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Efficacy of phosphatidylinositol-3 kinase inhibitors with diverse isoform selectivity profiles for inhibiting the survival of chronic lymphocytic leukemia cells

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Pharmacological inhibition of phosphatiylinositide-3-kinase (PI3K)-mediated signaling holds great promise for treating chronic lymphocytic leukemia (CLL). Therefore we assessed three structurally related PI3K inhibitors targeting the PI3K-δ isoform for their ability to inhibit the survival of freshly isolated CLL cells. The purely PI3K-δ-selective inhibitor idelalisib was compared to copanlisib (BAY 80-6946) and duvelisib (IPI-145), with isoform target profiles that additionally include PI3K-α or PI3K-γ, respectively. The concentrations leading to half-maximal reduction of the survival of CLL cells were more than ten-fold lower for copanlisib than for idelalisib and duvelisib. At concentrations reflecting the biological availability of the different inhibitors, high levels of apoptotic response among CLL samples were attained more consistently with copanlisib than with idelalisib. Copanlisib selectively reduced the survival of CLL cells compared to T cells and to B cells from healthy donors. In addition copanlisib and duvelisib impaired the migration of CLL cells towards CXCL12 to a greater extent than equimolar idelalisib. Similarly copanlisib and duvelisib reduced the survival of CLL cells in co-cultures with the bone marrow stroma cell line HS-5 more strongly than idelalisib. Survival inhibition by copanlisib and idelalisib was enhanced by the monoclonal CD20 antibodies rituximab and obinutuzumab (GA101), while antibody-dependent cellular cytotoxicity mediated by alemtuzumab and peripheral blood mononuclear cells was not substantially impaired by both PI3K inhibitors for the CLL-derived JVM-3 cell line as target cells. Taken together, targeting the α- and δ- p110 isoforms with copanlisib may be a useful strategy for the treatment of CLL and warrants further clinical investigation.

Chronic lymphocytic leukemia (CLL) is a common B cell malignancy that usually occurs at advanced age and follows a highly variable course.[1] Although management of the disease has been steadily improved, with chemo-immunotherapy by fludarabine, cyclophosphamide and rituximab (FCR) as the current treatment standard for patients without co-morbidity,[2] there is a need for more efficient and less toxic therapies, since CLL can only be cured by allogeneic stem cell transplantation. Therefore, novel targeted agents are being developed, some of which show great promise and might replace chemotherapy regimens in the near future.[3]

Key words: chronic lymphocytic leukemia, kinase inhibitors, apoptosis, monoclonal antibodies

Abbreviations: ADCC: L antibody-dependent cell-mediated cytotoxicity; BTK: Bruton's tyrosine kinase; CLL: chronic lymphocytic leukemia; FCR: fludarabine, cyclophosphamide and rituximab; IC50: concentration inhibiting 50%; LDH: lactate dehydrogenase; PBMCs: peripheral blood monoculear lymphocytes; PH: pleckstrin homology; PI3K: phosphatidylinositide-3-kinase

Additional Supporting Information may be found in the online version of this article.

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What's new?

Pharmacological inhibition of phosphatidylinositide-3-kinase (PI3K)-mediated signaling holds great promise for treating chronic lymphocytic leukemia (CLL). Here, the authors assessed the PI3K-delta-selective inhibitor idelalisib alongside two novel PI3K-delta inhibitors, which additionally target the PI3K-alpha (copanlisib) or gamma (duvelisib) isoforms. They found copanlisib to inhibit the survival of CLL cells at least ten-fold more efficiently than idelalisib and duvelisib. Survival inhibition by copanlisib was cell-type selective, accompanied by strong reduction of chemotaxis, and efficacious in the presence of stroma cell support. Survival inhibition by copanlisib was also enhanced by monoclonal antibodies, while antibody-dependent cell-mediated cytotoxicity for a CLL cell line remained undisturbed.

According to current understanding, the major driving forces for CLL pathogenesis consist in a critical dependence on antigen stimulation and micro-environmental stimuli, particularly those exerted in the lymph nodes, spleen and bone marrow rather than in peripheral blood.[4] Especially kinase signaling pathways downstream of the B cell receptor, like the PI3K/Akt signaling cascade, are considered as attractive therapeutic targets.[5,6] The apoptosis resistance of CLL cells is causally linked to constitutively activated PI3K/Akt signaling.[7,8]

Class I phosphatidylinositide-3-kinases catalyze the formation of the lipid second messenger phosphatidylinositide(3,4,5)triphosphate (PIP3), which is capable of binding to the pleckstrin homology (PH) domain of important signaling mediators, *e.g.,* the serine threonine kinase Akt or Bruton's tyrosine kinase (BTK).[9,10] Binding of PIP3 to the PH domain recruits the respective proteins to the cytoplasmic membrane, where they receive activating phosphorylation, *e.g.,* at Ser-473 and Thr-308 in the case of Akt, and in turn propagate signaling cascades by phosphorylating their cognate substrates. Consequently these signaling cascades can be interrupted by binding of small molecule inhibitors to the ATP binding cleft of the catalytic p110 subunit of PI3K thus preventing PIP3 formation.[11–16] The natural compound wortmannin and the quercetin derivative LY294002 were the first PI3K inhibitors and soon followed by more selective compounds targeting PI3K and mTOR simultaneously or separately, some of which were also tested on CLL cells.[17–22] In addition, PI3K inhibitors exhibit various degrees of isoform specificity, which influences their effectiveness on CLL cells.[7,19] In particular it is an attractive option to specifically target the p110-δ isoform, which dominates B cell receptor signaling and is preferentially expressed in CLL cells.[23,24] Apart from directly inhibiting the survival of CLL cells *ex* vivo[25,26] the p110- δ-specific inhibitor idelalisib potently disrupts tumor host interactions.[24,27] In a recent phase III trial idelalisib in combination with rituximab strongly improved the progression-free survival of CLL patients with co-morbidity.[28] Moderate survival effects of idelalisib on CLL cells in *vitro* despite its clinical success together with the occurrence of transient lymphocytosis during idelalisib treatment point to microenvironment interactions, *e.g.,* chemokine networks, as important mechanistic targets of PI3K.

In comparison to idelalisib we pre-clinically assessed the direct cytotoxicity on CLL cells of two PI3K-δ-selective candidate substances that are in clinical development (Supporting Information Fig. S1). The duvelisib molecule structurally closely resembles idelalisib, but biochemically targets p110-γ in addition to p110-δ. With this isoform selectivity profile, duvelisib suppressed the activity of auto-immune and inflammatory disease models[29] and recently entered phase I clinical trials for CLL.[30] Copanlisib, which like idelalisib is a derivative of the quinazoline scaffold, is a pan-class I-PI3K inhibitor with prominent selectivity for p110–α and p100-δ isoforms, as becomes evident from the at least sevenfold stronger biochemical inhibition of α- or δ- than of β- or γ-isoforms and from sub-nanomolar IC_{50} for p110-α and p110- δ (Supporting Information Table S1). It shows high *in vitro* and *in vivo* potency against multiple myeloma and various tumor cell lines.[31–33] The intravenous PI3K inhibitor copanlisib has also been investigated in phase I/II trials for activity against solid and hematological tumors.[34–36]

Apart from their action as single agents we investigated combinations of PI3K inhibitors with the CD20 antibody rituximab, which is part of the successful clinical application of idelalisib[28] and of the standard chemo-immunotherapeutic regimen for first line treatment of CLL.[2] As an alternative immunoreagent in combinations we used the glyco-engineered type II CD20 antibody obinutuzumab, which in combination with chlorambucil resulted in a significant progression-free survival benefit in elderly CLL patients compared to rituximab.[37] In addition we investigated whether PI3K inhibitors with different isoform selectivity profiles interfered with antibody-dependent cell-mediated cytotoxicity (ADCC).

Here, we compared inhibition of the survival and chemotaxis of freshly isolated CLL cells by the predominantly p110-δ-selective PI3K inhibitors idelalisib, duvelisib and copanlisib and explored possible synergistic effects in combinations with CD20 antibodies.

Material and Methods

Patient samples

Peripheral blood samples were obtained from patients who were previously diagnosed for CLL according to standard criteria. The study was performed according to the World Medical Association Declaration of Helsinki and was approved by the local ethics committee at the University of Cologne (Approval No. 11-319). The clinical and biochemical characteristics of the investigated patient samples are summarized in Supporting Information Table S2.

Inhibitors

The chemical structures of idelalisib (CAL-101, GS 1101), copanlisib (BAY 80–6946) and duvelisib are depicted in Supporting Information Figure 1. Copanlisib was kindly provided by Bayer; idelalisib, duvelisib and GDC-0941 were purchased from Selleck, Houston, TX. Rituximab and obinutuzumab were kindly provided by Roche-Glycart, Schlieren, Switzerland. Alemtuzumab was purchased from the hospital pharmacy.

Cell isolation and culture

The B cell lymphoma cell line SUDHL-5 and the CLL-derived, EBV-transformed lymphoblastoid, CLL-derived cell line JVM-3 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the human bone marrow stromal cell line HS-5 from the American Type Culture Collection (LGC Promochem, Teddington, UK). Isolation of CLL cells and *in vitro* cell culture were performed as detailed in the Supporting Information Methods.

Proliferation assay

As a surrogate of cell number determination, metabolic activity was determined by following photometrically the reduction of XTT [sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] as detailed in the Supporting Information Methods.

Determination of cellular survival

5×10^5 cells were collected after 48 hr of culture in the presence or absence of inhibitors and washed once before the determination of annexin V-binding. The cells were stained with FITC-labeled annexin V and 7-amino-actinomycin (BD Biosciences, Heidelberg, Germany) and analyzed using a FACS-Canto flow cytometer (BD Biosciences). The percentages of annexin V-negative, viable cells were recorded. Relative cell survival was calculated from the percentages of viable cells in drug-treated samples relative to untreated controls. CLL samples showing $< 50\%$ annexin V-negative cells in the untreated controls after 48 hr of culture were excluded from the analysis.

Staining by anti-CD3-fluoresceine and anti-CD19-phycoerythrin (Miltenyi Biotech, Bergisch Gladbach, Germany) allowed the differentiation of T and B lymphocytes among peripheral blood mononuclear cells (PBMCs). The percentages of annexin V-allophycocyanine binding, apoptotic cells were determined flow cytometrically in the gated PBMC subpopulations.

Analysis of signaling pathways

To analyze phosphorylation of Akt at serine 473 and cleavage of poly(ADP) ribose polymerase (PARP), preparation of cell lysates and detection of signaling molecules in Western blots using the Odyssey imager (Licor, Bad Homburg, Germany) were performed as previously described[38] and detailed in the Supporting Information Methods.

Chemotaxis assay

The effect of PI3K inhibitors on the migration of CLL cells towards the chemokine CXCL12 was determined in Boyden chamber experiments as previously described[27] and detailed in the Supporting Information Methods. Cell migration to 100 ng/ml CXCL12 during 3 hr was assessed from cell numbers in the bottom chambers.

Determination of antibody-dependent cell-mediated cytotoxicity (ADCC)

ADCC assays were performed with cultured JVM-3 cells as target and freshly isolated PBMCs from a healthy donor as effector cells by 4 hr co-cultivation at a 15:1 effector:target-ratio and subsequent detection of lactate dehydrogenase (LDH) release as detailed in the Supporting Information Methods.

Statistics

Error bars indicate standard deviations. Significance levels were determined by Student's *t* test and indicated as n.s.: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Unless indicated otherwise two-tailed, paired T-tests were used. In box plots, the middle quartiles of distributions are represented by boxes, while the maximal and minimal ranges indicated by whiskers are limited to 1.5 interquartile ranges.

Results

PI3K-inhibitor effects on B cell lymphoma cell lines

As a comparative estimate of inhibitor efficacy for CLL, the effects of PI3K-delta-inhibitors on the metabolic activity of the lymphoblastoid cell line JVM-3 were determined (Fig. 1a). In a concentration range up to 25 μM, idelalisib and duvelisib led to only marginal growth inhibition, while the concentration inhibiting 50% (IC_{50}) for copanlisib in the XTT assay was 2 μM. Owing to low idelalisib sensitivity of JVM-3 cells, the diffuse large B cell lymphoma cell line SU-DHL-5 was examined after inhibitor treatment for effects on metabolic activity and phosphatidylserine exposure (Supporting Information Fig. S2). The XTT assay after 24 hr indicated IC_{50} concentrations for idelalisib and copanlisib of 15 and 0.15 μM, respectively, while the corresponding IC_{50} concentrations for annexin V binding after 48 hr were 2.4 μM and 30 nM, *i.e.,* approximately five times lower. Taken together, higher cytotoxicity of copanlisib than idelalisib was observed in JVM-3 and SUDHL-5 cells. In the latter cell line, the inhibitor potencies differed by a factor of 80-100.

Effects of PI3K-δ-selective inhibitors on the survival of CLL cells

Similarly as in lymphoma cell lines, we compared the cytotoxicity of duvelisib and copanlisib with idelalisib in primary CLL cells (Figs. 1b and 1c). Administration of six concentrations of the PI3K inhibitors copanlisib and idelalisib to randomly selected CLL patient samples showed significantly higher sensitivity of CLL lymphocytes for copanlisib than for

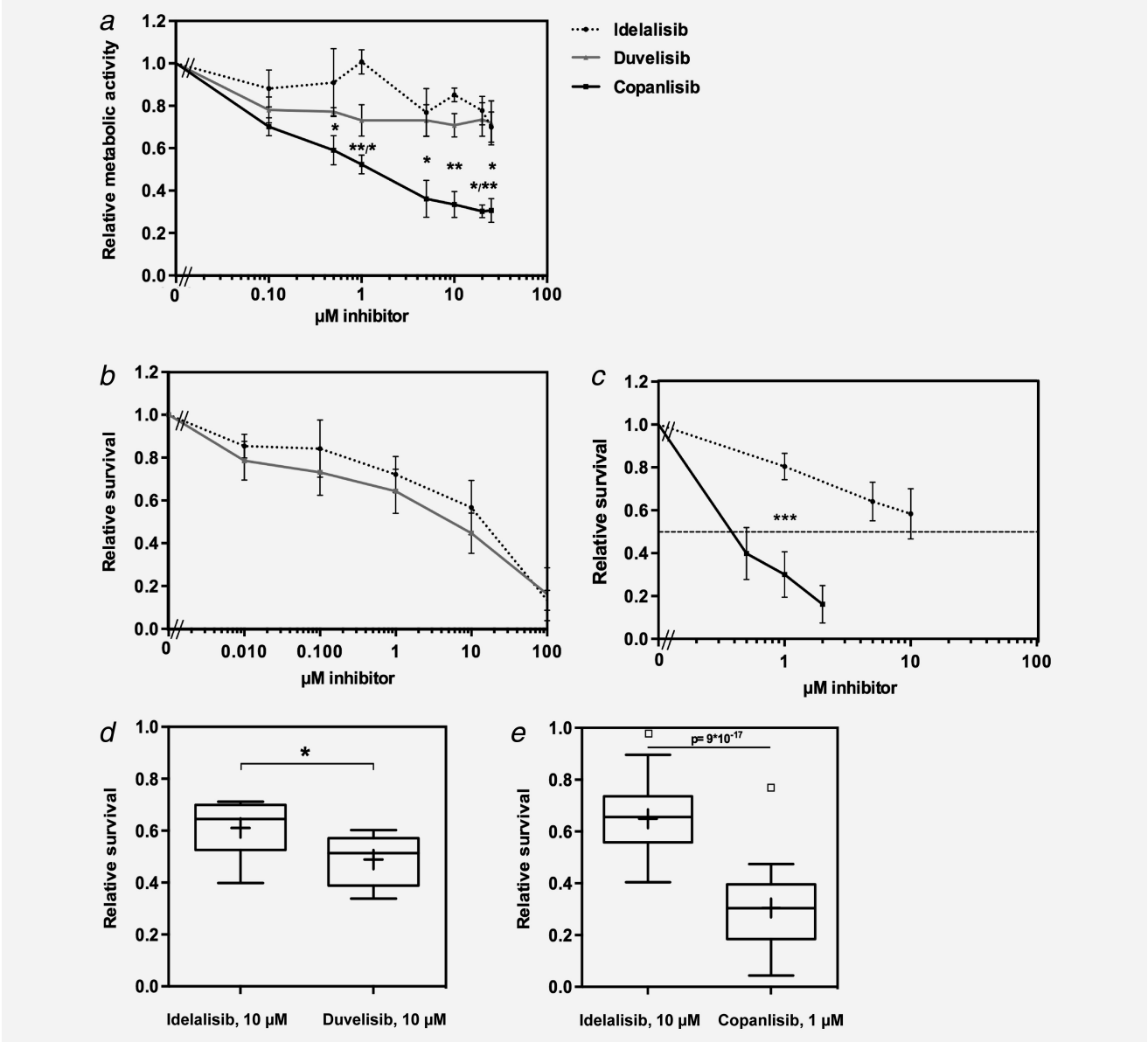

Figure 1. Sensitivity of CLL cells for PI3K inhibitors. (*a*) The sensitivity of JVM-3 cells for PI3K inhibitors was determined in three independent XTT assays after 48 hr of incubation. Asterisks indicate significantly higher efficacy of copanlisib *versus* idelalisib and duvelisib at common inhibitor concentrations according to paired Student's *t* test. (*b, c*) Two different sets of six CLL samples were treated with duvelisib (*b*) or copanlisib (*c*) for 48 hr with idelalisib as a common reference and examined for phosphatidylserine exposure. (*d,e*) Survival inhibition by duvelisib (*d*) or copanlisib (*e*) was compared to idelalisib at fixed inhibitor concentrations reflecting pharmacologically achievable levels in 6 or 32 CLL samples, respectively. Means are indicated by plus signs, outliers by empty squares.

idelalisib (Supporting Information Fig. S3) and allowed to set the concentration ranges for a pair-wise comparative assessment of inhibitor effects on identical samples. Two different sets of CLL patient samples were monitored for concentration-dependent reduction of cellular survival by the PI3K inhibitors duvelisib or copanlisib, respectively, compared to idelalisib (Supporting Information Fig. S4). While duvelisib reduced the survival of CLL cells only slightly more efficiently than idelalisib (Fig. 1b), copanlisib inhibited the survival of CLL cells with significantly greater efficacy than idelalisib in all samples, as reflected by mean IC_{50} concentrations of 450 nM and above 10 μM for copanlisib and idelalisib, respectively (Fig. 1c). At the common inhibitor concentration of 1 μM, cytotoxicity of copanlisib greatly surpassed that of idelalisib. Survival inhibition in freshly isolated CLL cell by the pan-class IA PI3K inhibitor GDC-0941 occurred at even higher IC_{50} concentrations than by idelalisib (Supporting Information Table S1). In summary, copanlisib reduced the survival of CLL cells approximately 20-fold more potently than idelalisib, the efficacy of which was only marginally surpassed by duvelisib. This finding suggests that the efficacy of PI3K-δ inhibitors for reducing the survival of CLL cells was enhanced by additionally targeting PI3K-α, but not PI3K-γ.

Distribution of inhibitor sensitivity among CLL patient samples

PI3K inhibitors were assessed in individual CLL samples at fixed concentrations reflecting pharmacologically achievable levels, namely 10 μM of idelalisib and duvelisib and 1 μM of copanlisib, to compare the distributions of inhibitor sensitivity among CLL samples (Figs. 1d and 1e). At these concentrations, the cytotoxic response of duvelisib surpassed that of idelalisib slightly, but significantly and showed a similar distribution (Fig. 1d; Supporting Information Fig. S5A). In a different set of CLL samples, copanlisib on the average inhibited the survival of CLL cells twice as strongly as idelalisib, corresponding to a survival difference of 35% (Fig. 1e; Supporting Information Fig. S5B). While copanlisib inhibited cellular survival stronger than idelalisib in all investigated CLL samples, also the range of inhibition by copanlisib surpassed that by idelalisib in all samples with one exception. The unusually low response to copanlisib in samples from this patient was confirmed at two additional occasions. Excluding this outlier sample with regard to copanlisib sensitivity, the response of individual CLL samples to 1 μM copanlisib was not correlated with that to 10 μM idelalisib ($r = 0.63$; $p = 0.38$). Compared to idelalisib, the mean difference in survival inhibition by 10 μM duvelisib was approximately one third of that observed for 1 μM copanlisib. The cytotoxic response to the reference treatment with idelalisib was highly similar in the two examined sets of samples. Unfavorable prognostic markers, *e.g.,* deletion of chromosomes 17p or 11q, or previous treatment by chemotherapy did not exclude high cytotoxic response to PI3K inhibitors (Supporting Information Table S2). With regard to direct reduction of cell survival in two sets of CLL samples, duvelisib and copanlisib showed slightly *versus* remarkably increased efficacy compared to idelalisib.

Inhibition of the PI3K/akt signaling cascade

To determine whether the cytotoxic effects observed upon treatment with the investigated PI3K inhibitors were due to specific interruption of the PI3K/Akt signaling cascade, we examined Akt phosphorylation in CLL cell lysates with and without inhibitor treatment (Fig. 2a). Anti-IgM-induced phosphorylation of Akt at serine 473 was substantially reduced in freshly isolated CLL cells after treatment with the investigated PI3K inhibitors. As a link of the PI3K/Akt signaling cascade with the apoptotic response, we examined CLL lysates for caspase activity detected by an increase of the cleaved PARP fragment of 89 kda and accompanying reduction of 116 kda uncleaved PARP (Fig. 2b). PARP cleavage was increased in lysates of CLL samples after treatment with PI3K inhibitors indicating that copanlisib and idelalisib in CLL cells efficiently prevented Akt activation downstream of PI3K and ensuing pro-survival signaling cascades.

Inhibition of CLL cell chemotaxis towards cxcl12

Since the survival of tumor cells alone might not adequately reflect clinically relevant inhibitor action, we additionally addressed the issues of tissue retention and mobilization of CLL cells by assessing the impact of PI3K inhibitors on the chemotaxis of CLL cells (Fig. 2c). Without addition of PI3K inhibitors, 1.4–2.8 times more CLL cells migrated to the bottom chamber in the presence than in the absence of CXCL12 (Supporting Information Table S3 and Supporting Information Fig. S6). In the present CLL samples, cell migration with and without chemokine differed significantly only in the absence of PI3K inhibitors. The average inhibition of CXCL12-dependent migration by 1 μM of idelalisib, duvelisib or copanlisib was 40%, 85% and 97%, respectively, indicating stronger impairment of chemotaxis by the novel PI3K inhibitors than by idelalisib.

Cell type-selectivity of PI3K inhibitors

To differentiate cell type-specific effects of PI3K inhibitors, PBMCs from healthy donors and CLL patients were treated with copanlisib or idelalisib and examined for phosphatidylserine exposure in B or T cells (Supporting Information Fig. S7, Fig. 3a). The percentages of viable cells were lower in B than T cells and showed remarkably wider variation among CLL samples than among healthy controls (Supporting Information Fig. S7). Treatment with copanlisib led to significantly reduced survival exclusively in B lymphocytes from CLL patients. In this population the cytotoxic efficacy of 1 μM copanlisib surpassed that of 10 μM idelalisib in a similar manner as in isolated CLL cells. In contrast, the PI3K inhibitors did not substantially affect the survival of the T cell population from healthy donors' and CLL patients' blood. The survival relative to untreated controls was significantly lower in copanlisib-treated B cells from CLL patients than in those from healthy donors or in equally treated T cells (Fig. 3a). Taken together, the investigated PI3K inhibitors preferentially targeted CLL cells as compared to normal B cells and left T cells unaffected.

In addition, we examined the inhibitor sensitivity of HS-5 cells representing bone marrow stromal fibroblasts (Supporting Information Fig. S8). In a concentration range from 5 to 20 μM copanlisib inhibited the metabolic activity of HS-5 cells significantly more strongly than idelalisib and duvelisib. On the other hand, HS-5 cells were approximately 50 times less sensitive for copanlisib than JVM-3 cells in the XTT assay (Fig. 1a). Thus, all three investigated PI3K inhibitors had stronger impact on CLL cells than on HS-5 cells.

Cytotoxicity of PI3K inhibitors in the presence of stroma cell support

To assess the influence of the bone marrow microenvironment on the cytotoxicity of PI3K inhibitors, isolated CLL cells from four different patients were cultured in medium alone or on feeder layers of the bone marrow stromal cell line HS-5 concomitant with inhibitor treatment (Fig. 3b). With and without co-culture alike, cytotoxicity of the investigated PI3K inhibitors increased from idelalisib over duvelisib to copanlisib. Co-culture improved the viability of

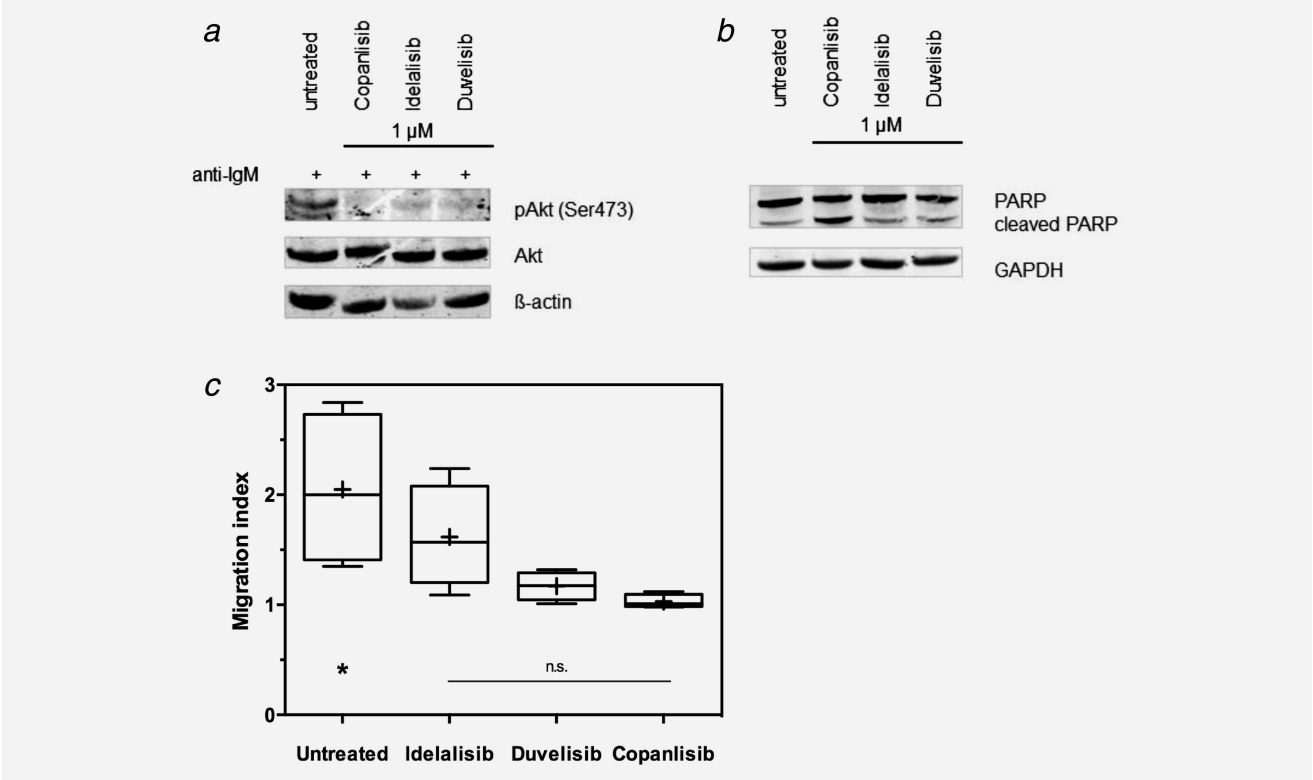

Figure 2. Impact of PI3K inhibitors on signaling and chemokine networks. (*a*) Anti IgM-induced Akt phosphorylation at Ser473 in CLL cells without or with inhibitor treatment for 3 hr was assessed by Western Blot analysis. (*b*) PARP cleavage was examined after 48 hr in the absence or presence of PI3K inhibitors in lysates of inhibitor-treated CLL cells. (*c*) Chemotaxis of four CLL samples to CXCL12 was determined after three hours and expressed as the ratio of migrated cells in the presence *versus* absence of CXCL12. Counts of migrated cells with or without CXCL12 were compared by paired *t* test.

untreated and idelalisib-treated CLL cells, increasing their relative survival on the average by 15%. In contrast, stroma cell support did not improve the mean viability of CLL cells treated with duvelisib or copanlisib. Consequently the significance of differences in viability after treatment with duvelisib or copanlisib compared to idelalisib was consistently higher with than without stroma cell support. Similarly, in the present set of samples at two inhibitor concentrations, all three investigated PI3K inhibitors significantly reduced the survival of CLL cells without co-culture compared to the untreated controls, but only duvelisib and copanlisib also in the presence of stroma cell support. In summary, duvelisib and copanlisib led to more efficient killing of CLL cells than idelalisib in co-cultures with HS-5 bone marrow stromal cells.

Combinations of PI3K inhibitors and monoclonal antibodies

Due to their independent and complementary modes of action, it is promising to combine kinase inhibitors and monoclonal antibodies (mAbs). For this purpose clinically achievable concentrations of PI3K inhibitors and of anti-CD20 mAbs were assessed for their direct cell killing capacity as single agents and in combination (Fig. 4a). In a set of nine CLL samples including the previously mentioned sample with exceptionally low copanlisib sensitivity, idelalisib or copanlisib reduced the mean relative survival of CLL cells by 45 or 56%, respectively, and thus much more efficiently than rituximab or obinutuzumab with 7 or 15% inhibition. In combinations, CD20 antibodies significantly enhanced the much stronger effects of PI3K inhibitors despite their low direct cytotoxicity. In agreement with higher single agent activities of copanlisib than idelalisib and obinutuzumab than rituximab, the combination of copanlisib and obinutuzumab led to the highest observed cytotoxicity among the investigated drug combinations and reduced the relative survival of CLL cells by 74%. The additive or synergistic type of enhancement was determined by estimating combination indices for individual CLL samples treated with the investigated combinations (Supporting Information Fig. S9). The distribution of combination indices showed approximately additive mean enhancement in combinations with rituximab, and more than additive enhancement in the majority of samples in combinations with obinutuzumab. In summary, at clinically relevant concentrations, CD20 antibodies significantly enhanced the cytotoxicity of PI3K inhibitors, which resulted in drug combinations showing high cell killing.

Apart from direct cell killing, the mechanisms of therapeutic antibodies involve complement-dependent and cell-

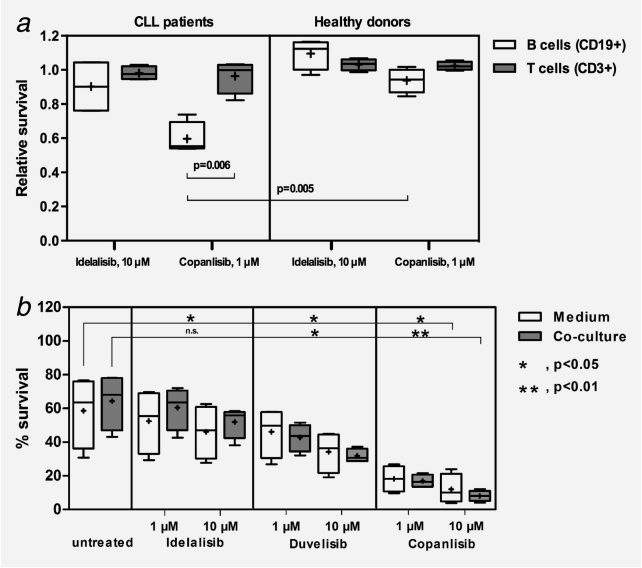

Figure 3. Cell type selectivity of PI3K inhibitors and influence of stroma cell support. (*a*) Relative survival of B and T cell subpopulations among PBMCs was determined *via* phosphatidylserine exposure by three-color flow cytometric analysis after 48 hr of incubation. B and T cell populations from four CLL patients and three healthy donors were compared by unpaired T-tests. (*b*) Percentages of viable CLL cells with or without co-culture on HS-5 stromal cells in the absence or presence of PI3K inhibitors were determined by flow cytometry *via* annexin V binding. The significance of survival reduction compared to corresponding untreated samples with or without co-culture was determined by paired *t* test ($n = 4$). In addition, the survival of CLL cells treated with duvelisib or copanlisib was compared to that of idelalisib-treated samples subjected to the corresponding inhibitor concentrations and culture conditions.

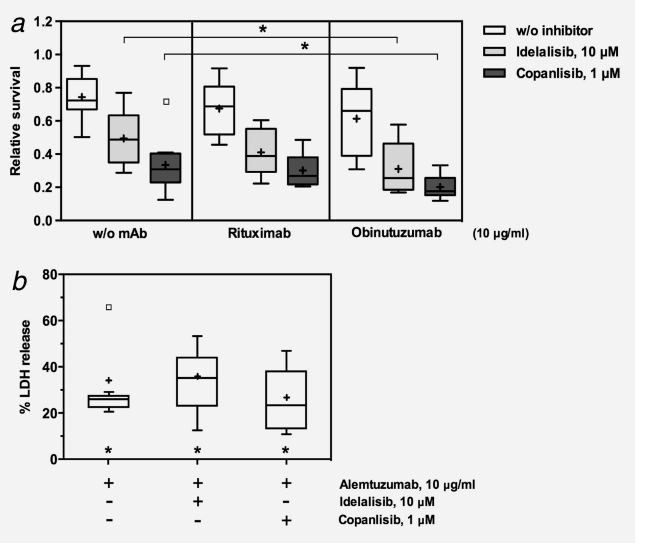

Figure 4. Direct cytotoxicity and ADCC in combinations of PI3K inhibitors and monoclonal antibodies. (*a*) In nine CLL samples treated for 48 hr with PI3K inhibitors and CD20 antibodies alone and in combination, percentages of annexin V-binding cells were determined by flow cytometry. (*b*) ADCC as percentages of maximal LDH release was determined for JVM-3 cells treated for three hours with alemtuzumab during co-incubation with four different preparations of healthy donor PBMCs as effector cells at 15-fold excess. The distribution of ADCC exceeding the LDH-release in the absence of alemtuzumab is shown. Samples with and without addition of alemtuzumab were compared by one-tailed paired *t* test. Means are indicated by plus signs, outliers by empty squares.

mediated cytotoxicity. To investigate, whether PI3K inhibitors interfere with cell-mediated response mechanisms, we assessed combinations of alemtuzumab with PI3K inhibitors for their capacity to exert ADCC against JVM-3 cells (Fig. 4b). Owing to high and stable expression of CD52 on lymphocytes, alemtuzumab that targets this glycoprotein was employed to elicit ADCC. Compared to CLL samples, the use of JVM-3 cells eliminated target cells as a source of inter-sample variation. Using four different PBMC preparations as effector cells, the mean ADCC mediated by alemtuzumab was approximately 35% of the LDH-release obtained by complete lysis of JVM-3 target cells. The distribution of ADCC obtained with different effector cell preparations was not significantly altered by addition of copanlisib or idelalisib. In turn, the LDH-release in co-cultures of target and effector cells was significantly different in samples with and without addition of alemtuzumab in the absence as well as in the presence of PI3K inhibitors. Despite slightly lower mean LDH-release in samples, to which copanlisib had been added, both investigated PI3K inhibitors did not significantly disturb the ADCC mediated by alemtuzumab and healthy donor PBMCs.

Discussion

Targeting p110-δ showed promising clinical efficacy for CLL treatment,[28] but could be further improved by PI3K inhibitors showing more potent direct tumor cell killing than idelalisib. Among the investigated PI3K-δ-selective inhibitors, the cytotoxicity for CLL cells of copanlisib by far surpassed that of idelalisib and duvelisib. To unravel the role of isoform selectivity for targeting malignant B lymphocytes, published selectivity profiles obtained *in vitro* with purified p110 isoforms were compared to the inhibitor sensitivity of CLL samples determined in the present study (Supporting Information Table S1). In biochemical assays the δ -specific PI3K inhibitors idelalisib and duvelisib with 500-fold stronger inhibition of the δ- than of the α-isoform can be distinguished from the pan-class IA inhibitors copanlisib and GDC-0941 with roughly equal inhibition of these isoforms. The cytotoxicity elicited in CLL cells by duvelisib *versus* copanlisib and the purely PI3K-δ-selective idelalisib points to major contributions to the survival of CLL cells of p110-α and p110-δ, but not p110-γ, in line with previous observations using different isoform-selective inhibitors.[19] In CLL cells, dual inhibition of the p110-δ- and γ-isoforms by duvelisib did not surpass the direct cytotoxicity obtained with solely δ-selective idelalisib, in contrast to roles for both isoforms in PTEN-deficient T cell acute lymphoblastic leukemia cell lines.[39] Contributions of the p110-α- and δ-isoforms to cell survival

were found in mantle cell lymphoma (MCL), where the dual p110-α- and p110-δ-selective inhibitor GDC-0941 induced stronger apoptosis than idelalisib.[40,41] The discrepant cytotoxicity of copanlisib and GDC-0941 for CLL cells despite a similar ratio of biochemical p110-δ and p110-α inhibition can be explained by the higher absolute potency of copanlisib for inhibiting these isoforms. In agreement with comparisons of inhibitor cytotoxicity for CLL and MCL cells with isoform selectivity profiles, RNA interference revealed that the survival of myeloma cell lines strongly depended on p110-alpha.[42] In contrast, co-culture-induced Akt phosphorylation of myeloma cells was reduced much more strongly by knockdown of p110- δ than of p110-α.[43] Taken together we show that the dual p110-α and p110-δ-selective inhibitor copanlisib leads to remarkable, potentially clinically useful inhibition of the survival of CLL cells, which by far surpasses that by idelalisib and duvelisib, as well as by other pan-class IA inhibitors.

The promising cytotoxicity for CLL cells of the quinozoline-based pan-class I-PI3K inhibitor copanlisib was, however, lower than that observed for the DLBL cell line SUDHL-5, as well as myeloma and other cancer cell lines.[31,32] With idelalisib as reference inhibitor we observed similar cytotoxity for SUDHL-5 cells as previously reported.[25] Copanlisib inhibited the survival of CLL cells approximately 15-fold more efficiently than idelalisib, which clearly exceeded the 3.6-fold higher cytotoxicity of buparlisib (BKM 120) compared to idelalisib.[21]

With regard to potential clinical application, we compared direct tumor cell killing by PI3K inhibitors at pharmacologically achievable concentrations. In this regard, duvelisib showed slightly higher direct cytotoxicity for CLL cells than idelalisib and therefore appears moderately superior to idelalisib due to and an approximately four-fold lower achievable plasma concentration. The cytotoxicity of copanlisib for CLL samples, however, by far and consistently surpassed that of idelalisib even at a ten-fold lower concentration reflecting the difference in clinically obtainable concentrations.[32] Similarly, while 1 μM copanlisib inhibited the survival of CLL cells by more than 50% in the vast majority of samples, buparlisib exhibited IC_{50} concentrations below the clinically obtainable plasma concentration of 5 μM only in one third of examined samples.[21] Also taking into account the tolerated plasma concentrations, the cytotoxic potency of copanlisib for CLL cells exceeded that of idelalisib, duvelisib and buparlisib or other kinase inhibitors, *e.g.,* dasatinib and sorafenib.[38]

Copanlisib exhibited pronounced cell type selectivity for CLL cells *versus* B lymphocytes from healthy donors. Like idelalisib and the pan-class I PI3K inhibitor LY294002, but in contrast to duvelisib,[44] it lacked significant cytotoxicity against T cells. HS-5 cells representing the bone marrow TME were not affected by idelalisib or duvelisib and showed approximately 50-fold lower sensitivity to copanlisib than the CLL-derived lymphoblastoid cell line JVM-3. Although copanlisib preferentially killed CLL cells, it showed cytotoxicity for HS-5 and normal B cells, which increases the likelihood of side effects in patients treated with this more toxic drug.

Apart from direct inhibition of PI3K signaling in CLL cells, the investigated inhibitors potentially also antagonize extrinsic activation of this pathway. Therefore, co-cultivation with protective bone marrow-derived stromal cells could afford a more relevant inhibitor assessment than suspension cultures.[45] Co-culture with HS-5 feeder cells enhanced the viability of untreated and idelalisib-treated CLL cells to a similar degree as previously described.[24,46] While similar protection of CLL cell survival by stroma cell support was observed after treatment with buparlisib,[21,22] duvelisib and copanlisib inhibited the survival of CLL more efficiently than idelalisib, both, without and with HS-5 co-culture. In the case of copanlisib, cytotoxicity for HS-5 cells may have contributed to impaired stroma cell protection.

The antibody component in the current standard immunechemotherapy for CLL by FCR[2] and in a pivotal idelalisib trial[28] prompted us to assess the direct cell killing capacity of PI3K inhibitors in combination with CD20 antibodies. At clinically achievable drug concentrations, we observed mutual enhancement of direct cytotoxicity in these combinations, which was more pronounced for obinituzumab than for rituximab owing to its higher cell killing capacity as single agent.[47] The considerably higher and more consistent mean cytotoxicity in combinations of obinutuzumab with PI3K inhibitors than with chemotherapeutic agents[47] suggests exploring its clinical potential in combination with PI3K inhibitors rather than with chlorambucil.[37]

For optimal application of monoclonal antibodies in combination with PI3K inhibitors, cell-mediated response mechanisms must be retained, *i.e.,* spared from the cytotoxic action of PI3K inhibitors. In this regard, the applicability of PI3K inhibitors depends on their cell type and isoform selectivity and idelalisib has been shown not to impair the ADCC mediated by alemtuzumab and natural killer (NK) cells.[24] Although a cell line was used instead of CLL samples, the ADCC observed *via* LDH-release showed high variance owing to the variable percentage and quality of effector cells, but was of similar size and heterogeneity as reported with a different detection method.[24] In the described assay system, idelalisib and copanlisib did not significantly interfere with the ADCC mediated by alemtuzumab. Similarly the ADCC mediated by CD20 antibodies has been reported not to be impaired by idelalisib, in contrast to other inhibitors of BCR pathways, *e.g.,* ibrutinib.[48,49] While duvelisib was reported to inhibit the survival of NK cells,[44] idelalisib and copanlisib in the present study did not interfere with alemtuzumab-mediated ADCC on the lymphoblastoid cell line JVM-3, which makes both appear appropriate for combination with immunotherapy.

Taken together, copanlisib showed superior efficacy in inducing apoptosis in CLL cells, when compared to idelalisib and duvelisib. These effects were further enhanced in combination with CD20 antibodies, rituximab and obinutuzumab. Finally, both isoform selectivity profiles did not negatively interfere with ADCC.

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