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CBP/Catenin Antagonist Safely Eliminates Drug Resistant Leukemia Initiating Cells

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Abstract

CBP and p300 are highly homologous transcriptional coactivators with unique, non-redundant roles that bind a wide array of proteins, including catenins – β and γ. ICG-001 is a small molecule inhibitor that specifically inhibits the CBP/catenin interaction. Importantly, ICG-001 does not inhibit the p300/catenin interaction. We demonstrate that specifically inhibiting the interaction between CBP and catenin with ICG-001, results in the differentiation of quiescent drug resistant chronic myelogenous leukemia initiating cells (CML-LICs), thereby sensitizing them to BCR-ABL tyrosine kinase inhibitors, e.g. Imatinib. Using ICG-001 in a NOD/SCID/IL2Rγ$^{-/-}$ mouse model of engrafted human chronic myelogenous leukemia, we now demonstrate the complete elimination of engrafted leukemia after only one course of combined chemotherapy. Combination-treated animals live as long as their non-engrafted littermates. Results from these studies demonstrate that specifically antagonizing the CBP/catenin interaction with ICG-001 can eliminate drug resistant CML-LICs without deleterious effects to the normal endogenous hematopoietic stem cell population.

Keywords

chronic myelogenous leukemia (CML); CBP; ICG-001; catenin

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Despite the stunning clinical success achieved to date in treating chronic phase chronic myelogenous leukemia (CML), responses in advanced phase patients treated with the BCR-ABL antagonist Gleevec/Imatinib (IM) are often short-lived, and patients generally undergo disease progression.[1] Even patients demonstrating complete response to IM therapy are not cured, as discontinuation of IM treatment frequently results in relapse. Furthermore, resistance to IM develops in 2% to 4% of patients annually and IM dose escalation is generally not sufficient to control the disease.[2] Several mechanisms that contribute to tyrosine kinase inhibitor (TKI) resistance have been proposed, including the insensitivity of quiescent CML stem cells to TKIs due to low expression of BCR-ABL.[3] Increased nuclear β-catenin has been observed with progression to blast crisis, [4] a hallmark of increased Wnt/β-catenin transcription.[5] LICs are insensitive to TKIs and additionally, genomic instability in this subpopulation is a significant concern.[6] Although deletion of β-catenin after CML initiation does not significantly affect survival in mice, deletion of β-catenin has been shown to synergize with IM and abrogate CML stem cells.[7]

Aberrant Wnt signaling is associated with many cancers including CML.[8] Many Wnt signaling related genes are upregulated in CML, especially in association with disease progression.[9] Genes that are critically involved in oncogenesis, including *survivin*, have been identified to be regulated by the activation of Wnt/catenin-mediated transcription.[10,11] Epigenetic silencing of negative regulators of the Wnt signaling cascade is also frequently associated with leukemias, including CML.[12] β-catenin signaling is also activated during the development of MLL leukemic stem cells.[13]

Wnt signaling is classically mediated through β-catenin,[14] which in turn recruits the Kat3 co-activators, CREB Binding Protein (CBP) or its closely related homolog EP300 (p300), as well as other components of the basal transcriptional apparatus.[15,16] The distinct roles of the co-activators CBP and p300 in self-renewal and differentiation respectively, has been documented.[17–20] This has also been confirmed at the genome wide level using ChIP-seq.[21] Using a hematopoietic stem cell model, Livingston and co-workers concluded that CBP, but not p300, is essential for HSC self-renewal, whereas p300 is critical for proper hematopoietic differentiation.[17,22] Additionally, Kawasaki *et al.* found that p300, but not CBP, is absolutely required for retinoic acid-induced F9 teratocarcinoma cell differentiation.[23] Utilizing the specific small molecule CBP/catenin antagonist ICG-001, we previously demonstrated that CBP and p300 have distinct functions in the regulation of β-catenin-mediated gene transcription including, *c-myc*, *Cyclin* D1,[24] *EphB2*,[25] and *survivin/BIRC5*,[10] the 4th most up-regulated transcript in cancer.[26] The molecular mechanism of ICG-001 has been previously described. Briefly, it modulates Wnt/catenin signaling by specifically binding to CBP, but not p300, thereby disrupting the CBP/catenin interaction and thus down-regulating CBP/catenin mediated transcription.[24,27,28] The high affinity, specific binding of ICG-001 to CBP subsequently leads to transiently increased p300/catenin interaction, and thereby upregulation of p300/catenin mediated gene transcription.[10,24,25,27–31] The minimal ICG-001 binding region on CBP has been mapped to amino acids 1–110 at the N-terminus; interestingly, the least homologous region between the two coactivators.

The controversial dichotomous behavior of Wnt/catenin signaling in controlling both maintenance of potency and differentiation in stem/progenitor cells can be readily explained through our model of differential coactivator usage by catenin – i.e. CBP usage by catenin leads to the transcriptional activation of cassettes of genes that are involved in the maintenance of stem/progenitor cell potency and self-renewal; whereas, the use of the coactivator p300, leads to activation of genes involved in the initiation of differentiation[28,29] (Supplementary Figure S1). The use of p300 by catenin as a coactivator can be pharmacologically induced by CBP/catenin antagonists e.g. ICG-001,[24,25] thereby initiating differentiation in a wide variety of systems[25,28,30,31] and eliminating cancer stem-like cells in both mouse genetic models[19] and drug resistant human primary B-ALL.[20]

In this report, we demonstrate that IM-resistant CML cells exhibit characteristics consistent with a quiescent LIC population. Our data also indicates that when the quiescent IM-resistant population is treated with the CBP/catenin antagonist ICG-001, it initiates a myeloid differentiation pathway. Treating this cell population with ICG-001 induces their differentiation, both *in vitro* and *in vivo*, thereby sensitizing these quiescent drug resistant cells to IM. Eliminating the LIC population via forced differentiation dramatically increases the effectiveness of BCR-ABL antagonist chemotherapy both *in vitro* and *in vivo* in murine models of CML. A CBP/catenin antagonist in combination with the BCR-ABL antagonist Nilotinib completely eliminates engrafted chronic myelogenous leukemia in NSG mice, without any apparent deleterious effects to the normal hematopoietic stem cell population as judged by both hematopoietic parameters and overall lifespan compared to their non-irradiated, non-engrafted, untreated littermates.

RESULTS

Imatinib Resistant CML Cells Are Enriched in LIC

The oncoprotein BCR-ABL is the molecular target for TKIs, such as IM and second generation agents Dasatinib and Nilotinib. However, the insensitivity of quiescent LICs to TKIs constitutes a significant problem. Rather than trying to prospectively identify LICs via specific cell surface markers,[4,32,33] we chose to initiate our investigations using primary CML patients' samples, which we treated *in vitro* with IM to identify drug resistant populations. IM resistance correlates with the emergence of drug resistant LICs, and is associated with increased nuclear catenin levels and enhanced Wnt/catenin transcription.[5] We anticipated that the drug resistant cell population would be enriched in LICs relative to the drug sensitive population. Treatment *in vitro* with 1μM IM for 6–12 days was used to select for resistant cells. IM treated versus control treated samples were analyzed by FACS. DAPI was used to exclude dead cells. We consistently observed an IM resistant population in all primary CML samples tested – both bone marrow and leukopheresis samples. This was true regardless of whether the patient had previously received IM chemotherapy, or was chemotherapy naive. The IM resistant cells were consistently characterized as being DAPI negative, low forward and low side scatter ($DAPI^{neg}/F^{low}/S^{low}$) (Figure 1a, upper panel). In contrast, the IM sensitive cells were DAPI negative, but exhibited both higher forward and side scatter ($DAPI^{neg}/F^{hi}/S^{hi}$). Enrichment of the IM resistant cell population could be achieved by treatment with IM in a dose dependent manner (Supplementary Figure S2A).

Cell cycle analysis revealed that approximately 65 times more IM sensitive cells compared to the resistant cells are in S phase (13% versus 0.2%, respectively). Furthermore, 96% of IM resistant cells were in the G0/G1 phase of the cell cycle versus 72% of the IM sensitive cells (Figure 1a, lower panel). BrdU incorporation and Ki67 staining were consistent with the cell cycle analysis (Figure 1b). These data are consistent with the IM resistant cells having a highly quiescent, blast-like phenotype.

Recent studies have revealed that multi-drug resistance genes, including MDR-1, ABCG2, and ABCA3 are intrinsically expressed in stem/progenitor cells from multiple adult tissues and that they contribute to the Side Population (SP) phenotype of malignant cells.[34,35] Wnt/catenin signaling appears to play an important role in *ABCB1*/MDR-1 transcription and multi-drug resistance[36] and in particular, CBP/catenin signaling is critical to maintain the SP.[37] We therefore utilized Hoechst 33342 dye exclusion to identify the SP cells in the same CML patient samples. Again, consistent with enrichment in LICs, the IM resistant cells contributed to the majority of the SP cells in the CML samples – 77% vs. 23% for IM resistant vs. IM sensitive (Supplementary Figure S2B).

We next examined the expression of BCR-ABL in both IM sensitive and resistant populations using real-time qRT-PCR. *BCR-ABL* gene expression is significantly (3 times) lower in the IM resistant cells compared with the IM sensitive cells (Figure 1c). This was anticipated, as it has been previously demonstrated that IM does not eliminate the CML leukemia initiating cell population. Both CML LICs and the IM resistant cell population do not depend on the BCR-ABL oncoprotein for their survival.[38]

We next sorted IM resistant (1.6×10^5) and IM sensitive (2×10^5) cells and engrafted them into NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice. After 6 months, the mice were sacrificed and peripheral blood, bone marrow and spleen were analyzed to determine the engraftment of the human leukemic cells. In five out of five mice that received the IM resistant cells, we could readily detect human $CD45^+$ cell engraftment (Figure 1d). In sharp contrast, none of the seven mice that received IM sensitive cells demonstrated any human $CD45^+$ cell engraftment at six months. *BCR-ABL* expression was confirmed by RT-PCR in all human $CD45^+$ samples collected. This confirmed that the IM resistant population was enriched in LICs compared with the IM sensitive population.

CML LICs do not require BCR-ABL for their survival,[38] however, as they differentiate and become transient amplifying cells and expand, they increase their expression of this oncoprotein. We therefore performed a series of experiments to determine whether the IM resistant cell population, upon differentiation and expansion, also increased its expression of BCR-ABL, like the leukemia initiating cell population. When IM resistant cells were cultured for 4 days on the murine stromal cell line M2-10B4, *BCR-ABL* expression was significantly increased by 10 fold (Figure 1e). In colony formation assays (CFC) using freshly isolated IM resistant cells, we could not detect any CFC activity, unlike the IM sensitive cells (Figure 1f). However, after co-culture on stroma for 5 weeks, IM resistant cells acquired significant colony forming activity (Figure 1g). We conclude that differentiating the primitive, quiescent IM resistant cell population on stroma generated

transient amplifying cells that expressed *BCR-ABL* and were competent to generate colonies.

Inhibiting the CBP/catenin interaction initiates differentiation in CML LIC

ICG-001 by binding to the amino terminus of the coactivator CBP blocks the interaction between CBP and catenin – both β and γ.[24,39] This results in decreased transcription of CBP/catenin dependent genes that are associated with tumor cell survival (e.g. *survivin*),[26] with a concomitant increase in the expression of genes associated with cellular differentiation (e.g. *EphB2*).[25] We examined 3 CML patients' samples, 2 BC and 1 IM resistant, for β- and γ-catenin expression in subcellular fractions by immunoblotting (Supplementary Figure S3). In two of 3 patients' samples, there is significantly increased nuclear γ-catenin. Next, we examined Wnt/catenin (β or γ-catenin) coactivator usage, either CBP or p300 – by co-immunoprecipitation assay in both IM resistant and IM sensitive cell populations. Treatment with IM has previously been shown to significantly increase γ-catenin expression.[10,39] After *in vitro* treatment of CML cells with IM, the majority of nuclear catenin was γ-catenin in the IM resistant cells and was essentially exclusively associated with CBP (Figure 2a, lane 7 vs. lane 8). Little or no catenin can be detected in association with either coactivator in the IM sensitive cells (Figure 2a, lanes 5 and 6).

When LICs differentiate, there is a concomitant upregulation of *BCR-ABL* expression.[40] ICG-001 is a potent initiator of differentiation in stem/progenitor cells,[19,20,27,31,41] we therefore evaluated *BCR-ABL* expression in the IM resistant LIC population. We treated blast crisis CML patient samples with ICG-001, and examined the expression of *BCR-ABL* and *survivin/BIRC5*. An inhibitor of apoptosis protein, *survivin* is the 4th most upregulated transcript in cancer and is associated with chemotherapy resistance, increased tumor recurrence, and poor prognosis.[11,42] As shown in Figure 2b, ICG-001 treatment resulted in a dramatic increase in *BCR-ABL* message in the IM resistant cells, with a 50% reduction in *survivin* message in the IM resistant population as well as a smaller reduction in the IM sensitive cells. Based on these results, we anticipated that ICG-001 would re-sensitize IM resistant CML cells to IM.[20]

Inhibition of CBP/catenin interaction re-sensitizes resistant cells to IM

CML patient samples were first treated with ICG-001 prior to IM treatment. As expected, ICG-001 treatment alone did not cause significant cell death (Supplementary Table 1). However, there was a significant increase in apoptosis in the dual treated cells (ICG-001 pre-treated, followed by IM), as compared to IM only treated cells (Figure 2c). Next, we tested 17 primary CML samples – 9 chronic phase patients, 4 blast crisis patients and 4 patients that had failed IM therapy. These samples were pre-treated with either ICG-001 or DMSO control for two days, and then exposed to low dose IM (0.2 μM) for two days. Again, ICG-001 treatment alone did not result in significant cell death. However, in both IM naïve samples, as well as samples from patients that had failed IM chemotherapy, cells pre-treated with ICG-001 responded to IM more effectively, compared with cells pre-treated with the DMSO-vehicle control as judged by increased apoptosis (Figure 2d). The down-regulation of CBP/catenin signaling with ICG-001 treatment, as anticipated, resulted in decreased *survivin* expression with a concomitant increase in *BCR-ABL* expression, thereby making

the cells BCR-ABL dependent, and thus more sensitive to IM. It is also important and worth noting that the blast crisis/failed IM samples were also more responsive to IM after ICG-001 treatment.

Inhibiting the CBP/catenin interaction eliminates CML LICs

We next investigated the effects of ICG-001 on colony forming activity. When primary unsorted patient samples were treated with 10μM ICG-001 for 2 days, the colony forming cell (CFC) numbers were significantly decreased compared to the DMSO control samples (Figure 2e, upper panel). When patient samples were combination-treated with IM and ICG-001, this essentially eliminated colony forming activity (Figure 2e, lower panel).

To determine *in vitro*, which subset of the CML cell population was most sensitive to ICG-001, we treated patient samples with IM alone, ICG-001 alone or in combination and analyzed the viability of the IM-resistant ($DAPI^{neg}/F^{low}/S^{low}$) and the IM-sensitive ($DAPI^{neg}/F^{hi}/S^{hi}$) populations. Whereas IM's effect, as anticipated, was manifested on the IM-sensitive ($DAPI^{neg}/F^{hi}/S^{hi}$) cell population, the effect of ICG-001 was more pronounced on the IM-resistant ($DAPI^{neg}/F^{low}/S^{low}$) cells. There was a strong synergistic effect in both cell populations treated with the combination of ICG-001 and IM (Figure 2f). Furthermore, as anticipated ICG-001 also decreased the side population in these samples (Figure 2g).

Inhibiting CBP/Catenin interaction eliminates CML LIC engraftment in NSG mice

Having determined that ICG-001 can sensitize drug resistant CML LICs to IM *in vitro*, we wanted to investigate the ability of ICG-001 to eliminate drug-resistant LIC *in vivo*. For these studies, we first selected two well characterized CML cell lines that were derived from blast crisis patients – K562 cells were sensitive to IM, whereas EM2 cells were resistant to IM (Supplementary Figure S4A). We initially characterized the effects of ICG-001 on K562 and EM2 cells *in vitro*. Similar to our results with the CML patient samples, ICG-001 treatment decreased survivin and increased BCR-ABL expression in both K562 and EM2 cells at both the mRNA (Figure 3a) and protein level (Figure 3b).

Similar to the biochemical specificity data previously reported in other cell types,[24] when CML cells were treated with ICG-001, the CBP/catenin interaction was specifically disrupted (Figure 3c, compare lanes 4 & 6), while the interaction between p300 and catenin was enhanced (Figure 3c, compare lanes 5 & 7). Furthermore, as demonstrated by ChIP, there was a decrease in CBP with concomitantly increased p300 occupancy at the human survivin promoter in the presence of ICG-001 (Figure 3d). Although ICG-001 treatment alone did not cause significant cell death in either line, treatment sensitized both K562 and EM2 cells to IM-induced apoptosis *after* ICG-001 treatment (Supplementary Figure S4B). ICG-001 treatment also significantly decreased CFC capacity and the side population (Supplementary Figure S4C and D).

We also evaluated the effects of ICG-001 on cell differentiation. Treatment of K562 cells with ICG-001 for 3 days increased the cell surface expression of myeloid and megakaryocyte markers examined – CD11b, CD16, CD33, CD56 and CD41 (Figure 3e). Taken together, the data demonstrates that ICG-001 by disrupting the CBP/catenin

interaction initiates a differentiative pathway with upregulation of BCR-ABL expression, thereby improving the efficacy of BCR-ABL antagonists.

Long term and secondary engraftment are hallmarks of both LICs and normal hematopoietic stem cells. To examine the ability of a CBP/catenin antagonist to eliminate drug-resistant LICs, we performed a series of experiments. We marked EM2 cells with a lentiviral luciferase expression vector, and then treated these cells *in vitro* with either ICG-001 or DMSO for 5 days prior to engraftment in NSG mice. ICG-001 and DMSO treated EM2 cells were subcutaneously transplanted into the first generation recipients. The transplanted cells were viable and proliferative as judged by imaging (Figure 3f). After 20 days, 5×10^4 leukemia cells were recovered from the engrafted primary recipients and transplanted into the bone marrow of secondary recipients. In the DMSO control group, the secondary transplantation was uniformly successful (4/4) and all of the mice were dead by day 53. In stark contrast, none of the mice in the ICG-001 pretreated group successfully engrafted, even after extended periods of observation (128 days) (Figure 3f). This data demonstrates that after only 5 days of *in vitro* treatment with ICG-001, the LIC population had been eliminated as judged by the lack of bone marrow engraftment, even though leukemic cell viability was seemingly not affected, based on *in vivo* imaging at day 20. This suggests that ICG-001 treatment eliminates the LIC population by differentiating the LIC to “bulk” CML cells, without killing this “bulk” population.

Furthermore, even allowing the cells to proliferate untreated for 20 days subcutaneously *in vivo*, there were no evidence of non-LICs reverting to an LIC phenotype. This experiment, together with previous studies with the CBP/catenin antagonist ICG-001, at least partially addresses concerns about the plasticity of the CSC/LIC phenotype and their therapeutic elimination.[20,37,43] However, as this experiment consisted of an *in vitro* pretreatment, concerns regarding the potential deleterious effects of CBP/catenin antagonists on the normal hematopoietic stem cell pool still needed to be addressed.

CBP/Catenin Antagonism Safely Eliminates CML LIC *In Vivo*

To alleviate these concerns and to monitor leukemia engraftment and progression *in vivo*, we marked K562 cells with the lentiviral luciferase expression vector and transplanted these cells into the bone marrow of NSG mice. Leukemia engraftment was detected by day 13 and subsequently the mice were randomized into 3 treatment groups – PBS only (control), PBS plus Nilotinib, and ICG-001 plus Nilotinib (Figure 4a). The second generation and more potent TKI Nilotinib was utilized in this experiment, due to its enhanced bioavailability in mice compared to IM.[44] All groups received one 28-day course of treatment (from post-transplantation day 13 to day 41). At the end of this treatment period, the control PBS treated mice showed continuously increasing levels of luciferase activity, whereas the luciferase signal in the PBS/Nilotinib as well as the ICG-001/Nilotinib treated groups had decreased to baseline levels (Figure 4a). However, by day 63, approximately 20 days after treatment had stopped, the luciferase signal in the PBS/Nilotinib group was detectable again, and the signal continued to increase (Figure 4a). This result was not surprising, and has also been observed clinically after cessation of treatment, as BCR-ABL antagonists are not able to eliminate the CML LIC population.[4] In sharp contrast, the ICG-001/Nilotinib

combination treatment group continued to show baseline luciferase levels, with no increase in luciferase signals even at the termination of monitoring.

The median life span post CML transplantation in the Nilotinib treated group was 235 days versus 73 days in the PBS control group. Nilotinib-only treated mice demonstrated significantly increased survival when compared to the PBS control-treated animals. However, all the mice in the PBS control group, as well as the Nilotinib only group were dead by Day 110 and Day 260 post transplantation, respectively (Figure 4b). Importantly, in the ICG-001/Nilotinib combination-treated group, all the mice survived *significantly longer* than the Nilotinib-only treated group. All the mice in the ICG-001/Nilotinib combination treatment group have essentially the same lifespan as their healthy littermate controls, which had never been engrafted with leukemia (Figure 4b).

To confirm the absence of the *BCR-ABL* transcripts in the peripheral blood of the ICG-001/Nilotinib combination-treated mice, PCR was performed using samples that were collected 330 days after cessation of treatment. As shown, no *BCR-ABL* transcripts were detectable in the combination-treated group (Figure 4c, lanes 1–5). Importantly, the mice from the ICG-001/Nilotinib combination treatment group also exhibited a normal hematopoietic profile as judged by red blood cell, white blood cell, platelet counts and hemoglobin measurements, similar to and essentially all within the normal range compared with their age matched, non-irradiated, non-engrafted, non-treated control littermates (Figure 4d).

Finally, we engrafted two primary human CML patient samples into NSG mice. Having confirmed the engraftment based upon FACS analysis at day 14 (Supplementary Figure S5), mice were randomized to Nilotinib or Nilotinib plus ICG-001 treatment groups, and a 28 day course of treatment was initiated. Similar to the results observed with the engrafted K562 cells, the combination treatment of ICG-001/Nilotinib demonstrated significant improvement in overall survival compared to the mice in the Nilotinib only group. The median survival time for the first Nilotinib only group was 108 days. However, all the mice in the ICG-001/Nilotinib group were still alive at the time of sacrifice almost 200 days post bone marrow transplantation, indicating the combination treatment significantly extended life span ($p=0.0018$) (Figure 4e, lower panel). The second sample group provided similar results. 190 days post leukemia transplantation, only 1 of 5 mice in the Nilotinib only group survived (median survival 169 days), while 5 of 6 mice in the ICG-001/Nilotinib treated group survived ($p=0.045$, Figure 4e, upper panel).

Discussion

The ability to safely eliminate the drug resistant LIC population, without damaging endogenous normal stem cell populations, is critical to the development of more effective chemotherapeutic strategies and the complete elimination of leukemia. We have recently reported that selective disruption of the CBP/β- and γ-catenin interactions using ICG-001 leads to differentiation of pre-B-ALL cells and loss of self-renewal capacity in primary drug resistant patient samples.[20] Similarly, ICG-001 initiates the differentiation of salivary gland tumor propagating cells in a mouse genetic model (Gain of Function Wnt signaling /Loss of

Function BMPR1 signaling) and decreases H3K4me3 at promoters of stem cell-associated genes *in vitro*.[19]

In this paper, we demonstrate that ICG-001 by specifically antagonizing the interaction between CBP and catenin in CML, results in the initiation of a differentiative pathway. This is manifested by the increased expression of the myeloid and megakaryocyte differentiation markers – CD11b, CD16, CD33, CD56 and CD41 (Figure 3e). CBP/catenin antagonists e.g. ICG-001, being initiators of differentiation are not cytotoxic and therefore only very limited apoptosis is observed when used as a single agent (Supplementary Table 1). The forced differentiation of LICs leads to decreased expression of the anti-apoptotic gene *survivin* and a concomitant increase in expression of the oncoprotein *BCR-ABL*, a hallmark of CML. These combined observed effects on LICs after treatment with ICG-001 – including downregulation of *survivin* and upregulation of *BCR-ABL* – are associated with elimination via forced differentiation of quiescent LICs into a “bulk” CML population that is sensitive to BCR-ABL antagonists, thereby enabling the complete elimination of the leukemia.

CML patient samples similarly show that ICG-001 leads to decreased *survivin* expression, particularly in the IM resistant but also albeit less, in the IM sensitive population. Pre-treatment of CML cells *in vitro* with ICG-001 eliminated, essentially irreversibly,[37] the LIC population, as judged by the lack of bone marrow engraftment into NSG mice, even though this pre-treatment did not decrease cell viability (Figure 3f). Importantly, ICG-001 in combination with Nilotinib, can safely eliminate engrafted leukemia of both primary CML patient samples, as well as K562 cells, in NSG mice, without any apparent deleterious effects to the normal endogenous hematopoietic stem cell population, as judged by normal hematopoietic parameters and normal life span.

We have shown that ICG-001, a specific CBP/catenin antagonist, by inhibiting the interaction between catenins and CBP, results in transient increased binding of p300/catenin, leading to the initiation of a differentiative pathway (Supplementary Figure S1).[27,28] Treatment with CBP/catenin antagonist leads to the differentiation of tumor initiating cells (TICs), as well as the downregulation of survivin levels. This renders the differentiated progeny sensitive to current chemotherapeutic agents. Modulation of this fundamentally critical and evolutionarily (at least from mouse to human) highly conserved protein/protein interaction decision point in stem cells[27,28,31,45] may provide a unique advantage in regards to drug resistance. Translationally, the unique ability of CBP/catenin antagonists to initiate the differentiation of drug resistant tumor initiating cells in multiple mouse models (genetic and primary patient cell engraftment) without any apparent deleterious effects on the normal endogenous stem cell populations, provides a new paradigm to effectively treat and eliminate TICs in a wide range of malignancies.[46,47]

The recently developed second generation CBP/catenin antagonist PRI-724 (IC_{50} ~150nM) has proven extremely safe in both IND enabling toxicology studies and in a Phase Ia clinical trial. In the IND enabling toxicology study, the NOAEL (no adverse effect dose level) in dogs given continuous i.v. administration for 28 days was 120 mg/kg/day with sustained plasma levels > than 200 times the IC_{50}. Furthermore, in the Phase Ia clinical trial for solid tumors, no maximum tolerated dose (MTD) was reached with escalation from 40–1280

mg/m^2/day with continuous infusion i.v. for 7 days. In addition, an inverse relationship was observed between dose level and *survivin/BIRC5* expression in patient circulating tumor cells.[48] The results reported here, in conjunction with the safety of CBP/catenin antagonists in the clinic to date, augur well for a new therapeutic paradigm to safely eliminate drug resistant tumor initiating cells. Clinical studies to evaluate this strategy are currently underway (www.clinicaltrials.gov NCT01606579).

MATERIALS AND METHODS

Cell culture and CML patient samples

Human CML cell lines K562, K562R (IM resistant), EM2 and M2–10B4 stromal cells were purchased from ATCC and cultured in RPMI 1640 medium (Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% FBS (GE Healthcare Life Sciences, Logan UT, USA). Cells were maintained at 37 °C, 5% CO_2 in a humidified incubator. K562R cells were cultured with medium containing 1μM IM. For *in vivo* studies, both EM2 and K562 cells were labeled with a lentiviral vector expressing firefly luciferase and GFP with pCCL-c-MNDU3c-LUC-PGK-EGFP viral supernatant. For high titer production of viral supernatant, the lentiviral expression plasmid was cotransfected using calcium chloride precipitation into HEK293T cells together with plasmid pMDG and pCMVΔ8.91. Transduced EM2 and K562 cells were FACS sorted for GFP bright population.

All samples from CML patients (bone marrow or peripheral blood) were obtained with written informed consent and the study was conducted according to the regulations of the institutional review board of the University of Southern California, Keck School of Medicine. 6 BC, 5 IM resistant, and 9 CP patients' samples were used in the study. Detailed de-identified patients' information is summarized in Supplementary Table 2. Primary CML cells were cultured in QBSF-60 medium (Quality Biological Inc, Gaithersburg, MD, USA) at a seeding concentration of 1–1.5×10^6/ml. Dead cells were removed using a dead cell removal kit (Miltenyi Biotec, San Diego, CA, USA) before treatment.

CFC assay and SP cell assay

Stromal cells were irradiated at 8000rad and then plated at 3×10^5 per well in 6 well plates. Then 1000 sorted IM-resistant (IM-R) or IM-sensitive (IM-S) cells were co-cultured with stromal cells in Stem Pro-34 SFM (Life Technologies Inc., Grand Island, NY, USA) supplemented with the following human growth factors: Arg-1 100ng/ml, TPO 300ng/ml, Flt3 300ng/ml, SCF 300ng/ml, GCSF 10ng/ml, IL-3 10ng/ml, IL-6 10ng/ml (PeproTech, Rocky Hill, NJ, USA). Cells were recovered for BCR-ABL expression or CFC assay.

CFC assays were performed according to the manufacturer's instructions. Briefly, cells were plated in triplicate on dishes containing methylcellulose medium (Methocult H4435, StemCell Technologies, Vancouver, Canada). Cells were pre-treated with different compounds or different compounds were added into the methylcellulose medium and 1000 (DMSO) to 2000 (ICG-001, or IM or ICG-001/IM) cells were plated. The plates were incubated at 37 °C with 5% CO2. Total colony numbers were counted 14 days after plating.

Side population assay was performed as previous described.[49] Cells were pre-treated with DMSO or ICG-001 (10μM) for 24 h before SP assay analysis was performed.

FACS analysis/sorting

Antibodies and isotype controls for immunostaining were purchased from eBioscience (San Diego, CA, USA): anti-human CD45 (clone 2D1, Cat# 8011-9459), CD11b (clone 1CRF44, Cat#47-0118), CD16 (clone CB16, Cat#9011-0168), CD33 (clone HIM3-4, Cat#12-0339), CD56 (clone CMSSB, Cat#17-0567), CD41 (clone HIP8, Cat#9011-0419) and CD61 (VI-PL2, Cat#17-0619), anti-BrdU (clone BU20A, Cat#11-5071), anti-ki67 (20Raj1, Cat#12-5699). Cell staining was performed according to the manufacturer's instructions and analyzed with a BD LSR Fortessa. To sort IM-R and IM-S cells, CML samples were treated with IM (1 or 5μM) for 4–6 days after which the cells were sorted using a BD AriaII Flow Cytometer. Annexin V and Caspase 3 analyses were performed according to manufacturer's instructions (BD Bioscience, San Jose, CA, USA).

Synthesis of ICG-001

ICG-001 was synthesized as previously described.[24]

RNA isolation and Real-time PCR

Total RNA was isolated with Trizol (Life Technologies) according to manufacturer's instructions and then treated with DNase before cDNA synthesis (iScript TM cDNA synthesis kit, Bio-Rad, Hercules, CA, USA). Quantitative real time PCR was performed with the SYBR Green PCR Master mix kit (Bio-Rad) using the following primers: Human survivin F 5'-AGCCCTTTCAAGGACCAC-3', R 5'-GGATCTTCATGAGGTAGTCAGTC-3'; BCR-ABL F 5'-GAGTCTCCGGGGCTCTATGG, R 5'- GCCGCTGAAGGGCTTTTGAA, human GusB F 5'- CGTCCCACCTAGAATCTGCT, R 5'- TTGCTCACAAAGGTCACAGG, human GAPDH F 5'-GGTGCTGAGTATGTCGTGGA, R 5'-ACAGTCTTCTGGGTGGCAGT. All results were normalized to the housekeeping gene GAPDH or Gus-B and expression was compared to control treated cells. Quantitation of gene expression was based on the cycle threshold (Ct) value for each sample. Delta Ct was calculated as (gene of interest Ct)-(housekeeping gene Ct). The relative quantity of mRNA expression was calculated by delta-delta Ct calculation as (treated sample delta Ct)-(control sample delta Ct). All experiments included negative controls consisting of no cDNA for each primer pair. Data represent the mean of at least two independent experiments (± S. D.).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously.[24] Briefly, cell lysates (50 – 100μg protein) were incubated with protein A agarose (Roche Applied Science, Mannheim, Germany) for 1h. The precleared samples were then incubated with specific antibodies at 4°C overnight. Normal rabbit IgG was used as negative control. Protein A agarose beads were incubated with protease inhibitors. The immunocomplexes were washed and separated on a 4–20% SDS-PAGE gradient gel (Invitrogen, CA) and analyzed by immunoblot. The antibodies utilized were CBP/p300 (Santa Cruz, SC-32244),

anti-β-catenin (BD Bioscience, Cat# 610153) and anti-γ-catenin (BD Bioscience, Cat# 610253). Antibodies for immunoblotting (50 μg total protein) were anti-BCR-ABL (clone 131), anti-CBP (clone SC-369), anti-p300 (clone SC-584), anti-survivin (clone SC-10811), all from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Anti-α-tubulin (clone CP06) was from Calbiotech (Spring Valley, CA). UN-SCAN-IT software was used to quantify the protein bands normalized to α-tubulin.

ChiP analysis of CBP/p300 occupancy at the human survivin promoter

ChiP analysis was performed as previously described.[10] In brief, K562 cells were cultured in T25 flasks with approximately 15 million cells in 10ml of complete growth medium containing DMSO, 10μM, or 20μM ICG-001 for 24 hours prior to cross-linking with 1% formaldehyde. Cells were then lysed and sonicated. Normal IgG (Santa Cruz, sc-2027), anti-CBP (A22, Santa Cruz, sc-369) or anti-p300 (N15, Santa Cruz, sc-584) were then added to the sheared chromatin. After 24 hours, agarose beads were added and DNA was eluted from beads. 1/10 of the elution was used for qPCR analysis on a BioRad MyIQ real time PCR detection system. The occupancy of CBP or p300 at the survivin promoter was determined by the Fold Enrichment Method (ThermoFisher Science, ChiP analysis). The primers used in the study were: forward: hu-Survivin ChIP-F 5'-CTC CAG GAC TCA AGT GAT GC-3'; and reverse hu-Survivin ChIP-R 5'-CCG CGG CCT TCT GGG AGT AG-3'

Animal studies

All animal studies were approved by the USC institutional IACUC committee. NOD.Cg-Prkdc^{scid}il2rg^{tm1wji}/SzJ (NSG) mice were purchased from Jackson Laboratory and subsequently bred and maintained at the University of Southern California animal facility. Female mice at age 8–10 weeks were used in the study. Animals were randomly assigned to each treatment group. Investigators were not blinded in the animal studies. Transduced EM2 or K562 cells were injected via tail vein into sublethally irradiated (300 cGy) NSG mice. Under general anesthesia with isoflurane, leukemia progression was monitored via *in vivo* utilizing an IVIS 100 bioluminescence/optical imaging system (Xenogen Corporation, Alameda, CA, USA). D-luciferin (Promega, Madison, WI, USA) dissolved in PBS was injected intraperitoneally at a dose of 2.5mg/mouse, 15 min before luminescence signal measurement. After confirmation of leukemia engraftment, mice received either saline/saline, Nilotinib/saline (100mg/Kg/day) or a combination of ICG-001 (50mg/Kg/day) and Nilotinib (100mg/Kg/day) treatment.

For the primary CML patients' sample study, leukemia cells were injected via tail vein into 300 cGy irradiated NSG mice and blood was collected for FACS analysis of human CD45 positive leukemia cells after 14 days to confirm leukemia engraftment. At the end of the experiment, the mice were sacrificed and bone marrow, blood, and spleen were collected for FACS analysis of human CD45 positive cells. To measure blood counts, mouse blood was collected in BD microtainer tubes with EDTA and analyzed with a Hemavet (Drew Scientific Inc, Miami, FL). ICG-001 was delivered via Alzet osmotic minipump (model 1004, Alzet, Cupertino, CA, USA) according to the manufacturer's instructions.

Statistical analysis

Student t test or log-rank (Kaplan-Meier) test was performed using GraphPad Prism 5. A minimum of 3 individual CML samples were used in gene expression and colony assays with at least 2 technical repeats (Table 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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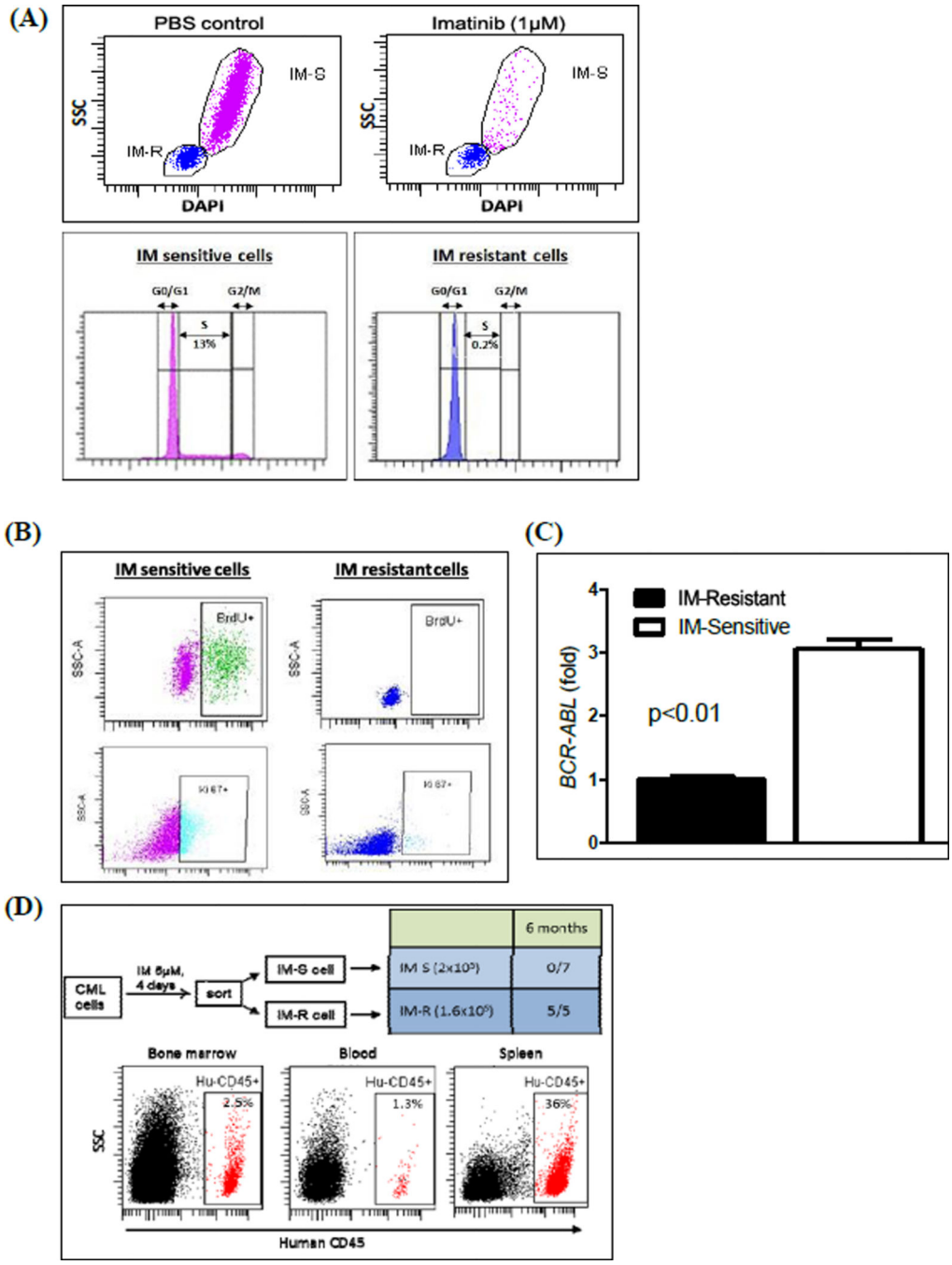


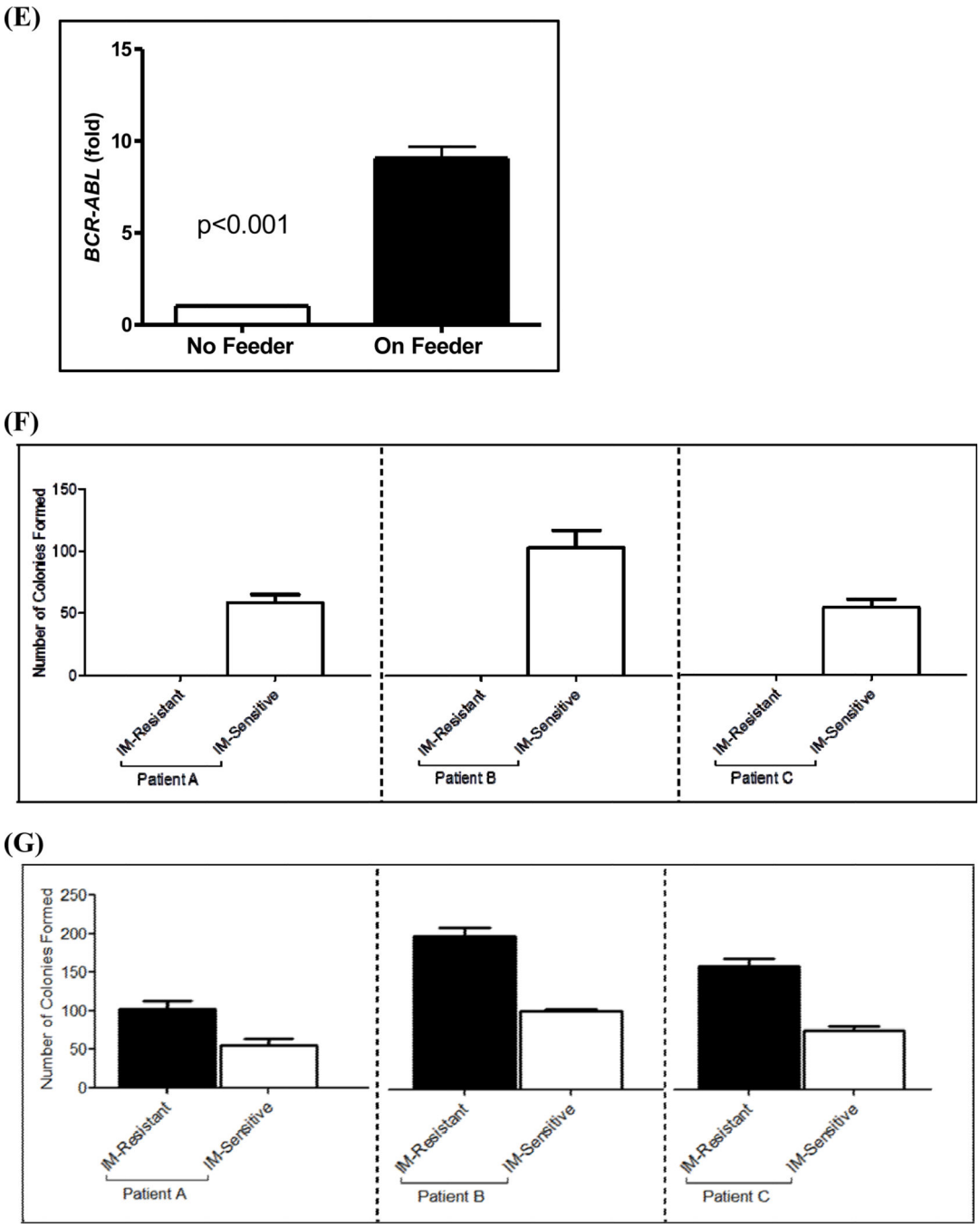


Figure 1.

(**A**) Primary CML cells were cultured in QBSF-60 serum free medium with or without IM (1μM) for 6–12 days. Cells were then analyzed by FACS for cell viability (DAPI was used for dead cell exclusion). The IM resistant population in all primary CML samples tested was consistently DAPI negative, low forward and low side scatter ($DAPI^{neg}/F^{low}/S^{low}$). The IM sensitive population was DAPI negative, but demonstrated both higher forward and side scatter ($DAPI^{neg}/F^{hi}/S^{hi}$) (*upper panel*). Cell cycle analysis using propidium iodide showed that there were approximately 65 times more cells in S phase of the cell cycle in the IM

sensitive population (13%) compared to the IM resistant cell population (0.2%). This analysis also demonstrated that 72% of the sensitive population was in G0/G1 phase compared to the IM resistant cell population in which 96% of the cells were in G0/G1 phase (*lower panel*). Representative results from a BC patient sample are shown. Similar results were obtained from 3 CML patient samples (Supplementary Table 2).

(**B**) After culture with IM for 6–12 days, CML cells were labeled with BrdU and stained for BrdU incorporation and Ki67 expression. The results confirm the quiescent nature of the IM resistant population. Representative results from 1 of 3 CML patient samples analyzed are presented.

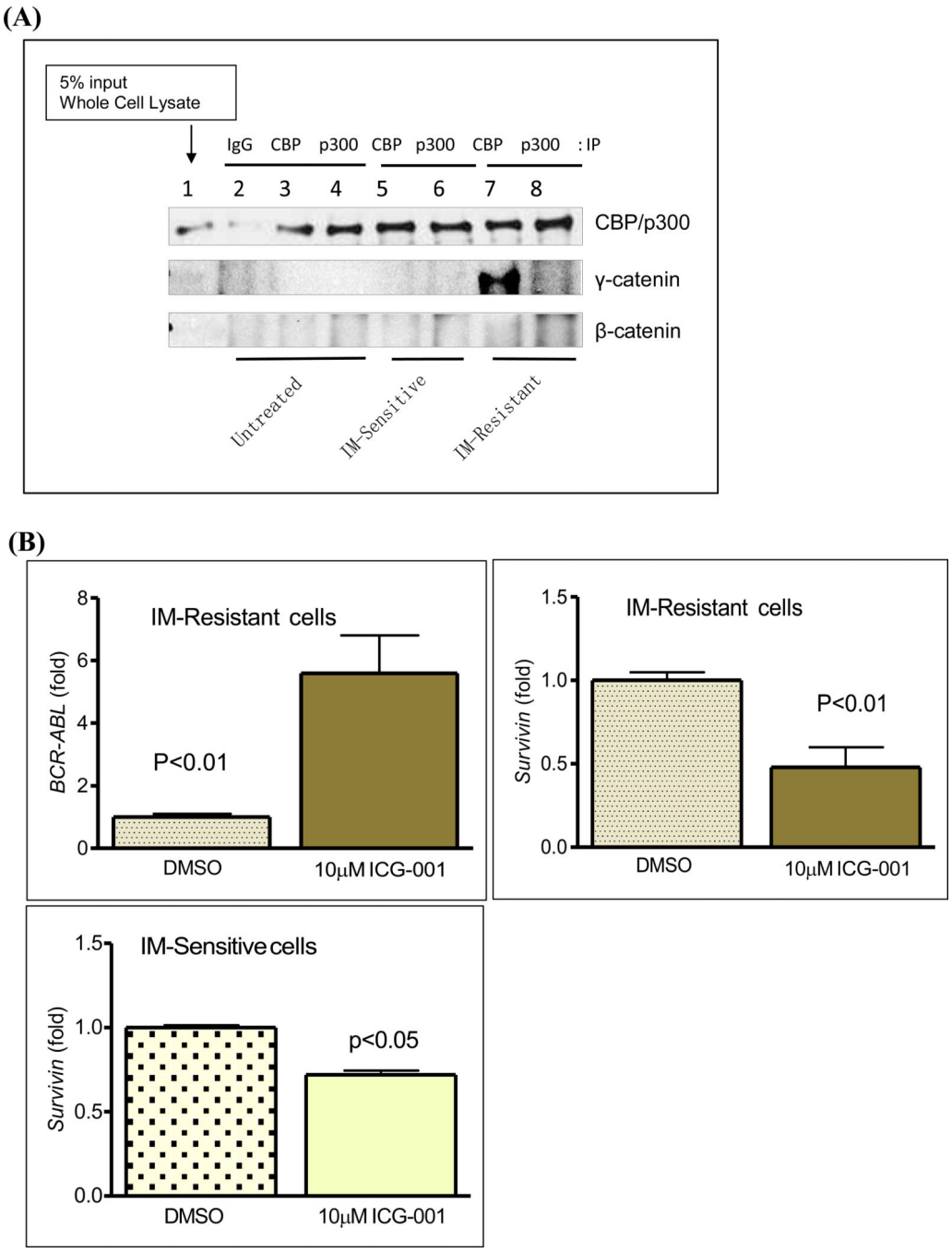
(**C**) After IM treatment for 6 days, cells were sorted and quantitative qRT-PCR for *BCR-ABL* was performed on cells from both the IM sensitive and IM resistant populations. The result presented is based upon analysis of 3 CML patient samples.

(**D**) One CML patient's (BC, IM naïve) cells were treated with IM (5μM) *in vitro* for 4 days and subsequently FACS sorted into IM-S and IM-R populations using the gates presented in Figure 1. The given number (inserted table in Figure 1D) of sorted cells were transplanted into NSG mice via tail vein injection. 6 months after engraftment, mice were sacrificed and donor cell (human CD45+) engraftment in bone marrow, blood and spleen was analyzed.

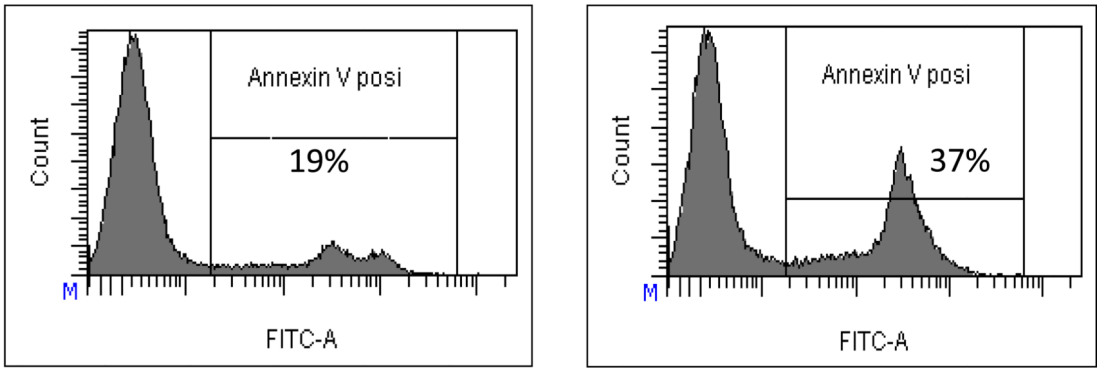
(**E**) Sorted IM-R cells dramatically upregulate *BCR-ABL* gene expression when cultured on stromal cells for 4 days. The result presented is based upon analysis of 3 patient samples.

(**F**) Freshly sorted IM-R cells do not form colonies in CFC assay, whereas IM-S cells readily form colonies under the same conditions. Results from 3 patient samples are presented.

(**G**) After co-culture on stromal cells, IM-R cells readily form colonies in CFC assay. Results from 3 patient samples are presented.

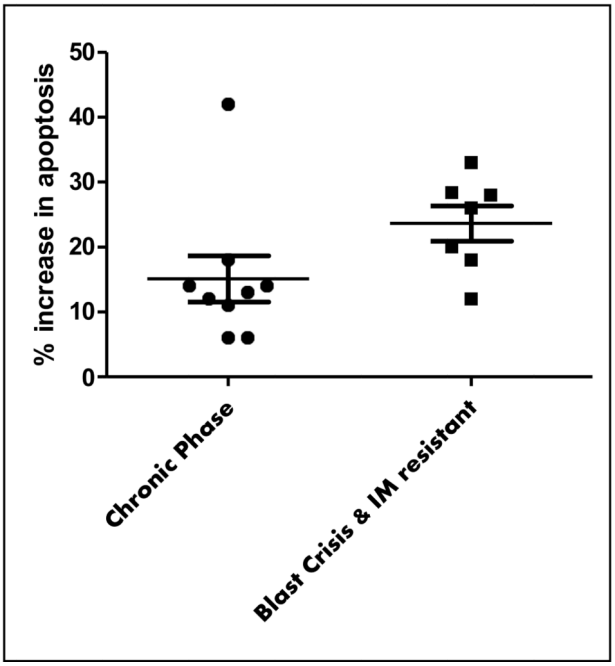


(C)

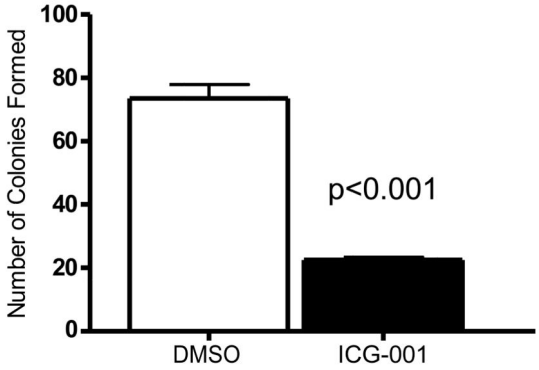


DMSO, Imatinib 0.2μM ICG-001, Imatinib 0.2μM

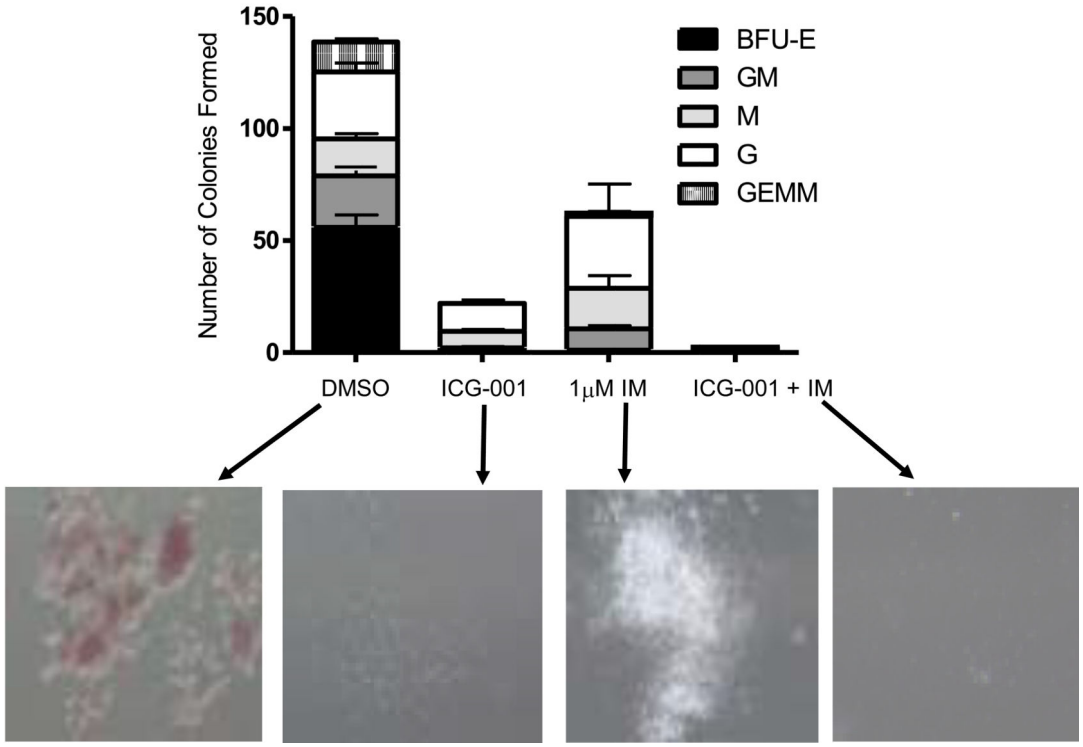
(D)



(E, *upper*)



(E, *lower*)



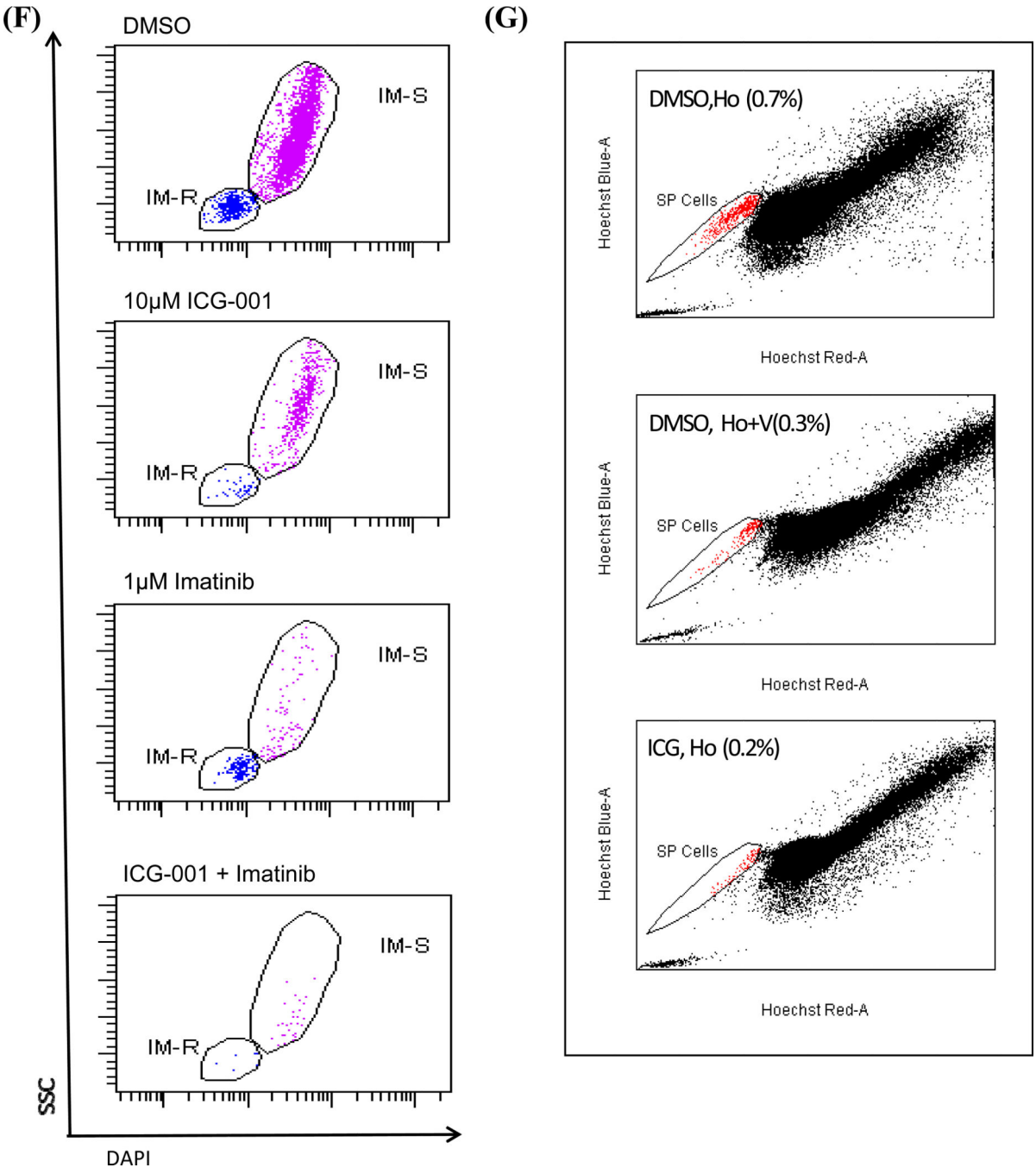


Figure 2.

(A) One CML patient's cells (BC patient, IM naïve) were treated with IM (1μM) for 4 days and then sorted into IM-R and IM-S populations for co-immunoprecipitation assay. After co-IP with antibodies specific for CBP and p300, the membrane was probed with the following antibodies: upper panel probed with an antibody recognizing both CBP and p300; the middle panel was probed with an antibody specific for γ-catenin; the lower panel was probed with an antibody specific for β-catenin. Only the IM-R and not the IM-S cells demonstrate a robust interaction between γ-catenin and CBP (middle panel, compare lanes 7 and 5). There

is no detectable β-catenin interaction with either CBP or p300 (lower panel). IP: immunoprecipitation; IB: immunoblotting.

(**B**) 48h post ICG-001 (10μM) treatment, FACS sorted IM-R cells demonstrate a significant increase in *BCR-ABL* expression, while *survivin/BIRC5* expression is significantly down-regulated in the IM-R as well as the IM-S cells, albeit less than in the IM-R cells. Result shown is a summary of 3 CML patient samples.

(**C**) Representative Annexin V analysis from the results shown in Figure 2D.

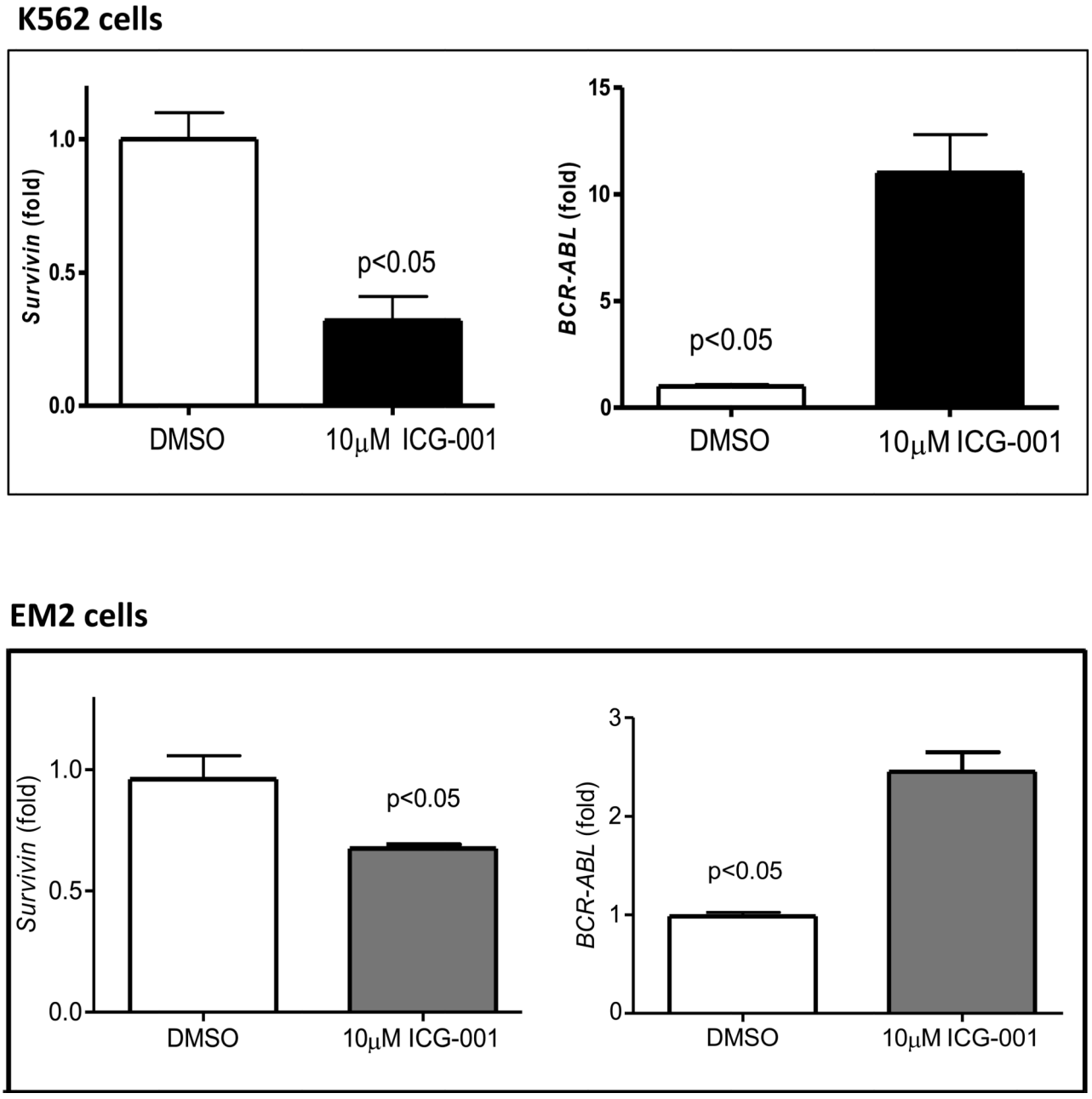
(**D**) ICG-001 pre-treatment results in increased sensitivity to IM induced apoptosis in primary CML patient samples: 9 CP, 4 BC, 4 IM failed. Each sample was divided into two wells, one treated with DMSO and the other with ICG-001 at 10μM for 2 days. Subsequently, Annexin V staining and analysis was used and dead cells were removed. The live cells were then treated with 0.2μM of IM for 24h and subsequently re-analyzed for Annexin V expression. There was no significant difference in Annexin V staining between DMSO and ICG-001 pretreated samples prior to IM treatment (data not shown).

(**E**) Primary CML cells were treated with ICG-00110μM or DMSO for 2 days. Subsequently, 500 cells were plated for CFC assay. As shown in the upper panel, ICG-001 treated cells gave significantly less colony formation. When different compounds were added in methycellulose medium, the combination of ICG-001(5μM) and IM (1μM) almost completely eliminated colony forming activity (1000 cells per well were plated). Colonies formed in samples treated with ICG-001alone were significantly smaller (lower panel). Data are representative of three individual CML patient samples.

(**F**) Primary patient samples treated with IM (1 μM) and ICG-001 (10 μM) eliminated both the IM-R and IM-S cells (representative results from 7 different patient samples).

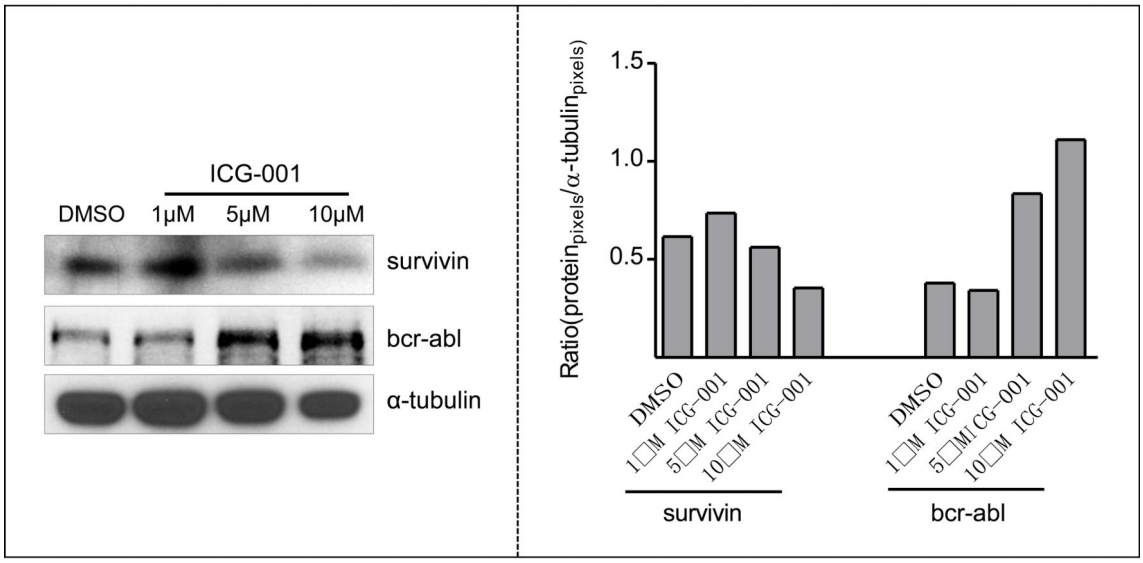
(**G**) CML cells were cultured in QBSF-60 serum free medium and treated with DMSO or ICG-001(10 μM) for 24 h. After treatment, side population analysis was performed by FACS. ICG-001 significantly decreased the SP in primary patient CML samples (V= Verapamil, Ho=Hoechst 33342, percentage of SP cells is labeled). The result shown is representative of 3 CML patient samples.

(A)

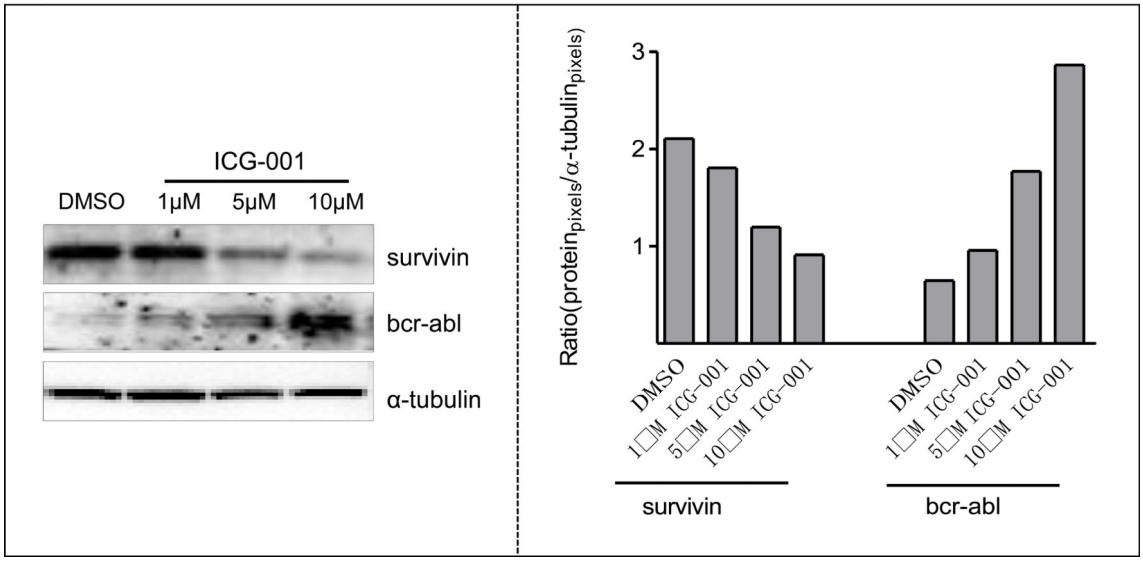


(B)

K562 cells



EM2 cells



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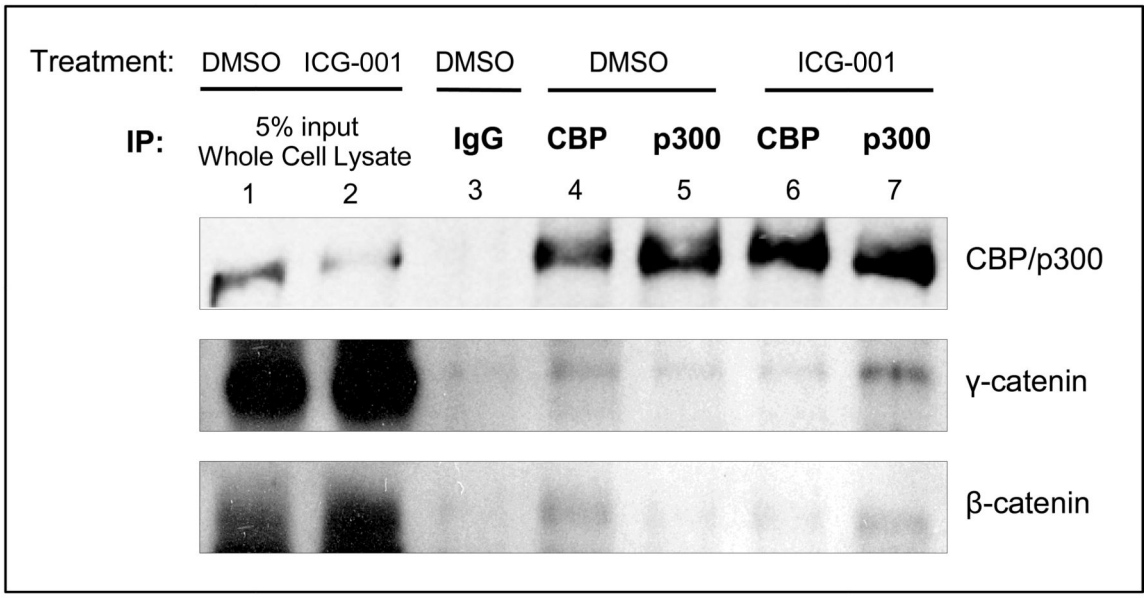

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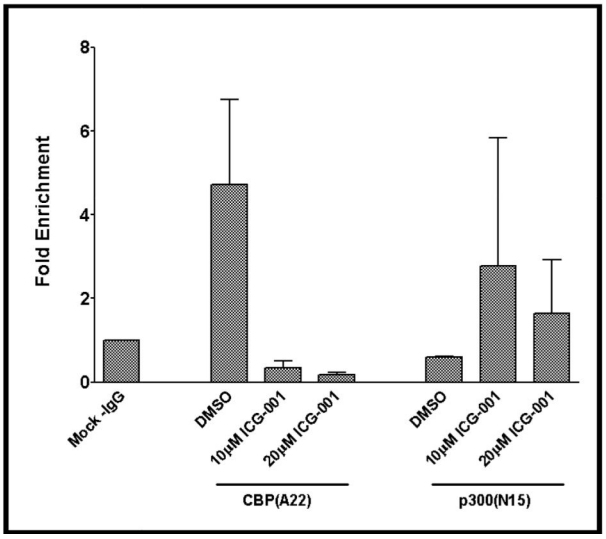

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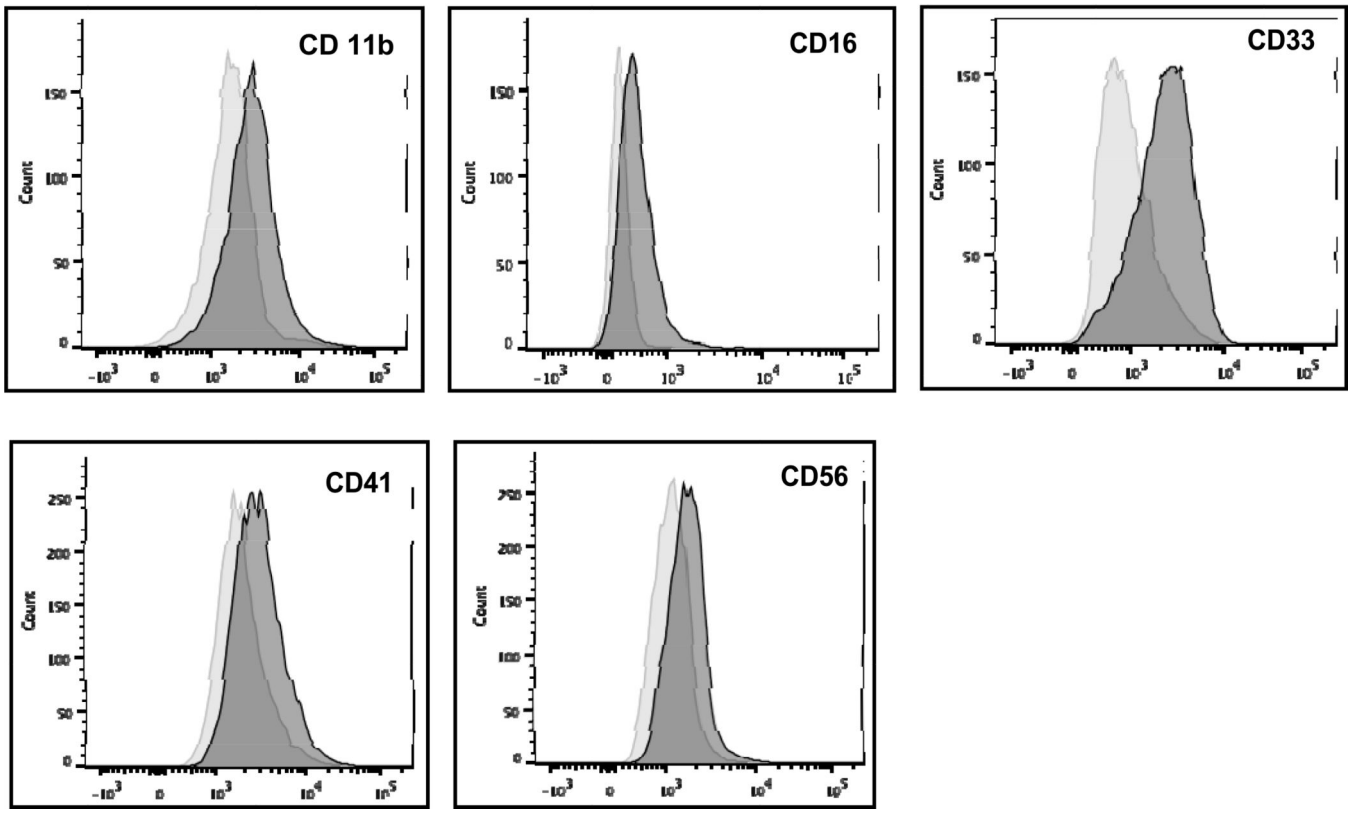


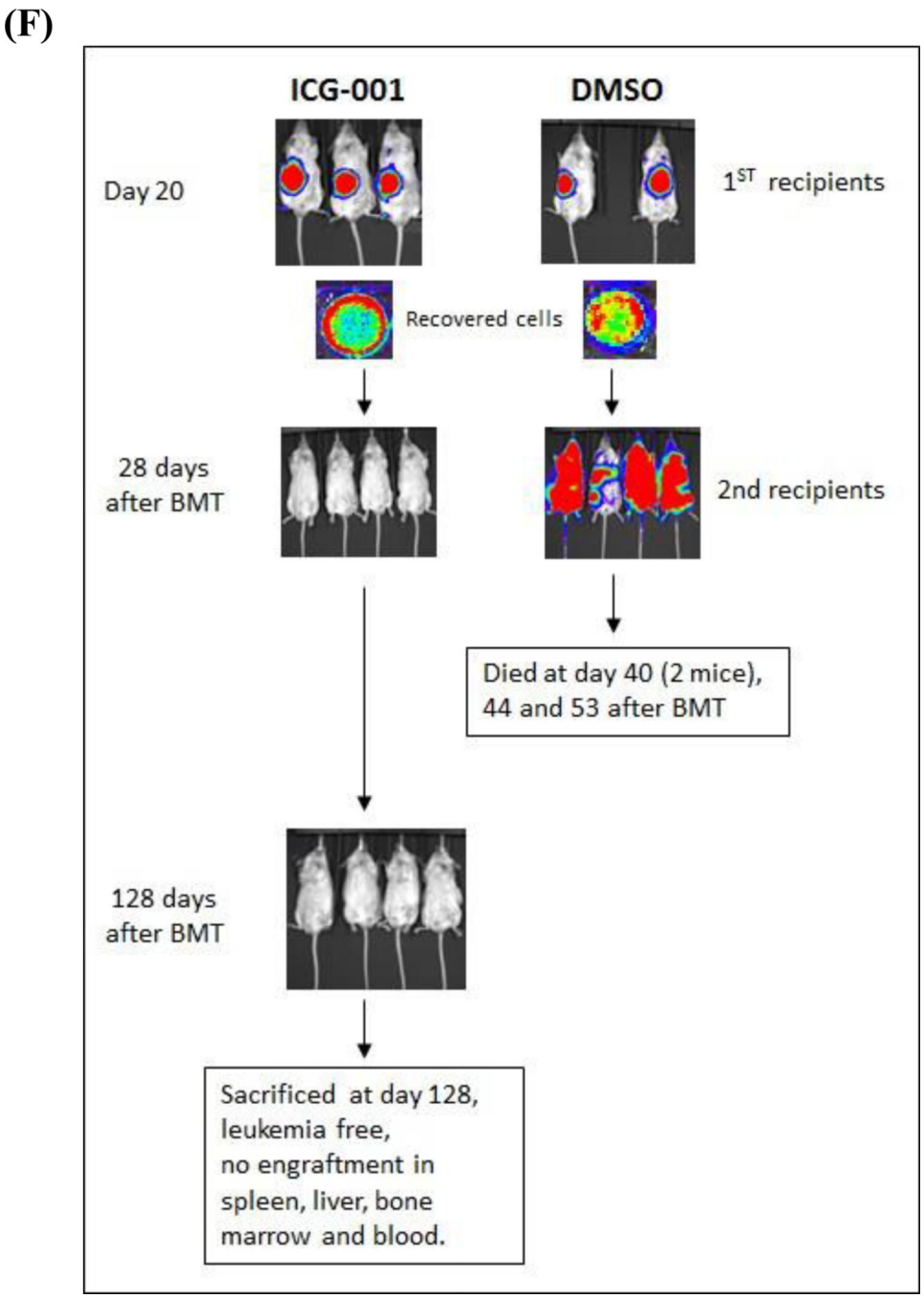


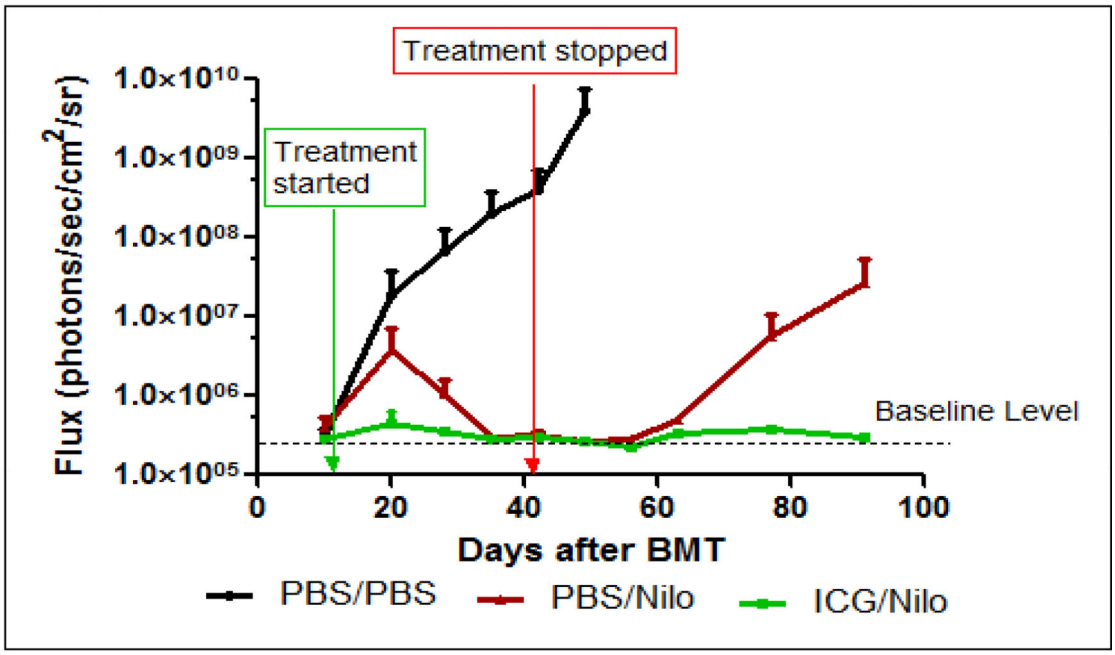
Figure 3.

(**A**) 48h treatment with ICG-001 significantly increases *BCR-ABL* gene expression in both K562 and EM2 cells while *survivin/BIRC5* gene expression is significantly down regulated. Results are a summary of three independent experiments.

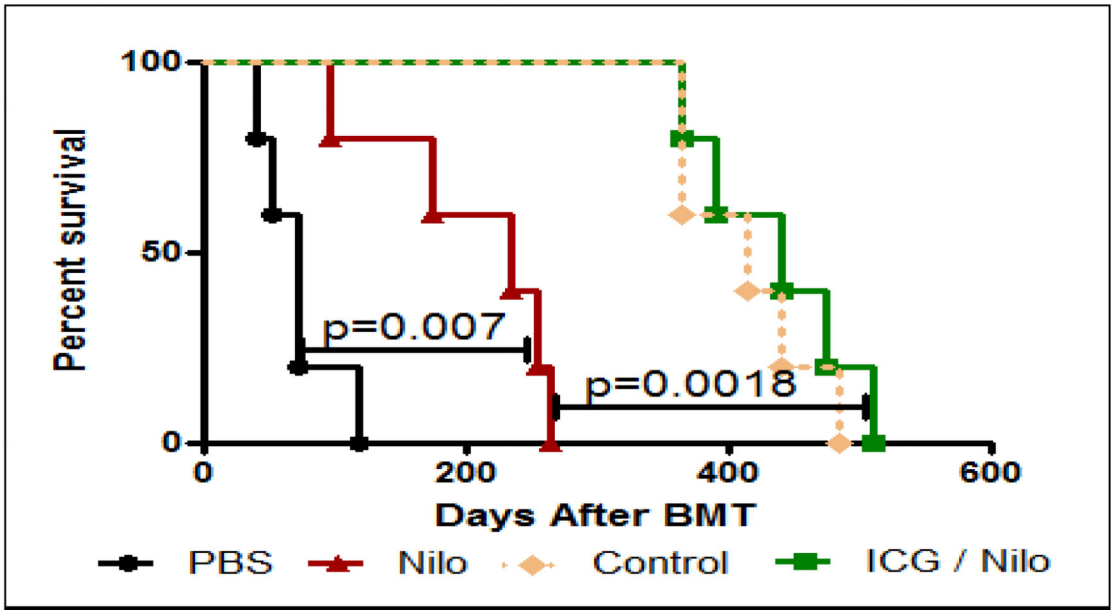
(**B**) At the protein level, expression of BCR-ABL is also significantly increased, while survivin is significantly down-regulated in both K562 and EM2 cells. The bar graphs on the right represent pixel numbers determined using UN-SCAN-IT software.

(C) In K562R cells, ICG-001 (10μM) treatment for 48h blocks both the β- and γ-catenin interaction with CBP (compare lanes 4 and 6) while increasing both β- and γ-catenin interaction with p300 (compare lanes 5 and 7). The upper panel was probed with an antibody recognizing both CBP and p300; the middle panel was probed with an antibody specific to γ-catenin; the lower panel was probed with an antibody specific to β-catenin .
(D) K562 cells were treated with DMSO or ICG-001 (either 10μM or 20μM) for 24 hours for ChiP analysis, using normal IgG, anti-CBP (A22) or anti-p300 (N15). qPCR was subsequently performed to determine the change in occupancy by CBP and p300 in the human survivin/BIRC5 proximal promoter region. ICG-001 decreased CBP, but increased p300 occupancy on the human survivin promoter. The results are the average of two experiments.
(**E**) K562 cells were treated with ICG-001 (10μM) for 3 days and then harvested for FACS analysis of multiple myeloid surface markers (CD11b, CD16, CD33, and CD56) and the megakaryocyte marker CD41. The light gray peaks represent the DMSO treated samples; the darker gray peaks represent the ICG-001 treated samples.
(**F**) EM2 cells marked with a lentiviral luciferase vector were pre-treated *in vitro* for 5 days with either DMSO control or ICG-001 (10μM) before being subcutaneously implanted into primary recipient NSG mice. The implanted cells expanded and were viable *in vivo* in the case of both treatments. After 20 days, 5×10^4 cells were recovered from the engrafted primary recipients and transplanted into the bone marrow of secondary recipient NSG mice via tail vein injection. In the DMSO group, secondary transplantation was uniformly successful (4/4) and all of the mice died by day 53. In stark contrast, none of the mice (4/4) in the ICG-001 pretreated group successfully engrafted even after 128 days of observation.

(A)



(B)



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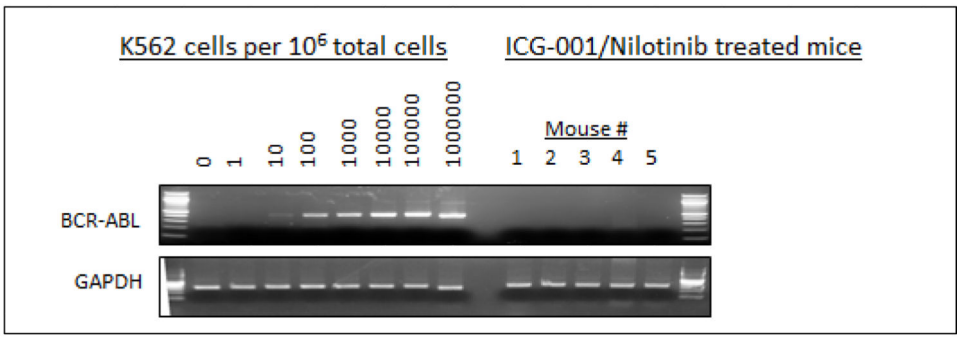
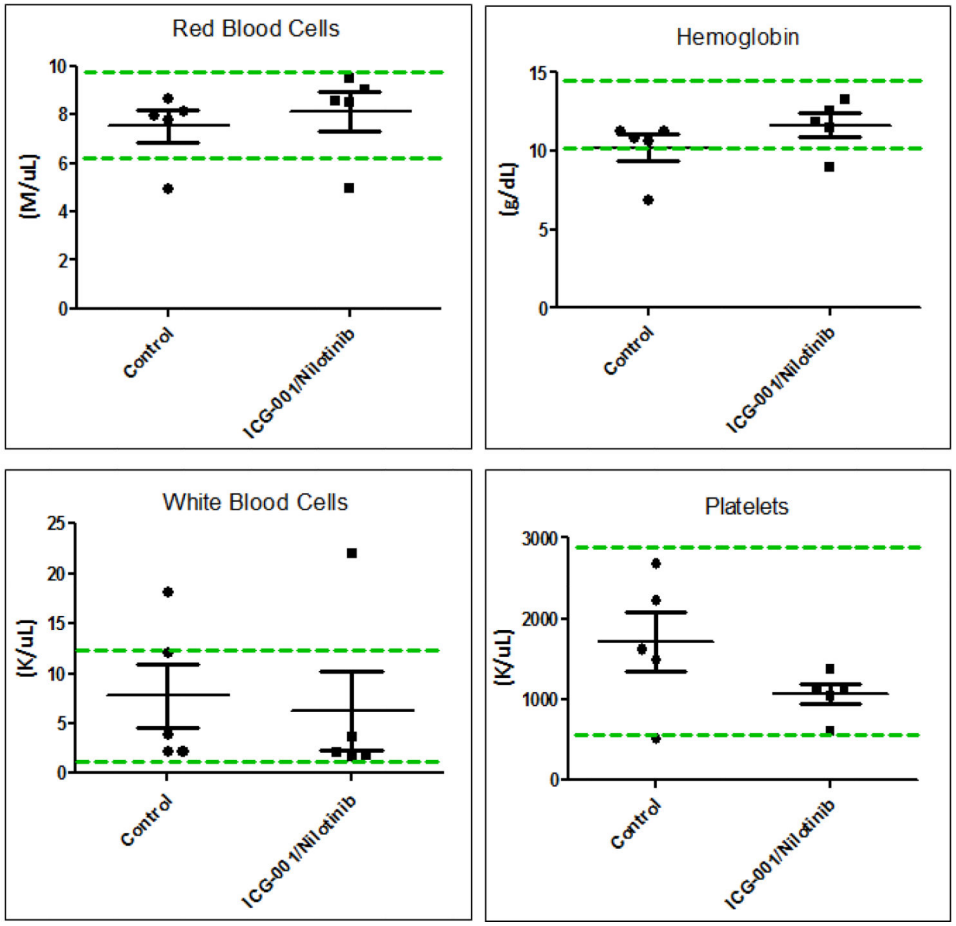


(D)



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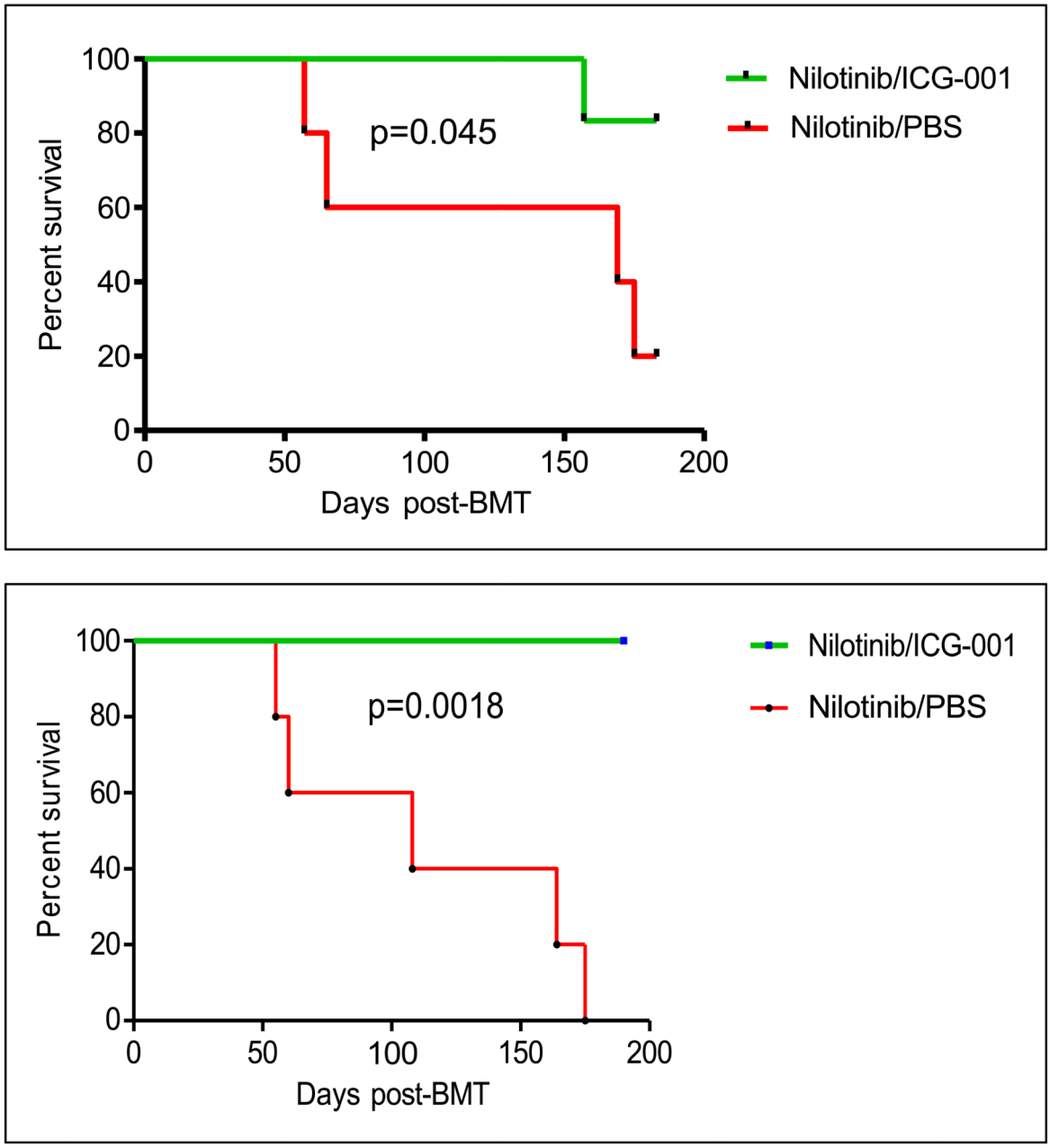

Figure 4.

(**A**) K562 cells marked with a lentiviral luciferase vector were engrafted into 15 NSG mice. Leukemia engraftment was detected at day 13 by bioluminescence imaging and the mice were subsequently randomized into 3 treatment groups – PBS/PBS, PBS/Nilotinib, and ICG-001/Nilotinib. ICG-001 or saline (control) was administered via Alzet osmotic minipump for 28 days. Nilotinib or saline (control) was administered by daily oral gavage. Leukemia development was monitored by bioluminescence imaging.

(**B**) Kaplan-Meier survival analysis demonstrated that mice from the ICG-001/Nilotinib treatment group survived more than 1 year post BMT and essentially as long as their nonleukemia engrafted, untreated littermates (p=0.0072 between saline and Nilotinib treament, p=0.0018 between Nilotinib/saline and Nilotinib/ICG-001 group). The control mice are the experimental mice littermates without leukemia engraftment.
(**C**) 330 days post treatment stoppage, qRT-PCR analysis for *BCR-ABL* was performed on peripheral blood from mice in the ICG-001/Nilotinib treatment group. Assay sensitivity was determined by serial dilution of K562 cells with mouse blood cells. With as little as 10 K562 cells per 10^6 mouse cells *BCR-ABL* can be detected. There was no *BCR-ABL* detected in any of the mice from the ICG-001/Nilotinib treatment group.
(**D**) 330 days post treatment stoppage, blood from the ICG-001/Nilotinib treatment group mice was analyzed. The green dotted lines indicate the normal range for each parameter compared to their non-irradiated, non-leukemia engrafted, untreated littermate controls.
(**E**) Two primary CML patient samples were transplanted into NSG mice and treated with either Saline/Nilotinib or ICG-001/Nilotinib. Kaplan-Meier survival analysis demonstrated a significant improvement in survival with the combination treatment.