catenin has to translocate to the nucleus where it activates transcription factors that modulate the expression of cell proliferation, migration, and survival genes, including *C-MYC* and *BIRC5*. Given the key role of β-catenin subcellular distribution, we wanted to analyze the localization of β-catenin after drug treatment. To detect the subcellular localization of β-catenin upon drug treatment, we performed subcellular fractionation in CEM-S and RPMI-8402 cells. Cells were treated for 24 hours with ICG-001 and ZSTK-474, either alone or in combination. Western blot analysis demonstrated that cytoplasmic levels of β-catenin were increased in response to combined treatment (Figure 5A).To corroborate western blot data, we performed immunofluorescence staining. Confocal fluorescence imaging confirmed that the combined treatment was more effective that single drugs in inducing β-catenin translocation from nucleus to cytoplasm (Figure 5B).

Combined treatment with ICG-001 and ZSTK increases cytarabine cytotoxicity

We next aimed to evaluate whether combined treatment with ICG-001 and ZSTK-474 would increase the cytotoxic effect of cytarabine (ARA-C), a drug used in polychemotherapy schemes for treating T-ALL patients (Wu and Li, 2018). Indeed, previous findings have documented how both Wnt/β-catenin and PI3K/Akt/mTOR pathways are involved in resistance of ALL cells to chemotherapeutic drugs (Chiarini et al., 2009; Dandekar et al., 2014).

ARA-C, when used together with both the inhibitors, was much more effective in reducing Jurkat and RPMI-8402 cell viability than when employed with either ICG-001 or ZSTK-474 alone (Figure 6A). To further demonstrate that ICG-001-dependent Wnt/β-catenin inhibition contributes to increase ARA-C cytotoxicity, we inhibited β-catenin expression using siRNA in Jurkat and RPMI-8402 cells (Figure 6B). We next treated cells with siRNA to β-catenin for 24 hours and then with ZSTK-474 and ARA-C alone or in combination for additional 48 hours. siRNA silencing of β-catenin enhanced ZSTK-474 and ARA-C cytotoxicity to a greater extent than scramble siRNA (Figure 6C).

Wnt/β-catenin and PI3K/Akt/mTOR signaling are upregulated by hypoxia

The connection between hypoxia and Wnt/β-catenin pathway has been observed in ALL (Chiarini et al., 2016). Hypoxia causes a stabilization of hypoxia-inducible factor (HIF) 1α that critically controls the expression of several genes involved in cellular adaptation to hypoxia (Schito et al., 2017). High levels of HIF1α in the hypoxic BM niche are found in different hematological malignancies, including ALL, and are related to disease progression, therapy resistance, relapse, and poor patient outcome (Schito et al., 2017). Moreover, it has been shown that β-catenin-dependent transcription is activated under hypoxic conditions through HIF1α stabilization and that β-catenin and HIF1α support LSC activity in T-ALL (Giambra et al., 2015).

We firstly aimed to determine whether Wnt/β-catenin and PI3K/Akt/mTOR pathways were activated in CEM-S and Jurkat cells cultured under hypoxic conditions (2% O_2) by performing western blot analysis. Under hypoxic conditions, the stabilization of HIF1α matched the increase in β-catenin levels as well as that of Ser473 Akt (Figure 7A). Moreover, T-ALL cells cultured in a semi-solid medium under hypoxic conditions displayed a higher clonogenic activity, suggesting that hypoxia promoted self-renewal capacity (Figure 7B).

Then, we analyzed cell viability of T-ALL cells in 2% O_2 versus 20% O_2 conditions (Figure 7C). CEM-S and Jurkat cells were treated for 48 hours with increasing concentrations of ICG-001 and ZSTK-474 alone or the combination of the 2 drugs and the effects of the inhibitors on cell viability were analyzed by MTT assay. Combined treatment with ICG-001 and ZSTK-474 displayed synergistic effects under both normoxic and hypoxic conditions (Figure 7C).

Furthermore, we analyzed by western blot the expression of β-catenin, HIF1α, Ser473 Akt and survivin under both normoxic and hypoxic conditions, in response to ICG-001 and ZSTK-474 treatment in Jurkat cells. In hypoxic cells, β-catenin, HIF1α, Ser473 Akt, total Akt, and survivin were more expressed compared with normoxic cells. Moreover, under both the culturing conditions, combined targeting of Wnt/β-catenin and PI3K/Akt/mTOR signaling pathways downregulated β-catenin, HIF1α, Ser473 Akt, and survivin levels more effectively than single drugs (Figure 7D).

Finally, we performed a colony formation assay to compare CFU activity under normoxic and hypoxic conditions. We found that combined treatment of Jurkat cells significantly reduced CFU activity when compared with either of single treatments (Figure 7E).

Discussion

The pathogenesis of hematological malignancies involves constitutive activation of different signal transduction cascades that play key roles in hematopoiesis and may induce transformation of hematopoietic stem or progenitor cells into LCSs (Pui et al., 2004).

It is well established that the PI3K/Akt/mTOR pathway is aberrantly activated in ~ 60% of T-ALL patients (Silva et al., 2008) and correlates with a poor outcome (Bongiovanni et al., 2017). Our group previously showed that PI3K inhibition displayed strong cytotoxic effects on both ALL cell lines and primary cells, demonstrating that targeting PI3K could be an attractive strategy for treating ALLs (Bressanin et al., 2012; Evangelisti et al., 2018a; Lonetti et al., 2014; Lonetti et al., 2015). This therapeutic approach for ALL has been supported by several clinical trials of PI3K inhibitors. For instance, BMK-120, a pan PI3K inhibitor, has entered clinical trials for patients with advanced acute leukemias (NCT01396499).

Several findings also highlighted that Wnt/β-catenin signaling controls the earliest steps of healthy T-cell development, while its dysregulation may lead to malignant transformation of T-cell progenitors (Bigas et al., 2013; Guo et al., 2008; Zhu et al., 2018).

PI3K/Akt/mTOR and Wnt/β-catenin pathways are interconnected at different levels. Firstly, Akt inhibits GSK3β via phosphorylation at Ser9, whereas active GSK3β, in turn, controls β-catenin phosphorylation at specific residues (Ser33, Ser37 and Thr41), leading to its ubiquitination and subsequent degradation via the proteasome (Beurel et al., 2015; Stamos et al., 2014). Moreover, deptor, an mTOR complex 1 (mTORC1) component, is a direct target of Wnt/β-catenin signaling in colorectal cancer cells, thereby reinforcing the crosstalk between the 2 signaling pathways (Wang et al., 2018a).

Although evidence demonstrated that PI3K/Akt/mTOR and Wnt/β-catenin pathways are involved in leukemogenesis, the effects of inhibiting both signaling networks have not been investigated in T-ALL so far. These premises prompted us to test the *in vitro* cytotoxicity of a CBP/β-catenin inhibitor and a PI3K inhibitor, administered alone or in combination, in pre-clinical model of T-ALL, and to investigate whether inhibition of both signaling pathways could achieve a synergistic effect. We used ICG-001, a potent antagonist of CBP/β-catenin-mediated transcription that displayed cytotoxic effects in different types of cancer cells (Arensman et al., 2014; Emami et al., 2004; Zhao et al., 2016) and the pan PI3K inhibitor ZSTK-474 that has anti-proliferative effects on T-ALL models (Lonetti et al., 2015). Importantly, ZSTK-474 has entered clinical trials in cancer patients (NCT01682473 and NCT01280487).

Ours results demonstrated that simultaneous inhibition of Wnt/β-catenin and PI3K/Akt/mTOR pathways was effective against T-ALL cells. Of interest, ICG-001 and ZSTK-474 synergistically acted in inducing apoptosis and clonogenic activity of T-ALL cells that displayed high levels of unphosphorylated (active) β-catenin and of active Akt. We demonstrated that combined treatment strongly correlated with a significant induction of apoptosis and cleavage of caspase 3 and PARP, most likely via downregulation of c-myc and survivin. Upon simultaneous inhibition of Wnt/β-

catenin and PI3K/Akt/mTOR signaling pathways, we observed a decrease in Ser9 p-GSK3β levels that was accompanied by a concomitant increase in p-β-catenin. This effect could be explained if we consider that β-catenin is inhibited directly by ICG-001 and indirectly through the action of ZSTK-474 on PI3K/Akt/GSK3β. Of note, our findings are in agreement with recently published papers about the synergistic antitumor effects of Wnt/β-catenin inhibitor in combination with a PI3K/Akt/mTOR inhibitor in PRL-3 high acute myelogenous leukemia cells (Zhou et al., 2018) and in tamoxifen-resistant breast cancer cells (Won et al., 2016).

C-MYC and *BIRC5* are Wnt/β-catenin target genes implicated in both survival and drug-resistance of ALL cells (Morrison et al., 2012; Park et al., 2011). The c-myc oncogene plays an important role also in cell cycle progression. Its hyperactivation contributes to cancerogenesis, including leukemogenesis (Dang, 2012) and it has been suggested that c-myc is a promising target for eradicating LSCs in T-ALL (Schubbert et al., 2014).

C-myc is regulated by both Notch1 and β-catenin (Sanchez-Martin and Ferrando, 2017). It has been demonstrated that the involvement of β-catenin in leukemogenesis may be dependent and independent from the Notch1 signaling network in T-ALL (Sanchez-Martin and Ferrando, 2017). Indeed, it has been documented that active Notch1 receptor can inhibit proteasomal degradation of β-catenin (Staal and Sen, 2008) but, in murine models and human T-ALL patients, overexpression of β-catenin targets could result in a highly aggressive leukemia form even in absence of Notch1 mutations, through c-myc amplification (Guo et al., 2007; Kaveri et al., 2013). In this context, our unpublished results have demonstrated that the T-ALL cell lines we employed for this study (HPB-ALL, Molt-4, Jurkat, CEM-S, and RPMI-8402 cells) have mutated (active) Notch1.

We observed that c-myc and survivin were significantly reduced at both transcript and protein levels upon ICG-001 treatment in T-ALL cells but not upon ZSTK-474 treatment. Importantly, downregulation of c-myc and survinin was more marked in samples incubated with both inhibitors compared with those treated with ICG-001 alone, implying that the 2 drugs had synergistic effects on c-myc and survinin expression levels. Gekas and colleagues previously demonstrated that c-myc expression was dependent on β-catenin in T-ALL cells (Gekas et al., 2016). Moreover, these authors demonstrated that Wnt/β-catenin signaling was fundamental for leukemia initiation and maintenance and that pharmacological inhibition of β-catenin was cytotoxic only for leukemic cells but not for normal hematopoietic cells, thus confirming the therapeutic potential of β-catenin pharmacological inhibition in T-ALL cells. Inhibiting c-myc activity has been traditionally difficult to achieve. Recently, however, Bromodomain and Extra-Terminal (BET) inhibitors have emerged as a promising class of drugs that are able to effectively target c-myc, also in hematological malignancies, including

T-ALL (Abedin et al., 2016; Loosveld et al., 2014) . However, our unpublished data show that ICG-001 was more effective in downregulating survivin expression than the clinical grade BET inhibitor, OTX-015 (Riveiro et al., 2016).

Aberrant Wnt/β-catenin signaling seems to be a common mechanism for drug-resistance in ALL (Dandekar et al., 2014; Gang et al., 2014; Park et al., 2011), suggesting that this hematological malignancy may be "Wnt addicted." In one example, pretreatment of relapsed patient ALL blasts with a Wnt/β-catenin inhibitor was sufficient to restore chemosensitivity (Dandekar et al., 2014). Our findings support the concept that simultaneous targeting of both Wnt/β-catenin and PI3K/Akt/mTOR pathway may further enhance the cytotoxic effects of chemotherapeutic drugs, allowing for a lower dosage of traditional therapeutics.

In agreement with others (Giambra et al., 2015), we observed an increase in HIF1α and total β-catenin expression as well as upregulated CFU activity when T-ALL cells were cultured under hypoxic conditions. We also observed an upregulation in the levels of active Akt and in those of survivin. It is important to highlight that the PI3K/Akt/mTOR pathway increases HIF1α in hypoxic cancer cells (Maynard and Ohh, 2007) and that HIF1α in turn activates PI3K/Akt/mTOR, thereby fostering a vicious circle (Alvarez-Tejado et al., 2001; Deguchi et al., 2009; Zeng et al., 2006). HIF1α upregulates β-catenin in hypoxic T-ALL cells (Giambra et al., 2015).

Of note, the 2 inhibitors maintained their cytotoxic efficacy also when used in cells cultured under hypoxic conditions and, when combined together, they were more effective in downregulating HIF1α, β-catenin, and survivin expression as well as CFU activity than when employed as single agents. These findings seem to be important in light of the fact that in a murine model of T-ALL leukemic cells with active Wnt/β-catenin signaling were shown to reside in hypoxic BM niches (Giambra et al., 2015) and that the hypoxic BM microenvironment can sustain LSCs and contribute to chemoresistance (Benito et al., 2011; Hira et al., 2017). It has previously demonstrated that both β-catenin and HIF1α are specifically required for LSCs maintenance in T-ALL (Giambra et al., 2015). Nevertheless, also the PI3K/Akt/mTOR pathway is important for T-ALL LSC survival (Schubbert et al., 2014). Therefore, simultaneous inactivation of Wnt/β-catenin and PI3K/Akt/mTOR signaling pathways may be a promising therapeutic approach to eradicate T-ALL LCSs residing in BM hypoxic niches.

In conclusion, we have demonstrated the efficacy of combined targeting of Wnt/β-catenin and PI3K/Akt/mTOR signaling networks in T-ALL. Despite initial concerns over specificity and thereby off-target toxicity of ICG-001 (Kim et al., 2017), the second-generation clinical grade CBP/β-catenin antagonist PRI-724 was well-tolerated by patients with HCV cirrhosis (Kimura et al., 2017).

Although similar evidence is still lacking in cancer patients, we believe that the capacity of CBP/β-catenin antagonists to safely treat acute leukemias via elimination of drug-resistant LSCs might provide a real cure for these malignancies, especially in combination with other targeted and classical therapeutics. Further pre-clinical and clinical investigations are needed to confirm this.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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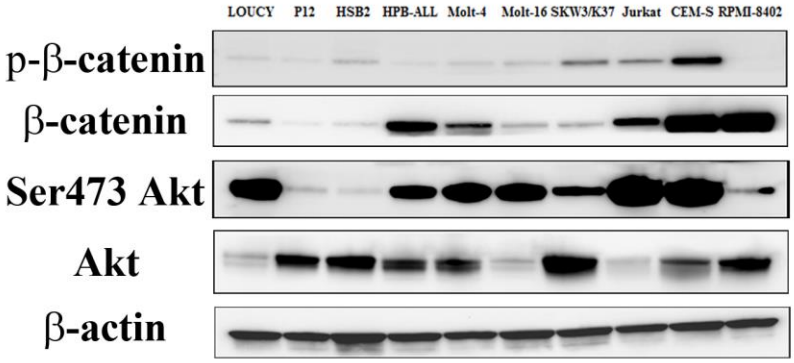
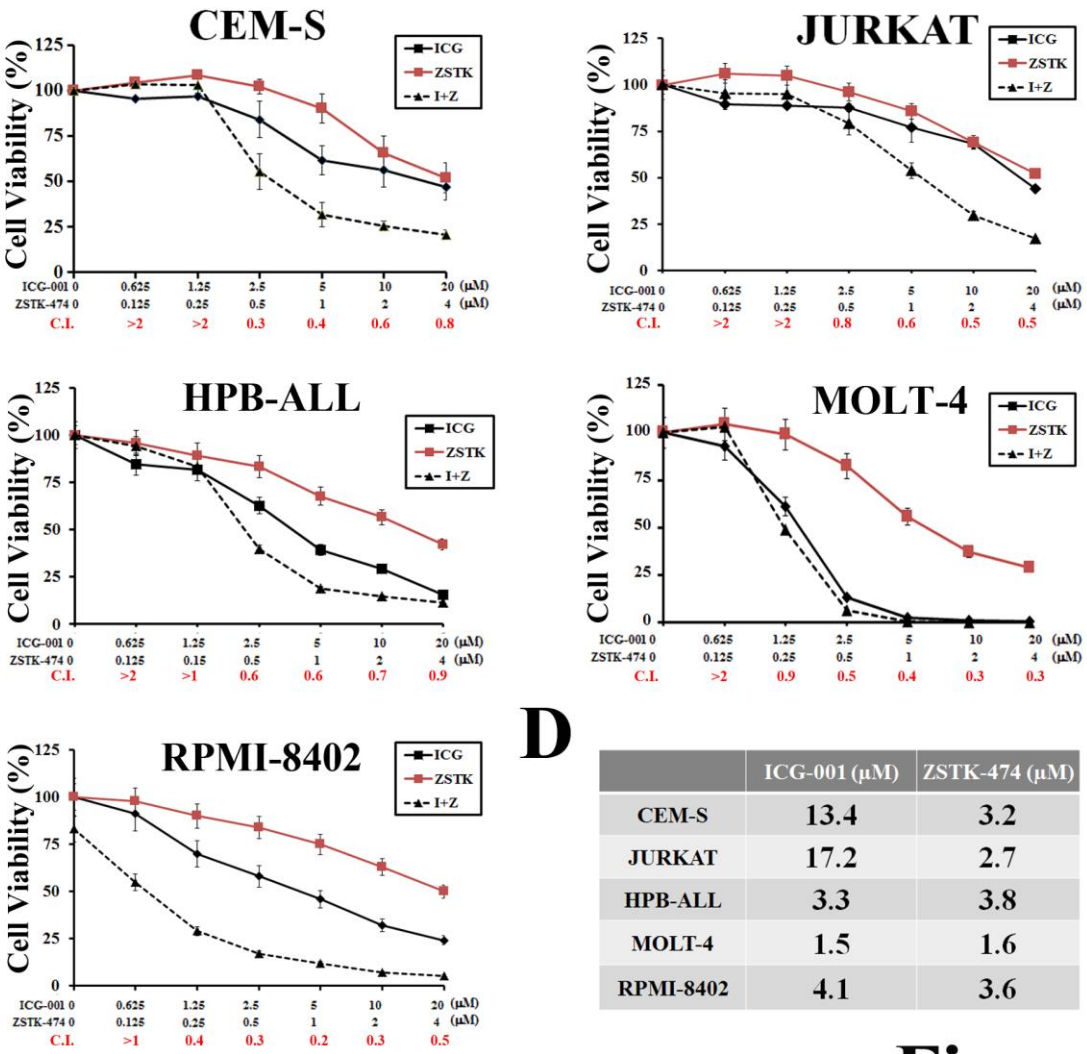
A



B

p-β-catenin/ β-catenin	MOLT-4	HPB-ALL	JURKAT	CEM-S	RPMI-8402
	0.05	0.33	0.04	0.12	0.00067

C



D

	ICG-001 (μM)	ZSTK-474 (μM)
CEM-S	13.4	3.2
JURKAT	17.2	2.7
HPB-ALL	3.3	3.8
MOLT-4	1.5	1.6
RPMI-8402	4.1	3.6

Figure 1

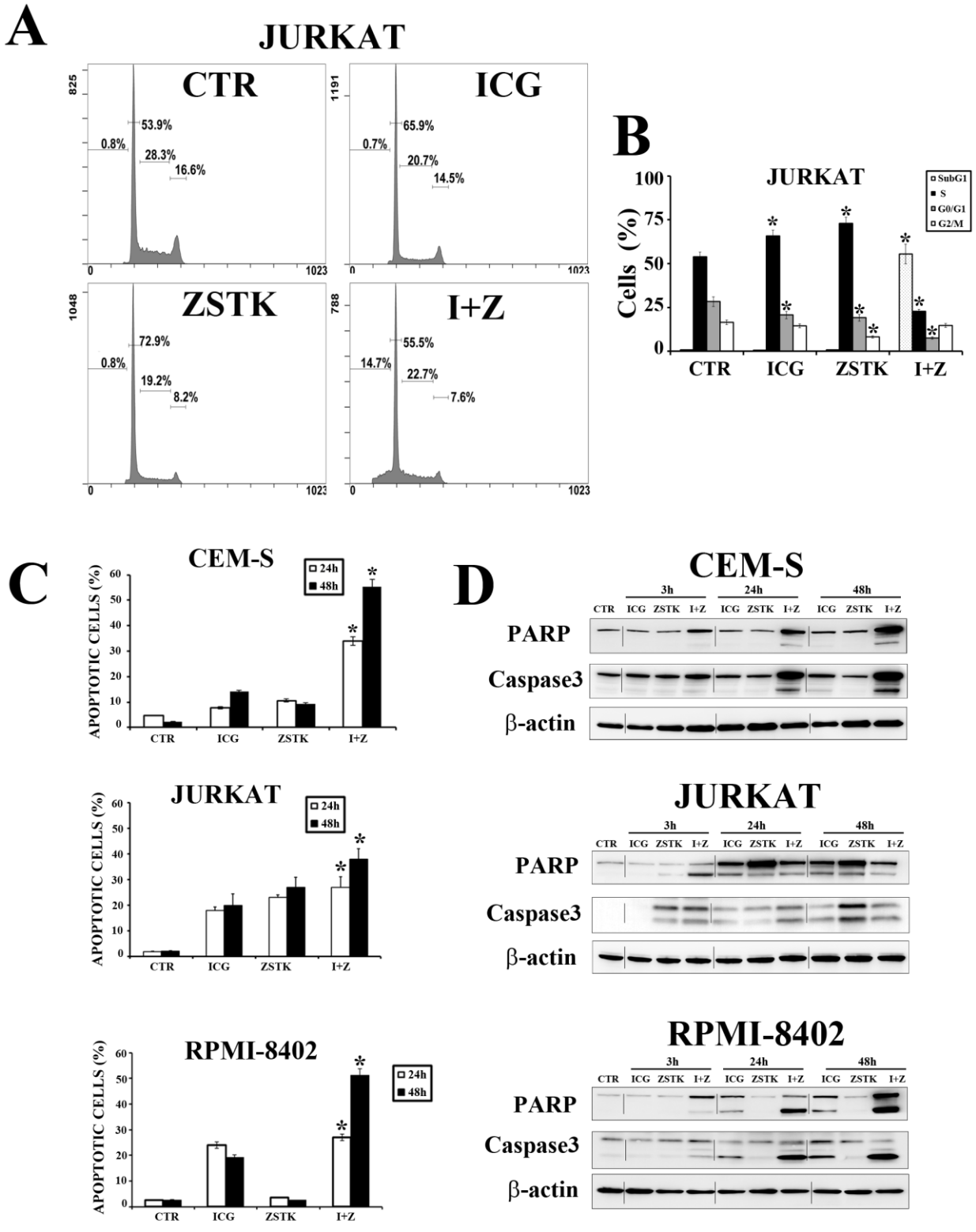

A
JURKAT
CTR
825
53.9%
0.8%
28.3%
16.6%
0
1023
ICG
1191
65.9%
0.7%
20.7%
14.5%
ZSTK
1048
72.9%
0.8%
19.2%
8.2%
I+Z
788
55.5%
14.7%
22.7%
7.6%
B
JURKAT
Cells (%)
SubG1
S
G0/G1
G2/M
CTR
ICG
ZSTK
I+Z
C
CEM-S
APOPTOTIC CELLS (%)
24h
48h
JURKAT
RPMI-8402
D
CEM-S
3h
24h
48h
CTR ICG ZSTK I+Z
PARP
Caspase3
β-actin
JURKAT
RPMI-8402

Figure 2

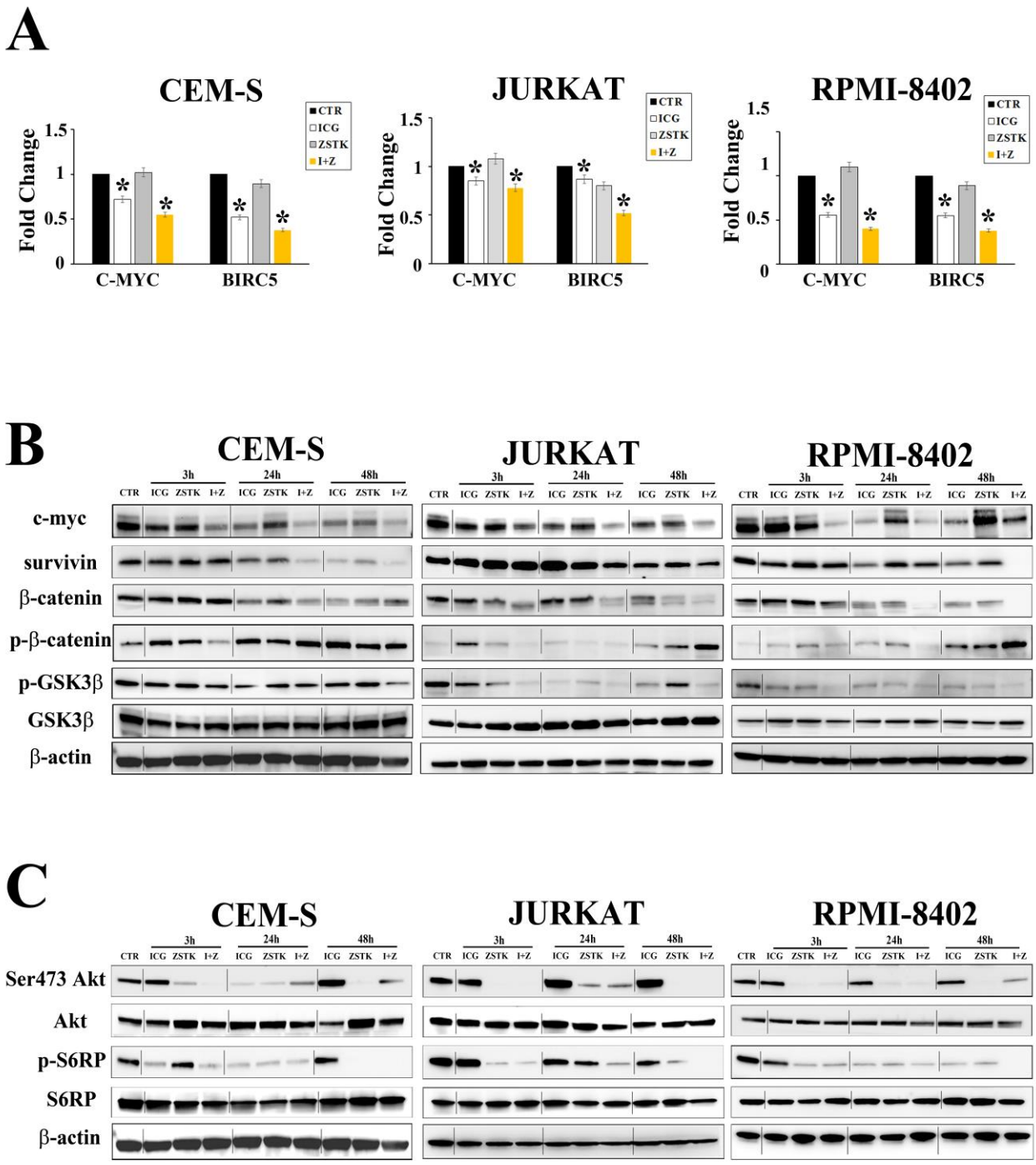

A
CEM-S
JURKAT
RPMI-8402
Fold Change
CTR
ICG
ZSTK
I+Z
C-MYC
BIRC5
B
CEM-S
JURKAT
RPMI-8402
3h
24h
48h
c-myc
survivin
β-catenin
p-β-catenin
p-GSK3β
GSK3β
β-actin
C
CEM-S
JURKAT
RPMI-8402
Ser473 Akt
Akt
p-S6RP
S6RP
β-actin

Figure 3

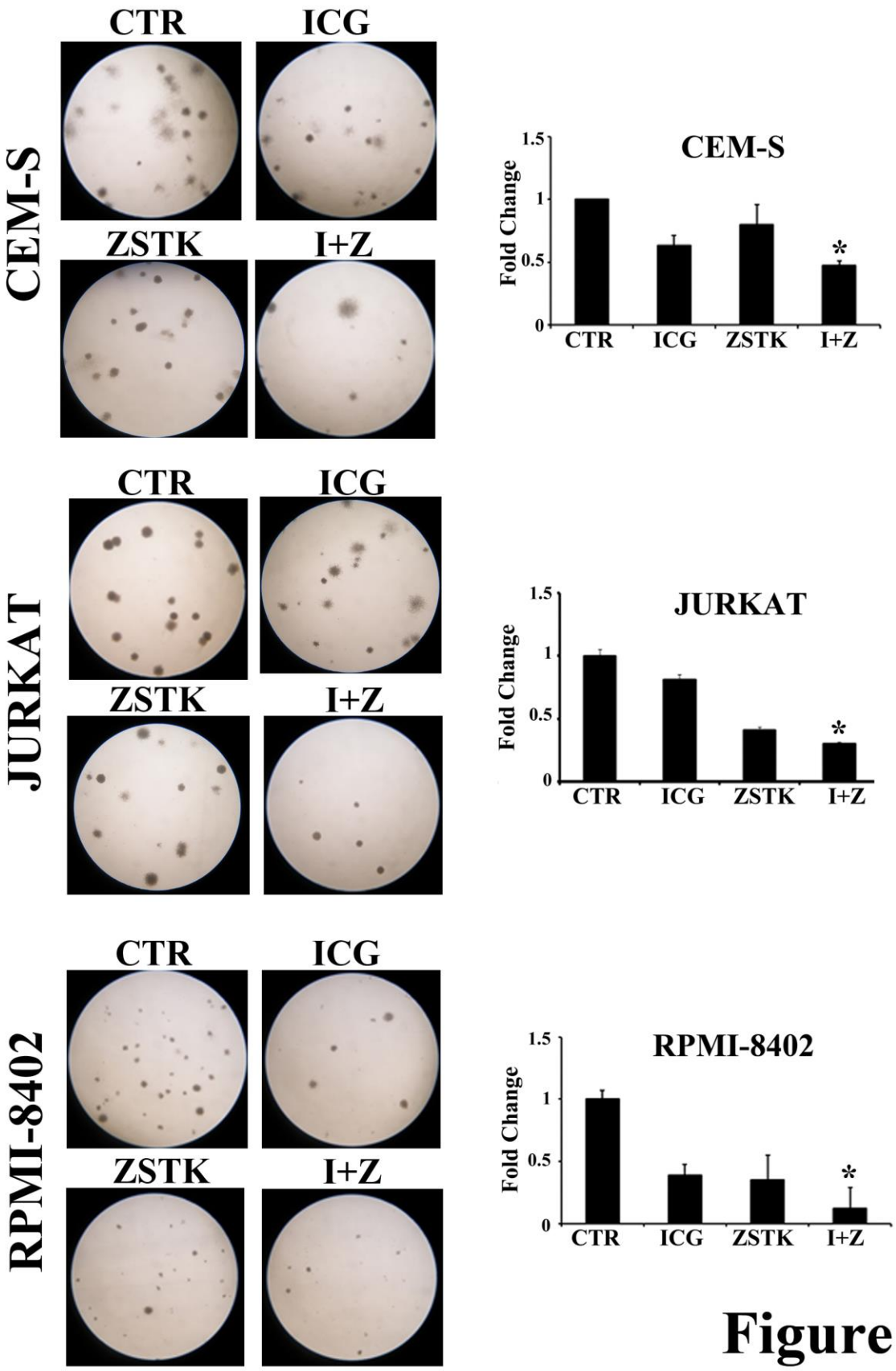

CEM-S
CTR
ICG
ZSTK
I+Z
CEM-S
Fold Change
1.5
1
0.5
0
CTR
ICG
ZSTK
I+Z
*
JURKAT
CTR
ICG
ZSTK
I+Z
JURKAT
Fold Change
1.5
1
0.5
0
CTR
ICG
ZSTK
I+Z
*
RPMI-8402
CTR
ICG
ZSTK
I+Z
RPMI-8402
Fold Change
1.5
1
0.5
0
CTR
ICG
ZSTK
I+Z
*

Figure 4

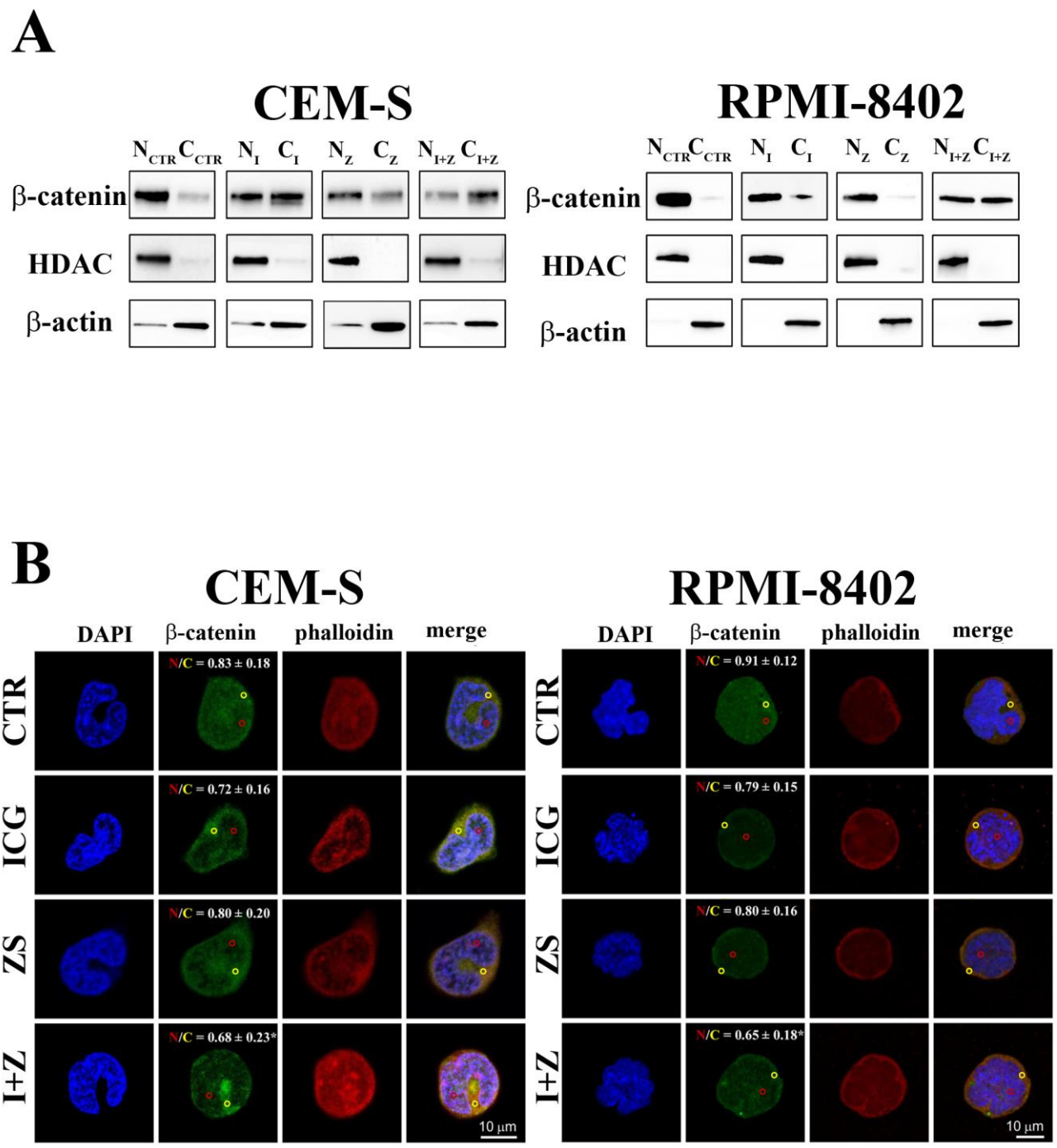

A
CEM-S
RPMI-8402
N_CTR C_CTR N_I C_I N_Z C_Z N_I+Z C_I+Z
β-catenin
HDAC
β-actin
B
CEM-S
RPMI-8402
DAPI
β-catenin
phalloidin
merge
CTR
ICG
ZS
I+Z
N/C = 0.83 ± 0.18
N/C = 0.72 ± 0.16
N/C = 0.80 ± 0.20
N/C = 0.68 ± 0.23*
N/C = 0.91 ± 0.12
N/C = 0.79 ± 0.15
N/C = 0.80 ± 0.16
N/C = 0.65 ± 0.18*
10 μm

Figure 5

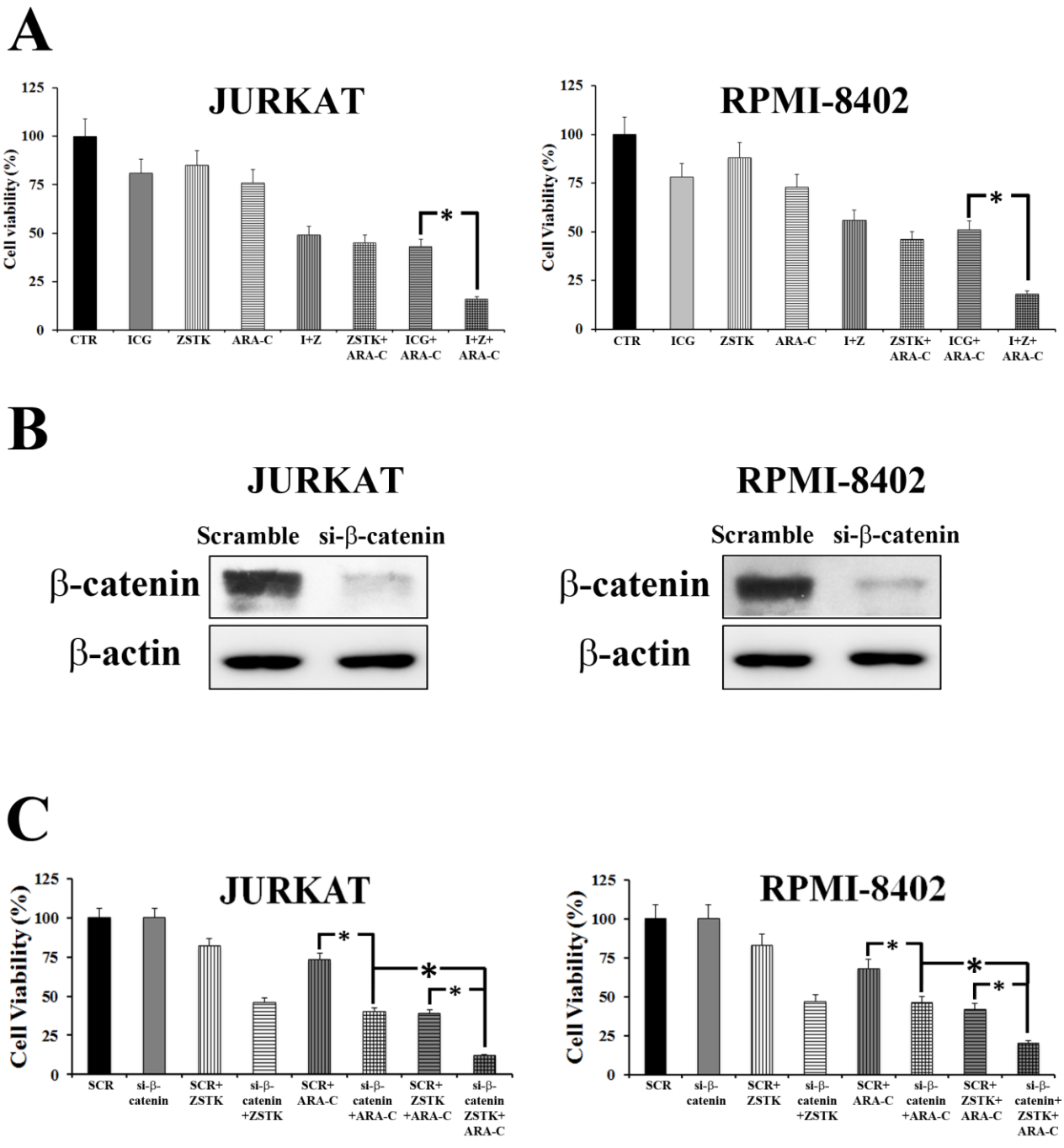

A
JURKAT
Cell viability (%)
125
100
75
50
25
0
CTR
ICG
ZSTK
ARA-C
I+Z
ZSTK+ ARA-C
ICG+ ARA-C
I+Z+ ARA-C
*
RPMI-8402
Cell Viability (%)
B
JURKAT
Scramble
si-β-catenin
β-catenin
β-actin
RPMI-8402
C
JURKAT
Cell Viability (%)
SCR
si-β-catenin
SCR+ ZSTK
si-β-catenin +ZSTK
SCR+ ARA-C
si-β-catenin +ARA-C
SCR+ ZSTK +ARA-C
si-β-catenin ZSTK+ ARA-C
RPMI-8402
SCR+ ZSTK+ ARA-C
si-β-catenin+ ZSTK+ ARA-C

Figure 6

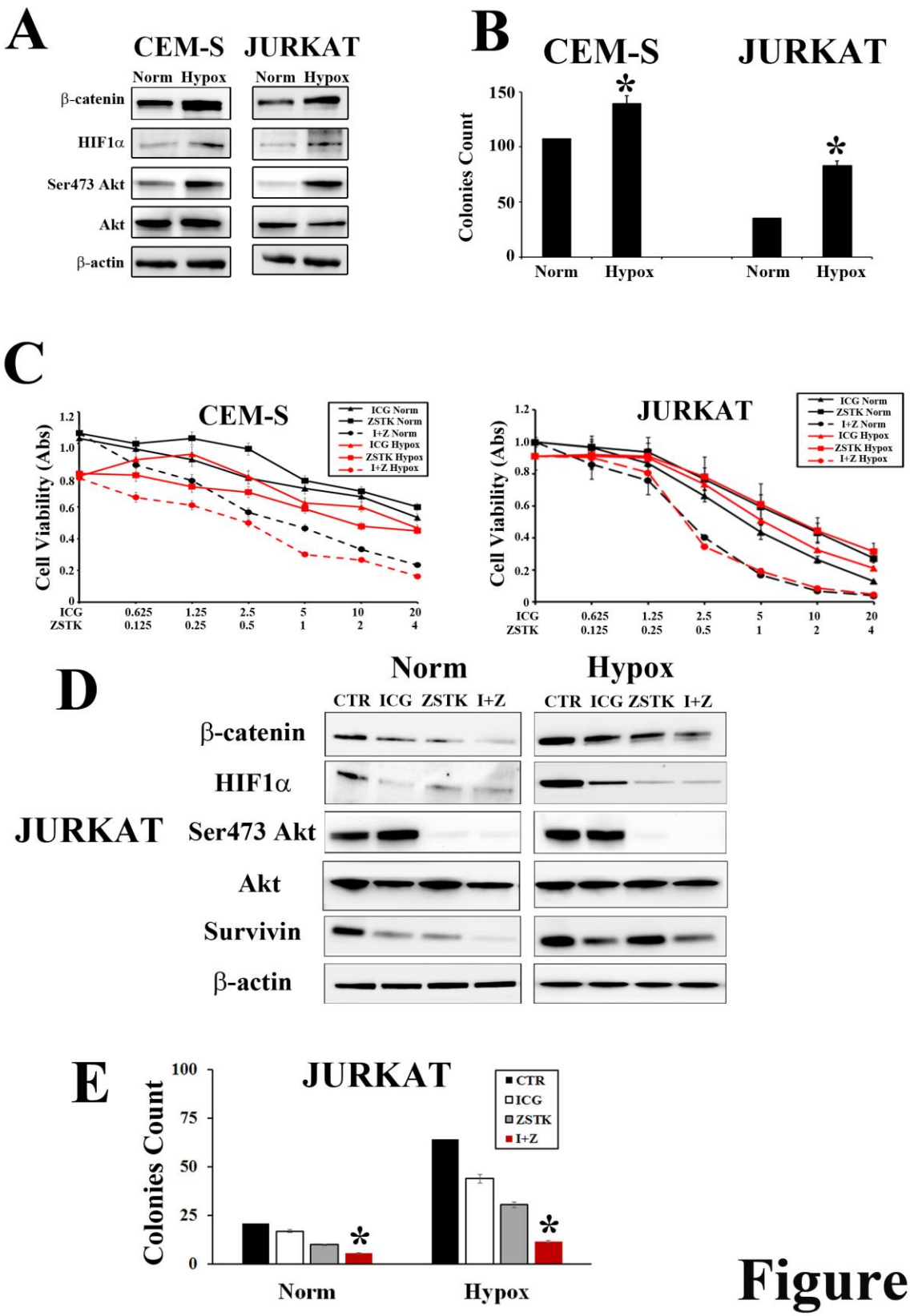

A
CEM-S
JURKAT
Norm Hypox
Norm Hypox
β-catenin
HIF1α
Ser473 Akt
Akt
β-actin
B
CEM-S
JURKAT
Colonies Count
150
100
50
0
Norm
Hypox
Norm
Hypox
C
CEM-S
JURKAT
Cell Viability (Abs)
ICG Norm
ZSTK Norm
I+Z Norm
ICG Hypox
ZSTK Hypox
I+Z Hypox
ICG
ZSTK
0.625
0.125
1.25
0.25
2.5
0.5
5
1
10
2
20
4
D
Norm
Hypox
CTR ICG ZSTK I+Z
CTR ICG ZSTK I+Z
JURKAT
β-catenin
HIF1α
Ser473 Akt
Akt
Survivin
β-actin
E
JURKAT
Colonies Count
100
75
50
25
0
CTR
ICG
ZSTK
I+Z
Norm
Hypox

Figure 7

Figure legends

Figure 1. ICG-001 and ZSTK-474 affect viability of T-ALL cell lines. (A) Western blot analysis of a panel of T-ALL cell lines showing the expression levels of p-β-catenin (Ser33/37/Thr41), β-catenin, p-Akt (Ser473Akt), and Akt. (B) Table showing phosphorylated β-catenin/non phosphorylated β-catenin ratio obtained by blot densitometric scanning. (C) MTT assays performed in T-ALL cell lines after 48 hours of treatment with increasing concentrations of ICG-001 (ICG) and ZSTK-474 (ZSTK) or the combination of the 2 drugs (I+Z). For each combination experiment, a Combination Index (CI) was calculated using the CalcuSyn software (Biosoft, Cambridge, UK). The results are the mean of at least three different experiments ± sd. (D) IC_{50} values for ICG-001 and ZSTK-474 obtained through MTT assays after 48 hours of treatment.

Figure 2. **ICG-001 and ZSTK-474 induce apoptosis.** (A) Flow cytometric analysis of cell cycle distribution in Jurkat cells treated with ICG-001 (ICG) and ZSTK-474 (ZSTK) or the combination of the 2 drugs (I+Z) for 24 hours. CTR: untreated cells. (B) Percent distribution of the different cell cycle phases. Asterisks indicate statistically significant differences with respect to untreated cells (CTR) (*$p < 0.05$). (C) T-ALL cell lines were treated for 24 or 48 hours with ICG-001 (ICG), ZSTK-474 (ZSTK) or the combination of the 2 drugs (I+Z). Then, cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry. CTR: untreated cells. Asterisks indicate statistically significant differences with respect to untreated cells (*$p < 0.05$). (D) Western blot analysis for PARP and cleaved caspase 3 (17/19-kDa) in T-ALL cell lines treated for the indicated times with ICG-001, ZSTK-474 or the combination of the 2 drugs. CTR: untreated cells.

Figure 3. ICG-001 and ZSTK-474 interfere with Wnt/β-catenin and PI3K/Akt/mTOR signaling. (A) qRT-PCR expression profiling of *C-MYC* and *BIRC5* genes. T-ALL cells were treated for 24 hours with ICG-001 (ICG) or ZSTK-474 (ZSTK) or the combination of the 2 drugs (I+Z). Histograms show differently expressed genes versus untreated samples (CTR). Results are the mean of three separate experiments. Asterisks indicate statistically significant differences with respect to untreated cells (*$p < 0.05$). (B-C) Western blot analysis in response to drug treatment for the indicated time. CTR: untreated cells.

Figure 4. ICG-001 and ZSTK-474 impair clonogenic activity of T-ALL cells. Samples were treated with ICG-001 (ICG), ZSTK-474 (ZSTK) or the combined drugs (I+Z) and plated in semi-solid methylcellulose-based media. Colonies were counted 12 days after seeding. Results are

displayed as the fold change in the number of CFU growing from drug-treated cells compared with untreated cells (CTR). Experiments were performed in triplicates and the data are representative of three independent experiments. Asterisks indicate statistically significant differences with respect to untreated cells (*$p < 0.05$).

Figure 5. **Drug treatment increases β-catenin cytoplasmic levels.** (A). Western blot analysis. CEM-S and RPMI-8402 cells were treated for 24 hours with ICG-001 (I), ZSTK-474 (Z) or their combination (I+Z). After subcellular fractionation, equal amounts of protein (20 μg) from cytoplasmic (C) and nuclear (N) fraction was separated by SDS-PAGE. Membranes were probed with antibodies to β-catenin, histone deacetylase (HDAC; nuclear marker) or β-actin (cytoplasmic marker). CTR: untreated cells. (B) Confocal microscopy analysis. Cells were treated with drugs as for western blot analysis. Samples were then were stained with antibody to β-catenin (green), while cytoplasm and nucleus were stained with phalloidin (red) and DAPI (blue), respectively. N/C: nuclear/cytoplasm mean fluorescence intensity ratio relative to β-catenin, as assessed by imaging analysis. Representative region of interests of nucleus (in red) and cytoplasm (in yellow). The ratio N/C was quantified and reported as mean ± SEM; t-test; *$P < 0.05$.

Figure 6. Combined treatment with ICG-001 and ZSTK increases ARA-C cytotoxicity. (A) T-ALL cell lines were treated for 48 hours with ICG-001 (ICG, 2.5 μM), ZSTK-474 (ZSTK, 0.5 μM), cytarabine (ARA-C, 0.15 μM) or their double (I+Z; ZSTK+ARA-C; ICG+ARA-C) or triple combination (I+Z+ARA-C). Cell viability was assessed by MTT assay. CTR: untreated cells. Asterisks indicate a significant difference (*$p<0.05$). Results are the mean of three separate experiments ± sd. (B) Jurkat and RPMI-8402 cells were treated with either scramble siRNA or ON-TARGET plus siRNA-β-catenin for 24 hours. β-catenin silencing was confirmed by Western blot analysis. Equal amounts of protein (20 μg) was separated by SDS-PAGE. Antibody to β-actin served as loading control. (C) Cells with β-catenin downregulated via siRNA (si-β-catenin) for 24 hours were subsequently treated for 48 hours with ZSTK-474 (0.5 μM) or ARA-C (0.15 μM) or their combination. Cell viability was assessed by MTT assay. SCR: cells treated with scramble siRNA. Asterisks indicate a significant difference (*$p<0.05$). Results are the mean of three separate experiments ± sd.

Figure 7. **Effects of hypoxia on Wnt/β-catenin and PI3K/Akt/mTOR signaling.** (A) Western blot analysis of β-catenin, HIF1α, Ser473 Akt, and Akt under normoxic (Norm: 20% O_2) or hypoxic (Hypox: 2% O_2) conditions in T-ALL cells. (B) CEM-S and Jurkat cells were plated in semi-solid

methylcellulose-based media under normoxic and hypoxic conditions. Colonies were counted under the microscope 12 days after seeding. Experiments were performed in triplicate and the data are representative of three independent experiments. Asterisks indicate statistically significant differences (*$p < 0.05$). (C) MTT assay was performed in CEM-S and Jurkat cells after 48 hours of drug treatment [ICG-001 (ICG) and ZSTK-474 (ZSTK) or their combination (I+Z)] under normoxic or hypoxic conditions. The results are the mean of at least three different experiments ± sd. (D) Jurkat cells were treated with the drugs for 48 hours under normoxic or hypoxic conditions. Then, western blot analysis for β-catenin, HIF1α, Ser473 Akt, Akt, and survivin was performed. CTR: untreated cells. (E) Jurkat cells were treated with drugs and plated in semi-solid methylcellulose-based media for 12 days, under normoxic or hypoxic conditions. Colonies were counted 12 days after seeding. Experiments were performed in triplicate and the data are representative of three independent experiments. Asterisks indicate a significant difference (*$p < 0.05$). CTR: untreated cells.