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Research paper

CDK4/CDK6 inhibition as a novel strategy to suppress the growth and survival of *BCR-ABL1*^{T315I}+ clones in TKI-resistant CML

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ABSTRACT

Purpose: Ponatinib is the only approved tyrosine kinase inhibitor (TKI) suppressing *BCR-ABL1*^{T315I}-mutated cells in chronic myeloid leukemia (CML). However, due to side effects and resistance, *BCR-ABL1*^{T315I}-mutated CML remains a clinical challenge. Hydroxyurea (HU) has been used for cytoreduction in CML for decades. We found that HU suppresses or even eliminates *BCR-ABL1*^{T315I}+ sub-clones in heavily pretreated CML patients. Based on this observation, we investigated the effects of HU on TKI-resistant CML cells *in vitro*.
Methods: Viability, apoptosis and proliferation of drug-exposed primary CML cells and *BCR-ABL1*+ cell lines were examined by flow cytometry and ^{3}H-thymidine-uptake. Expression of drug targets was analyzed by qPCR and Western blotting.
Findings: HU was more effective in inhibiting the proliferation of leukemic cells harboring *BCR-ABL1*^{T315I} or T315I-including compound-mutations compared to cells expressing wildtype *BCR-ABL1*. Moreover, HU synergized with ponatinib and ABL001 in inducing growth inhibition in CML cells. Furthermore, HU blocked cell cycle progression in leukemic cells, which was accompanied by decreased expression of CDK4 and CDK6. Palbociclib, a more specific CDK4/CDK6-inhibitor, was also found to suppress proliferation in primary CML cells and to synergize with ponatinib in producing growth inhibition in *BCR-ABL1*^{T315I}+ cells, suggesting that suppression of CDK4/CDK6 may be a promising concept to overcome *BCR-ABL1*^{T315I}-associated TKI resistance.
Interpretation: HU and the CDK4/CDK6-blocker palbociclib inhibit growth of CML clones expressing *BCR-ABL1*^{T315I} or complex T315I-including compound-mutations. Clinical studies are required to confirm single drug effects and the efficacy of `ponatinib+HU´ and ´ponatinib+palbociclib´ combinations in advanced CML.
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1. Introduction

In most patients with chronic myeloid leukemia (CML) in chronic phase (CP), imatinib treatment results in long-lasting deep molecular responses [1–3]. However, resistance against imatinib may develop, often in the context of *BCR-ABL1* point mutations or because of activation of BCR-ABL1-independent signaling pathways [4–7]. In these patients, second-generation tyrosine kinase inhibitors (TKI), such as nilotinib, dasatinib or bosutinib, are usually prescribed [8,9]. One

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Research in context

Evidence before this study

Tyrosine kinase inhibitor (TKI)-resistant chronic myeloid leukemia (CML) represents a clinical challenge, especially when clonal cells display T315I mutant forms of *BCR-ABL1*. Ponatinib is a multi-targeted TKI that blocks BCR-ABL1^{T315I}. However, resistance or intolerance against ponatinib may occur.

Added value of this study

We here demonstrate that drugs regulating CDK4/CDK6 expression or function in CML cells, such as hydroxyurea (HU) or palbociclib, exert strong anti-neoplastic effects on CML cells expressing *BCR-ABL1*^{T315I} or T315I-including compound mutations. The growth-inhibitory effects of HU on CML cells exhibiting *BCR-ABL1*^{T315I} was demonstrable *in vivo* in heavily pretreated and highly resistant CML patients as well as *in vitro* in various cell line models. Furthermore, synergistic growth-inhibitory effects of the drug-combinations HU+ponatinib, palbociclib+ponatinib, and HU+ABL-001, were demonstrable.

Implications of all the available evidence

Patients with CML who develop TKI-resistance in *BCR-ABL1*^{T315I}-mutated sub-clones have a grave prognosis. Ponatinib may overcome this form of resistance in a subset of patients whereas others have or develop resistance against ponatinib. We are the first to provide evidence that simultaneous targeting of BCR-ABL1 and CDK4/CDK6 is a reasonable strategy to overcome drug resistance in CML, an observation that may help develop therapeutic concepts for advanced, multi-resistant CML. Our findings are relevant clinically, since, apart from stem cell transplantation, effective therapies for these patients are lacking and the drug combination HU+ponatinib can easily be applied as both drugs are FDA-approved and available. Furthermore, our data suggest that the application of novel drug combinations, such as ABL001+HU or ponatinib+palbociclib in multi-resistant CML expressing *BCR-ABL1*^{T315I} or T315I-including compound mutations, may represent effective new therapies, which should be tested in clinical studies.

major problem in these patients is the outgrowth of sub-clones harboring the T315I-mutated variant of *BCR-ABL1*, which confers resistance against all second-generation TKI [9–11].

As of now, ponatinib is the only approved TKI that exerts major growth-inhibitory effects against sub-clones bearing *BCR-ABL1*^{T315I} [12,13]. Indeed, ponatinib was found to induce clinically meaningful responses in a high proportion of patients with *BCR-ABL1*^{T315I}-positive CML [13]. However, occurrence of *BCR-ABL1*^{T315I} still represents a clinical challenge. First, ponatinib has been reported to cause severe cardiovascular side effects and may therefore not be an optimal drug for long-term treatment in all patients, especially in elderly patients or in patients exhibiting cardiovascular risk factors [13–16]. Second, sub-clones bearing T315I-positive compound mutations of *BCR-ABL1* or the E255V mutation are usually resistant against ponatinib therapy [17–20]. In these cases, therapeutic options are very limited. One treatment option for such advanced CML patients is allogeneic hematopoietic stem cell transplantation (HSCT) [21–23]. However, HSCT can only be offered to a smaller number of patients who are fit and can tolerate such intensive therapy. In addition, prior to HSCT, sufficient debulking is often required. Overall most of the TKI-resistant patients have to be managed using continuous drug therapy which is associated with side effects. One strategy is to apply lower doses of ponatinib or to test new targeted drugs directed against mutant forms of BCR-ABL1, such as asciminib (ABL001) or PF-114 [24–29]. However, not all patients may respond and little is known about long-term side effects and toxicity profiles of these novel BCR-ABL1-targeting drugs [27–29]. Therefore, current research is seeking additional therapeutic strategies to control *BCR-ABL1*^{T315I}+ CML.

Hydroxyurea (HU) is a ribonucleotide reductase-inhibitor that is used for the treatment of sickle cell disease and palliative cytoreduction of end-stage (resistant) myeloid leukemias, including CML as well as palliative therapy of *BCR-ABL1*-negative myeloproliferative neoplasms [30–34]. In CML, HU has been described to exert major effects on cell cycle progression and proliferation of leukemic cells [30–33,35,36]. However, the mechanisms of action of HU on CML cells are not well understood. In addition, the effects of HU on growth and survival of TKI-resistant CML sub-clones harboring various *BCR-ABL1* mutations have not yet been investigated.

We here describe that HU exerts major anti-leukemic effects on leukemic sub-clones expressing *BCR-ABL1*^{T315I} in patients with TKI-resistant CML. In addition, we questioned whether HU would produce cooperative effects with other drugs as single drug effects may not be sufficient to overcome resistance in multi-mutated TKI-resistant CML cells. Indeed, we were able to show that HU and ponatinib synergize in inhibiting growth of leukemic cells in TKI-resistant CML. We also examined the mechanisms and potential targets involved in HU-induced effects on CML cells. In these studies, we found that CDK4 and CDK6 may serve as potential targets of therapy in *BCR-ABL1*^{T315I}+ CML. Finally, we provide evidence that the CDK4/CDK6 inhibitor palbociclib blocks the growth and survival of *BCR-ABL1*^{T315I}+ CML cells in the same way as HU. These data have clinical implications and may lead to the development of new therapeutic concepts in advanced multi-resistant *BCR-ABL1*^{T315I}+ CML.

2. Subjects and methods

2.1. Patients

Four CML patients receiving HU for cytoreduction were analyzed retrospectively. CML CP was diagnosed between 1999 and 2004. Three patients received imatinib as initial therapy and one patient received imatinib after HSCT. After 2–4 years of imatinib therapy, all 4 patients had lost their hematologic and molecular response. In one of these patients, a *BCR-ABL1*^{T315I}+ sub-clone developed during imatinib therapy. In the other 3 patients, therapy had been switched to a second-generation TKI (nilotinib and/or dasatinib) and *BCR-ABL1*^{T315I} was detected in their second (#1 and #2) or third (#3 and #4) relapse. Of note, *BCR-ABL1*^{T315I} was detected in all patients between 2006 and 2011. Ponatinib treatment was not available at that time. In 2 patients (#1 and #4), additional *BCR-ABL1*-mutations were detected. One patient (#3) had progressed to blast phase (BP), whereas 3 patients were still in CP. The patients' characteristics are summarized in Table 1. Treatment response was evaluated following European LeukemiaNet guidelines [9,37,38]. After detection of *BCR-ABL1*^{T315I}, HU (1000–3000 mg/day) was prescribed to suppress growth of leukemic cells.

For *in vitro* studies, a total of 23 primary leukemic cell samples were obtained from the peripheral blood (PB) or bone marrow (BM) of additional patients with CML as summarized in Table 2. Furthermore, control BM cells were obtained from 6 lymphoma patients without BM involvement. All investigations were approved by the local ethics committee of the Medical University of Vienna (ethic vote number: 224/206). Informed consent was obtained from all patients.

Table 1
Patients´ characteristics (HU-treated patients).

Patient no.	Gender	Age at diagnosis (years)	Disease duration before HU-treatment (months)	Therapies received before HU-treatment	CML-phase at start of HU-treatment	*BCR-ABL1* mutations detected	WBC at HU-start (G/l)	HU dose (mg/day)	Duration of HU treatment (months)	Follow up - outcome and duration (months)
#1	m	41	22	imatinib, cytarabin, 6-mercaptopurine, dasatinib	CP	T315I G250E E255K	11.6	1500–2000	18	HSCT MMR (20)
#2	m	65	108	interferon-α, cytarabine, imatinib	CP	T315I	9.6	1000–3000	11	PD, † (13)
#3	m	53	104	HSCT, DLI interferon-α imatinib, dasatinib, nilotinib	BP	T315I	29.9	1500	2	PD, † (2)
#4	m	51	92	imatinib, dasatinib, nilotinib, interferon-α	CP	T315I E255K	80.0	500–3000	4	HSCT MMR

HU, hydroxyurea; m, male; HSCT, allogenic hematopoietic stem cell transplantation; DLI, donor lymphocyte infusion; CP, chronic phase; BP, blast phase; MMR, major molecular response; PD, progressive disease.
† Deceased.

2.2. Laboratory investigations

During follow-up, routine blood investigations, including serial determinations of blood counts and differential counts, were performed in certain time intervals (1–12 weeks). In addition, *BCR-ABL1* mRNA levels were quantified in the peripheral blood (PB) in 1–6 month intervals. The *BCR-ABL1* transcript burden was quantified by real-time PCR according to the International Scale (IS) [39]. Screening for mutations in the *BCR-ABL1* tyrosine kinase domain (TKD) was performed essentially as described [40]. To quantify the mutant allele burden of *BCR-ABL1*^{T315I}, ligation-dependent PCR (LD-PCR) was employed using the thermocycler AB-9600 (Applied Biosystems, Foster City, USA) as reported [41]. The percentage of *BCR-ABL1*^{T315I} was expressed as percent of total *BCR-ABL1* mRNA [41].

2.3. Reagents

Reagents used in this study are described in the Supplemental file and Supplemental Table S1.

2.4. Cell lines and culture conditions

The human CML cell lines KU812, KCL22 and K562 were used in this study. KU812 cells were kindly provided by Kenji Kishi (Niigata University, Niigata, Japan). KCL22 and K562 cells were purchased from the German Collection of Microorganism and Cell Culture (DSMZ, Braunschweig, Germany). In case of KCL22, a *BCR-ABL1*^{T315I}+ sub-clone was generated by culturing cells in medium containing imatinib and dasatinib essentially as described (KCL22^{T315I}) [42]. In

Table 2
Patients´ characteristics: CML samples used for *in vitro* studies.

Patient no.	Age (years)	Gender	Source	CML phase	*BCR-ABL1* mutations	Therapy before cell sampling	HU IC_{50} (μM)	Palbociclib IC_{50} (nM)	`HU + ponatinib´
#5	64	m	PB	CP	n.t.	HU	175	n.t.	n.t.
#6.1	48	m	PB	CP	n.t.	None	74	n.t.	Cooperative
#6.2	48	m	PB	BP	T315I	imatinib, dasatinib (dis), nilotinib (dis)	116	31	n.t.
#7	34	m	BM	CP	n.t.	None	50	n.t.	n.t.
#8	50	m	PB	CP	n.t.	None	46	n.t.	Cooperative
#9	59	m	PB	CP	n.t.	None	73	n.t.	n.t.
#10	63	m	BM	CP	n.t.	None	75	n.t.	Cooperative
#11	54	f	PB	CP	n.t.	None	96	n.t.	Cooperative
#12.1	48	m	BM	BP	G250E	imatinib (res), dasatinib (res), bosutinib (res), ponatinib (res), HU	236	n.t.	n.t.
#12.2	48	m	BM	BP	G250E E255V	imatinib (res), dasatinib (res), bosutinib (res), ponatinib (res), HU	30	n.t.	n.t.
#13	78	f	PB	BP	F317L, L248V, K274del	interferon-alpha, imatinib (res), dasatinib (res), bosutinib (res), ponatinib (res), HU, rapamycin	285	n.t.	n.t.
#14	55	m	BM	CP	n.t.	None	50	n.t.	Cooperative
#15	48	f	PB	CP	n.t.	None	56	n.t.	Cooperative
#16	81	f	BM	CP	n.t.	imatinib (dis)	36	5	n.t.
#17	29	f	BM	CP	n.t.	None	33	11	n.t.
#18	69	f	PB	CP	n.t.	None	41	2	n.t.
#19	71	m	BM	BP	n.t.	None	148	338	n.t.
#20	67	m	BM	CP	n.t.	None	43	5	n.t.
#21	34	f	PB	CP	n.t.	None	72	16	Cooperative
#22	78	m	BM	CP	n.t.	None	31	19	n.t.
#23	18	m	BM	CP	n.t.	None	14	1	Cooperative
#24	59	f	BM	CP	n.t.	None	30	48	n.t.
#25	34	f	PB	CP	n.t.	None	n.t.	n.t.	Cooperative

Abbreviations: CML, chronic myeloid leukemia; HU, hydroxyurea; m, male; f, female; PB: peripheral blood; BM: bone marrow; CP, chronic phase; BP, blast phase; n.t., not tested; none, no therapy (diagnostic sample); res, resistant; dis, discontinued due to intolerance; μM, micromolar. Responses of cells to HU, palbociclib and `HU + ponatinib´ were assessed by ^{3}H-thymidine uptake.

addition, we employed untransfected (BCR-ABL1-negative) Ba/F3 cells, Ba/F3 cells harboring wild type (WT) *BCR-ABL1* (Ba/F3p210^{WT}) or *BCR-ABL1*^{T315I} (Ba/F3p210^{T315I}) [43]. Ba/F3 cells expressing T315I-based compound mutations *(BCR-ABL1*$^{T315I/E255K}$, *BCR-ABL1*$^{T315I/F311L}$, *BCR-ABL1*$^{T315I/F359V}$, *BCR-ABL1*$^{T315I/G250E}$) were generated as described recently [44]. Primary PB and BM mononuclear cells (MNC) were kept in culture as reported [45]. All cell lines and primary cells were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) and antibiotics. KCL22^{T315I} cells were kept in the presence of 5 μM imatinib. Untransfected Ba/F3 cells were kept in the presence of 0.1 ng/ml IL-3. For analysis of CDK4 and CDK6 expression, cell lines were kept in RPMI 1640 medium supplemented with 1% FCS and antibiotics for up to 72 h.

2.5. Measurement of proliferation and competitive outgrowth, apoptosis and cell cycle arrest of BCR-ABL1+ cells

To examine proliferation, cell lines and primary cells were incubated with control medium (Co) or in various concentrations of drugs (HU, ponatinib, ABL001, palbociclib, cytarabine, homoharringtonine and interferon alpha (IFNα) alone or in (two- or three) drug combinations at 37 °C for 48 h. Then, ^{3}H-thymidine-uptake was measured as described [45]. To determine the effects of HU, ponatinib, and the combination 'HU+ponatinib' on clonal outgrowth of mutant-bearing sub-clones, experiments were performed using mixtures of BCR-ABL1+ Ba/F3 cells. In these experiments, Ba/F3p210^{WT} cells (labeled with Venus fluorescent protein), Ba/F3p210^{T315I} cells (labeled by green fluorescence protein, GFP) and Ba/F3p210$^{T315I/E255V}$ cells (labeled by tdTomato fluorescent protein) were mixed in a 1:1:1 ratio and incubated together in control medium or in the presence of HU (100 μM), ponatinib (10 nM) or a combination of both drugs for 72 h. Thereafter, cell viability was measured by trypan blue exclusion, and the relative percentage of 'sub-clone cells' in each condition was measured on a FACScan (Becton Dickinson, San Diego, CA). Three independent experiments were performed.

2.6. Evaluation of apoptosis and cell cycle arrest in drug-exposed cells

For analysis of apoptosis, CML cell lines were kept in control medium or in the presence of HU or/and ponatinib for 48 h. Then, apoptosis was determined by staining for AnnexinV-FITC and propidium iodide (PI) or AnnexinV-FITC and 4′,6-diamidino-2-phenylindole (DAPI). For analysis of cell cycle progression, cells were kept in the presence or absence of various concentrations of HU or palbociclib for 24 h. Thereafter, PI was added and cell cycle distribution was analyzed as described previously [45]. In all cases, 3 independent experiments were performed.

2.7. Western blotting and qPCR

To study the effects of HU on expression of CDK4, CDK6, retinoblastoma gene product (Rb) and phosphorylated Rb (pRb), Western blot experiments were performed on CML cell lines. Cells were kept in RPMI 1640 medium supplemented with 1% FCS and antibiotics in the presence or absence of HU (500 μM) or palbociclib (0.5 μM) for 24–72 h. After incubation, cells were recovered and lysed in lysing buffer and then examined by Western blotting essentially as described [45] using antibodies against CDK4, CDK6, pRb, Rb, as well as β-tubulin and β-actin (loading controls). To determine drug-induced apoptosis, antibodies directed against cleaved poly-ADP-ribose polymerase (PARP) and total PARP were employed. A list of antibodies is provided in the supplemental file (Supplemental Table S2). To determine mRNA expression levels in CML cell lines, cells (as indicated) were kept in control medium or in the presence of HU (at the same condition as for Western blot analyses). RNA was isolated from CML cell lines using the RNeasy MinEluteCleanupKit (Qiagen, Hilden, Germany). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA), random primers, first strand buffer, dNTPs (100 mM), and RNasin (all from Invitrogen) according to the manufacturer's instructions. PCR was performed as reported [46] using primers specific for CDK4 and CDK6. A list of PCR primers used in qPCR experiments is provided in the supplemental file (Supplemental Table S3). mRNA levels were quantified on a QuantStudio 3 PCR System (Applied Biosystem, Foster City, CA, USA) using iTAq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA). mRNA expression levels were normalized to ABL1 mRNA levels and expressed as percentage of ABL1. Calculations were based on standard curves established for CDK4, CDK6 and ABL1 mRNA expression. All Western blotting and qPCR-experiments were repeated three times.

2.8. shRNA induced knockdown of CDK4 and CDK6

To learn more about the role of CDK4 and CDK6 on proliferation and survival of CML cells, K562 and KCL22 cells were transfected simultaneously with shRNA constructs directed against CDK4 and CDK6 or with control shRNAs as described in the supplemental file (Supplemental methods and Supplemental Table S4).

2.9. Statistical analysis

The Student's *t*-test (two-sided) for dependent samples was applied. Results were considered statistically significant when p was <0.05. All data analysed for statistically significance using the *t*-test met the assumptions of the test. In drug combintion experiments, drug interaction-types were determined by calculating combination index (CI) values using Calcusyn software as reported [47].

3. Results

3.1. Response to HU treatment in 4 patients with BCR-ABL1^{T315I}+ CML

We examined 4 patients with advanced *BCR-ABL1*^{T315I}+ CML who were treated with HU for bridging to HSCT ($n = 2$) or for palliative cytoreduction ($n = 2$). HU treatment resulted in stabilization of the leukocyte counts in 3 of 4 patients, but failed to induce a molecular response (Fig. 1). However, surprisingly, the percentage of *BCR-ABL1*^{T315I} compared to total *BCR-ABL1*, assessed by LD-PCR, decreased significantly in all 4 patients during HU treatment, and in 3 of the 4 patients, the T315I mutant was no longer detectable after therapy (Fig. 1). These data suggest that HU is able to eliminate *BCR-ABL1*^{T315I}+ leukemic cells. After 2 months of HU therapy, HSCT could be performed in 2 patients (#1 and #4). These patients remained in complete hematologic and molecular remission during the observation period (20 and 40 months) (Fig. 1). In patient #2 (palliative HU) a clinically and hematologically stable disease (leukocytes: 3400–15,000/μL) was observed over 18 months. Thereafter, the patient developed a *BCR-ABL1*^{T315I}-negative BP of CML and died. In patient #3 in whom HU was started at the time of BP, no substantial decrease in blood leukocyte, blasts or total *BCR-ABL1* could be observed during HU treatment, despite a temporary suppression of *BCR-ABL1*^{T315I} from 94% to 7.3% of total *BCR-ABL1* (Fig. 1). This patient died 2 months after the start of HU treatment. Together, these observations suggest that HU is able to suppress or even eliminate mutant CML sub-clones harboring *BCR-ABL1*^{T315I}+ *in vivo* in patients with CML CP.

3.2. HU suppresses proliferation and survival of TKI-sensitive and TKI-resistant BCR-ABL1+ cells in vitro

To explore the effects of HU on drug-resistant CML cells in more detail, we performed a series of *in vitro* experiments. As visible in Fig. 2(A) and Table 2, HU suppressed the proliferation of primary cells isolated from the PB or BM of 20 patients with CML, including 4 TKI-resistant cases (one exhibiting *BCR-ABL1*^{T315I}) with IC_{50}-values ranging between 25 and 300 μM (mean IC_{50}: 85.81 μM). In normal BM

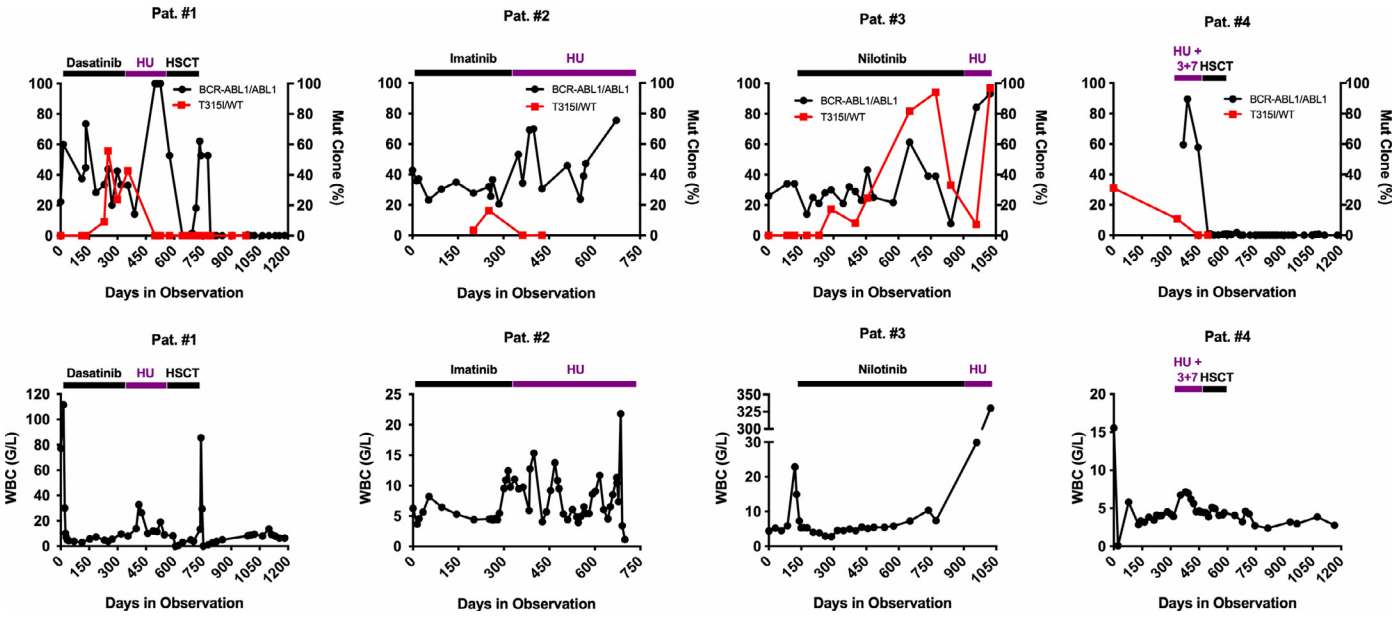

Fig. 1. Hydroxyurea (HU) induces molecular response and suppresses the T315I-positive sub-clone(s) in advanced CML. Four heavily pretreated CML patients (#1–#4) were treated with HU, BCR-ABL1 tyrosine kinase inhibitors (imatinib, 400 mg/day; dasatinib, 100 mg/day; nilotinib, 2 × 400 mg/day per os), polychemotherapy, or hematopoietic stem cell transplantation (HSCT) as indicated. The percentage of *BCR-ABL1* mRNA relative to *ABL1* mRNA (according to the international scale) as well as the percentage of *BCR-ABL1*^{T315I} mRNA relative to *BCR-ABL1* mRNA (*BCR-ABL1*^{T315I}/*BCR-ABL1*, determined by ligase-dependent PCR) are shown in the upper panels. The white blood count (WBC) of the same patients are shown in the lower panels. 3 + 7: combined chemotherapy following the 3 + 7-protocol consisting of daunorubicine (60 mg/m^2 per day, days 1–3) and cytosine arabinoside (200 mg/m^2 per day, days 1–7).

MNC isolated in 6 donors, HU was found to inhibit proliferation at higher concentrations (mean IC_{50}: 176.7 μM; Fig. 2(B)). These data are in line with the clinical observation that HU (compared to other cytoreductive drugs) exerts only moderate myelo-suppressive effects and is therefore considered to be a relatively save drug. HU effects were also seen in KCL22^{T315I} cells, Ba/F3 cells expressing *BCR-ABL1*^{WT}, and Ba/F3 cells harboring *BCR-ABL1*^{T315I} (Fig. 2(C)). Moreover, HU was found to inhibit the proliferation of Ba/F3 cells expressing T315I-including BCR-ABL1 compound mutants (Fig. 2(C)). An intriguing observation was that HU was more effective in sub-clones harboring mutant *BCR-ABL1* than *BCR-ABL1*^{WT} (IC_{50} in Ba/F3 cells with mutant *BCR-ABL1*, including T315I-involving compound mutants: <100 μM; IC_{50} in Ba/F3p210^{WT}: 100–200 μM; IC_{50} in KCL22^{T315I} cells: <200 μM; IC_{50} in un-transduced KCL22 cells: 200–300 μM) (Fig. 2(C)). In untransfected Ba/F3 cells, IC_{50} values for HU were significantly higher than in Ba/F3 cells exhibiting various mutant forms of *BCR-ABL1* (Fig. 2(C)) which may point at a therapeutic window.

For comparison, three additional cytoreductive agents that have been used for the treatment of CML, namely cytarabine, homoharringtonine, and IFNα [48–50], were also tested in our Ba/F3 cells and in our human CML cell line models. In these experiments, cytarabine and homoharringtonine produced relatively strong anti-proliferative effects in all cell lines examined (Supplemental Fig. 1A and B), whereas IFNα did not produce significant effects at concentrations up to 5 × 10^4 U/mL (not shown). More importantly, contrasting HU, neither cytarabine nor homoharringtonine exerted more potent effects in cells harboring *BCR-ABL1*^{T315I} than in cells lacking *BCR-ABL1*^{T315I} (Supplemental Fig. 1A and B).

Finally, we examined the effects of HU on survival of CML cells. In these experiments, HU was found to induce apoptosis in all human CML cell lines tested (Supplemental Fig. S1C). Interestingly, the apoptosis-inducing effect of HU was stronger in KCL22^{T315I} cells than in KCL22 cells.

Collectively, these data show that HU inhibits growth and survival of CML cells, and that HU effects are stronger in CML cells expressing *BCR-ABL1*^{T315I} or T315I-including compound mutants of *BCR-ABL1* compared to CML cells expressing *BCR-ABL1*^{WT}.

3.3. HU cooperates with ponatinib in inducing growth arrest in primary CML cells and in BCR-ABL1+ cell lines

Drug combinations may be a suitable strategy to overcome drug resistance in patients with advanced CML who failed TKI therapy. We examined cooperative (potentially synergistic) effects of the drug combination 'HU+ponatinib'. As shown in Fig. 3(A), ponatinib and HU produced strong synergistic effects on proliferation in all TKI-sensitive and TKI-resistant CML cell lines, including KCL22^{T315I}. Synergism was confirmed using Calcusyn software (Supplemental Fig. S2A). For comparison, cytarabine, homoharringtonine and IFNα were also combined with ponatinib. Cooperative effects were observed when combining cytarabine or homoharringtonine with ponatinib (Supplemental Fig. 3A), whereas IFNα failed (even in combination) to produce anti-neoplastic effects (not shown). Furthermore, HU and ponatinib were found to cooperate in inducing apoptosis in all CML cell lines examined (Supplemental Fig. S3B). Next, we evaluated the effects of the drug combination in Ba/F3 cells harboring *BCR-ABL1*^{T315I} or T315I+ compound mutants. In these experiments, HU and ponatinib were found to produce synergistic growth-inhibitory effects in all sub-clones examined (Fig. 3(B) and Supplemental Fig. S2B). We also tested the drug combination in primary leukemic cells obtained from 9 patients with newly diagnosed CML CP. As visible in Fig. 3(C) and Table 2, the drug combination 'HU+ponatinib' produced additive or even synergistic growth-inhibitory effects in these cells. Collectively, these data suggest that HU and ponatinib exert synergistic anti-leukemic effects in CML cells and may overcome TKI resistance. Currently, ponatinib is the only approved TKI that blocks BCR-ABL1^{T315I}. However, other, novel BCR-ABL1 blocker, such as ABL001 (asciminib) [51], are currently tested in clinical trials and may be useful for third-line treatment of CML in the future. Therefore, we extended our experiments to ABL001. As a single drug, ABL001 (0.1–1 μM) was found to inhibit proliferation of Ba/F3 cells harboring *BCR-ABL1*^{WT}, *BCR-ABL1*^{T315I} or *BCR-ABL1*$^{T315I/G250E}$. However, cells harboring other T315I-including compound mutants showed relative resistance against ABL001 (Supplemental Fig. S3C). Next we combined ABL001 with HU. As visible in Fig. 3(D) and Supplemental Fig. S2C, synergistic

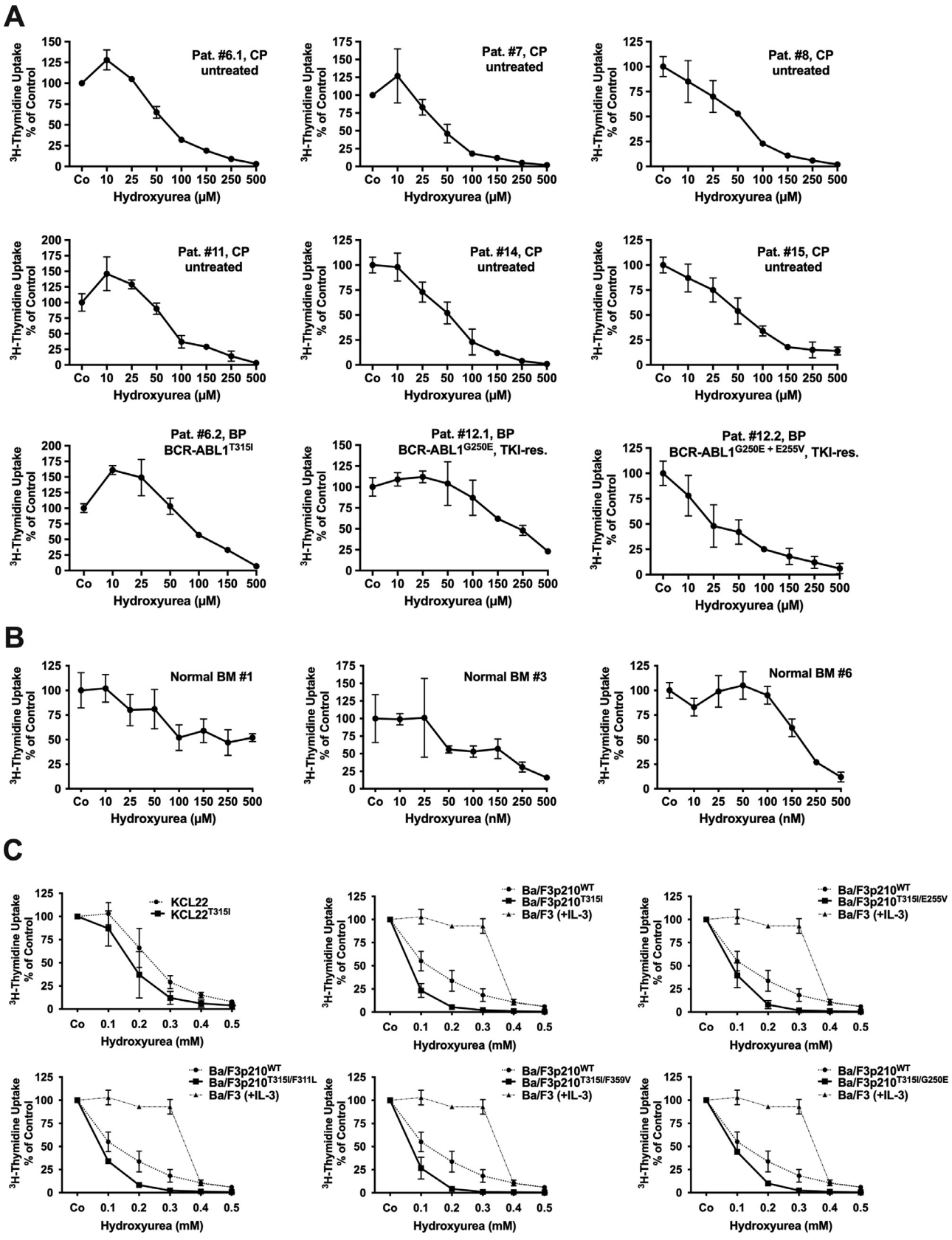

Fig. 2. Hydroxyurea (HU) inhibits the proliferation of primary CML cells and TKI-resistant cell lines harboring T315I-mutated *BCR-ABL1*. Primary leukemic cells obtained from CML patients (A), normal BM cells (B) and KCL22 cells expressing *BCR-ABL1*^{WT} (C, left upper image, dotted line), KCL22 cells expressing *BCR-ABL1*^{T315I} (C, left upper image, black line), untransfected (*BCR-ABL1*-negative) Ba/F3 cells (kept in 0.1 ng/ml IL-3) (C, upper right, middle and lower panels, stippled lines) and Ba/F3 cells expressing *BCR-ABL1*^{WT} (C, dotted lines) or various mutant forms of *BCR-ABL1* (B, black lines) were incubated in control medium (Co) or medium containing various concentrations of HU at 37 °C for 48 h. Thereafter, ^{3}H-thymidine-uptake was measured. (A and B) Results are expressed as percent of control and represent the mean±S.D. from triplicates. Patients' numbers refer to Table 2. TKI-res., TKI-resistant. (C) Results are expressed as percent of control and represent the mean±S.D. from 3 independent experiments.

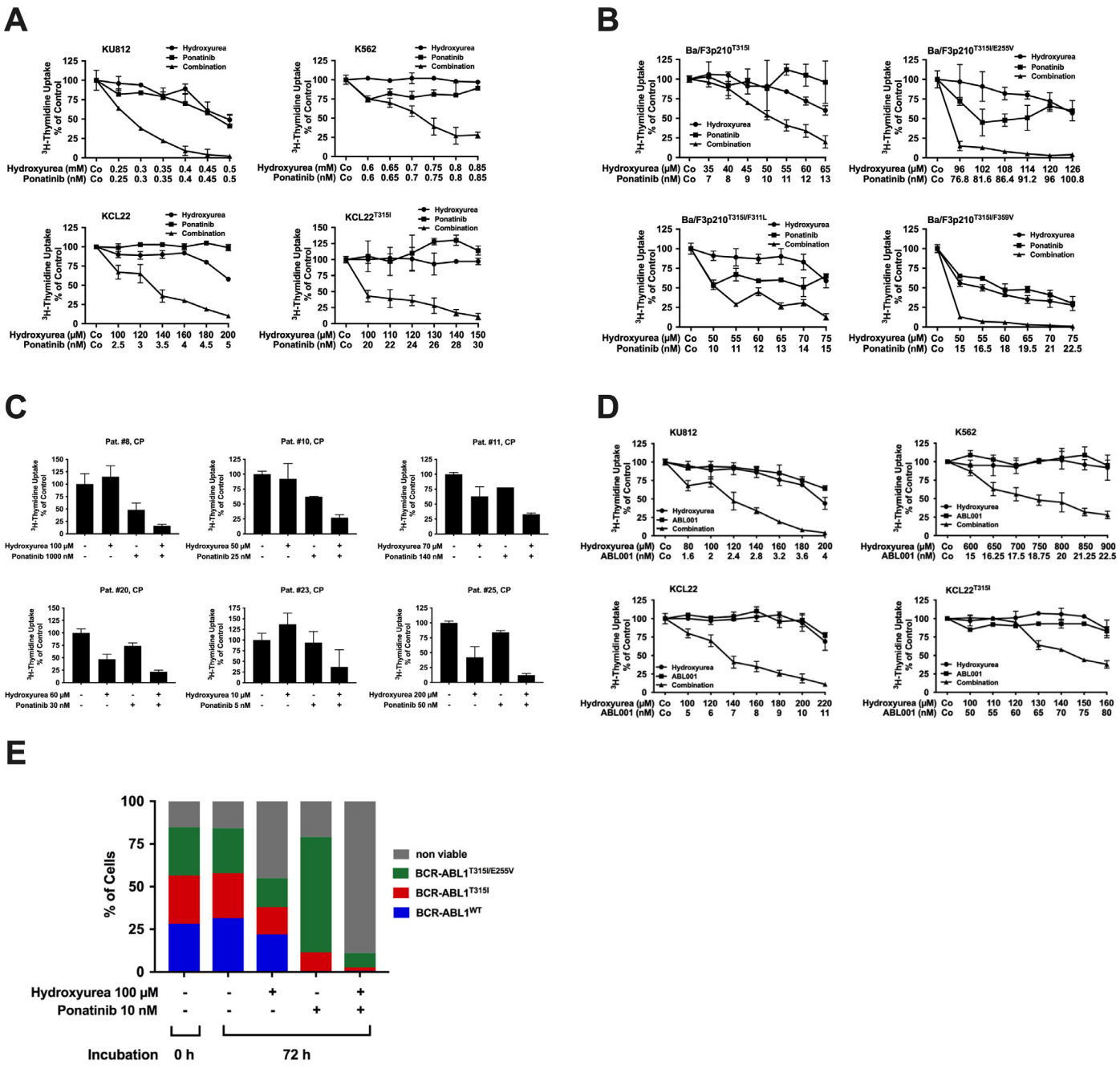

Fig. 3. Hydroxyurea (HU) synergizes with ponatinib in inducing growth inhibition in BCR-ABL1 positive cell lines and suppresses out-growth of cells harboring T315I-inculding BCR-ABL1 mutations. (A-C) Human CML cell lines (A), Ba/F3 cells expressing various mutant forms of *BCR/ABL1* (B), and primary leukemic cells obtained from CML patients (C), were incubated in control medium (Co) or in various concentrations of HU, ponatinib or the combination of both drugs as indicated at 37 °C for 48 h. Thereafter, ^{3}H-thymidine-uptake was measured. Results are expressed as percent of control and represent the mean±S.D. from triplicates. Patients' numbers in (C) refer to Table 2. (D) Human CML cell lines were incubated with control medium (Co) or medium containing various concentrations of HU, ABL001 or the combination of both drugs as indicated at 37 °C for 48 h. Thereafter, ^{3}H-thymidine-uptake was measured Results are expressed as percent of control and represent the mean±S.D. of triplicates. (E) Ba/F3p210^{WT} (labeled by Venus), Ba/F3p210^{T315I} (labeled by GFP) and Ba/F3p210$^{T315I/E255V}$ (labeled by tdTomato) were mixed in a 1:1:1 ratio and incubated together in control medium or in the presence of HU (100 μM), ponatinib (10 nM) or the combination of both drugs for 72 h (h). Thereafter, the ratio between clones in each condition was measured by flow cytometry. The percentage of non-viable cells was determined by trypan blue staining. Results show one typical experiment. Almost identical data were obtained in 2 other independent experiments.

growth-inhibitory effects were also obtained with the combination 'HU+ABL001' in K562, KU812, KCL22 and KCL22^{T315I} cells. Moreover, synergistic effects between ABL001 and HU were also observed in all Ba/F3 cell clones tested, including Ba/F3 cells harboring *BCR-ABL1*^{T315I} or T315I-including compound mutants of *BCR-ABL1* (Supplemental Fig. S3D). These data show that HU may be a suitable combination partner for various TKI used to treat CML.

3.4. The drug combination 'HU+ponatinib' suppresses the competitive outgrowth of sub-clones harboring T315I-inclusive compound mutants of BCR-ABL1

To explore whether the combination 'HU+ponatinib' can suppress the outgrowth of sub-clones expressing *BCR-ABL1* compound mutations, Ba/F3 cells expressing *BCR-ABL1*^{WT}, *BCR-ABL1*^{T315I} *or BCR-*

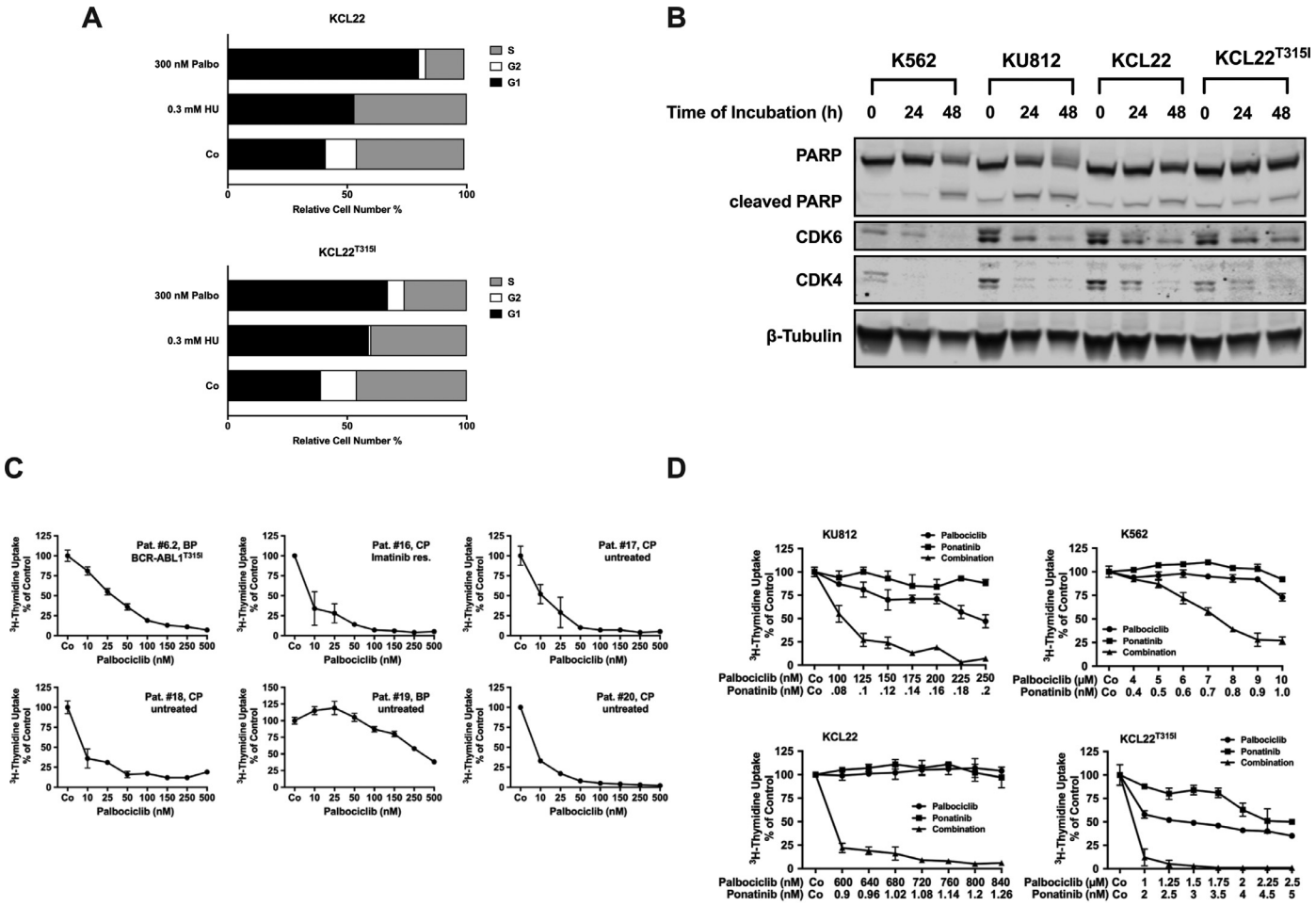

Fig. 4. Synergistic effects between HU and ponatinib are mediated by suppression of CDK4/CDK6. (A) KCL22 and KCL22^{T315I} cells were kept in control medium (Co) or incubated in various concentrations of hydroxyurea (HU) or palbociclib as indicated for 24 h. Thereafter, PI was added and cell cycle distribution was determined by flow cytometry. The percentage of cells in G1-phase, G2/M-phase and S-phase in each condition are shown. Results represent the mean of 3 independent experiments. (B) K562, KU812, KCL22 and KCL22^{T315I} cells were kept in RPMI medium supplemented with 1% fetal calf serum (FCS) in the absence ("0") or presence of 500 μM HU for 24 or 48 h as indicated. Thereafter, cells were subjected to Western blot analysis using antibodies against, PARP, CDK4, CDK6 or β-tubulin as indicated. (C) Primary CML cells were kept in control medium (Co) or in various concentrations of palbociclib (as indicated) for 48 h before ^{3}H-thymidine-uptake was measured. Results are expressed as percent of control and represent the mean±S.D. from triplicates. Patients' numbers refer to Table 2. res., resistant. (D) KU812, K562, KCL22 and KCL22^{T315I} were incubated in control medium (Co) or in various concentrations of palbociclib, ponatinib or the combination of both drugs (as indicated) at 37 °C for 48 h. Then, ^{3}H-thymidine-uptake was measured. Results are expressed as percent of control and represent the mean±S.D. from triplicates.

ABL1$^{T315I/E255}$ were mixed at a ratio of 1:1:1 and then exposed to HU, ponatinib or a combination of both drugs. As expected, ponatinib was found to be more effective in suppressing cells expressing *BCR-ABL1*^{WT} compared to cells expressing BCR-ABL1 mutants (Fig. 3(E)). By contrast, HU was found to be more effective against the outgrowth of cells harboring *BCR-ABL1*^{T315I} or *BCR-ABL1*$^{T315I/E255V}$ compared to *BCR-ABL1*^{WT}. Only the combination 'HU+ponatinib' was found to suppress the survival of all three co-cultured sub-clones (BaF3p210^{WT}, BaF3p210^{T315I}, BaF3p210$^{T315I/E255V}$) and to induce cell death in all these sub-clones (Fig. 3(E)). These data suggest that the combination 'HU+ponatinib' is effective in suppressing the outgrowth of TKI-resistant sub-clones in multi-resistant CML.

3.5. Effects of HU on cell cycle progression and on expression of the cell cycle regulators CDK4 and CDK6

Previous data suggest that HU inhibits cell survival by interfering with cell cycle progression. In addition, it has been described that HU suppresses the expression of the cell cycle regulator CDK6 [36,52]. To learn more about the mechanism of action of HU in TKI-resistant CML cells, cell cycle progression was analyzed. As visible in Fig. 4(A), HU induced cell cycle arrest in KCL22 cells. The same effect was seen with palbociclib, a more specific inhibitor of CDK4/CDK6 [53,54]. HU effects on cell cycle progression were more pronounced in KCL22 cells harboring *BCR-ABL1*^{T315I} than in cells expressing native *BCR-ABL1* (Fig. 4(A)). Next we examined the effects of HU on expression of CDK4 and CDK6 in CML cells. As shown in Fig. 4(B) and Supplemental Fig. S4A, HU decreased the expression of CDK4 and CDK6 at the mRNA and protein level in all CML cell lines tested, confirming previous data [36,52]. By contrast, cytarabine, homoharringtonine and IFNα failed to reduce CDK4/CDK6 expression in all CML cell lines tested (Supplemental Fig. S4B).

3.6. Effects of CDK4/CDK6 knock-down and palbociclib on growth and survival of CML cells

To learn more about the role of CDK4 and CDK6 as potential therapeutic targets in CML cells, we performed shRNA-mediated simultaneous knockdowns of CDK4 and CDK6 in K562 and KCL22 cells. In both cell lines, transfection with the 2 shRNA constructs resulted in decreased expression of CDK4 and CDK6 protein as assessed by Western Blotting (Supplemental Fig. S5A). Interestingly, knock-down of

CDK4 and CDK6 did not lead to induction of apoptosis but resulted in a growth-disadvantage in both cell lines when compared to cells treated with control shRNA (Supplemental Fig. S5B). These data suggest, that inhibition of CDK4/CDK6 interferes with proliferation in CML cells. Knock-down of either CDK4 or CDK6 did not produce any effect on proliferation of K562 and KCL22 cells (not shown). Next, we tested the effects of palbociclib – a pharmacologic inhibitor of CDK4/CDK6 - on growth and survival of TKI-resistant CML cells. Palbociclib profoundly suppressed the proliferation of primary leukemic cells obtained from 10 CML patients, including 8 with CP CML (IC_{50}: 1–48 nM) and 2 in BP (IC_{50}: 31 and 338 nM, respectively) (Fig. 4(C), Table 2). In addition, palbociclib inhibited the proliferation of all CML cell lines tested, although the concentrations required to block proliferation in cell lines were higher than in primary CML cells (Supplemental Table S5). Moreover, palbociclib was found to synergize with ponatinib in inducing growth inhibition in KU812, K562, KCL22 and KCL22^{T315I} cells in the same way as HU (Fig. 4(D), Supplemental Fig. S2D). These data suggest, that CDK4/CDK6-inhibitors sensitize CML cells against ponatinib. However, specific knock-down of CDK4/CDK6 failed to exert the same antineoplastic effects, suggesting, that additional targets of HU and palbociclib may play an certain role for synergistic drug interactions. As expected, palbociclib suppressed the phosphorylation of Rb in all CML cell lines tested (Supplemental Fig. S6A and B). By contrast, HU, albeit suppressing the expression of total CDK4 and CDK6, did not downregulate Rb phosphorylation (Supplemental Fig. S6A and B). Finally, we examined the effect of the drug combination ‘HU+palbociclib’. As shown in Supplemental Fig. S7A and B, palbociclib was found to synergize with HU in inhibiting the proliferation in all CML cell lines examined, including KCL22^{T315I}. Furthermore, the 3-drug combination `HU+ponatinib+palbociclib´ was found to produce stronger anti-proliferative effects than the single compounds (applied at very low concentrations) or the 2-drug combinations (Supplemental Fig. S7C and D).

4. Discussion

BCR-ABL1^{T315I} occurs in 20–30% of all TKI-resistant CML patients exhibiting *BCR-ABL1* mutations and thus represents a major clinical challenge [10–12]. Ponatinib suppresses BCR-ABL1^{T315I} but is not an optimal drug for all patients due to its side effects [12–15]. Therefore, other strategies to control CML sub-clones expressing *BCR-ABL1*^{T315I} or more complex *BCR-ABL1*-mutations are currently being developed. HU is a well-tolerated drug that has been used for palliative treatment of CML over decades [31,32]. We here report that HU inhibits the growth of CML cells and Ba/F3 cells expressing *BCR-ABL1*^{T315I} or T315I-involving compound mutations. In addition, we show that HU cooperates with ponatinib in suppressing the growth of leukemic cells expressing *BCR-ABL1*^{T315I} or T315I-containing compound mutants. We also show that the growth-inhibitory effect of HU on *BCR-ABL1*^{T315I}-positive cells is stronger than HU effects on CML cells displaying native *BCR-ABL1* and that HU treatment results in a decrease in expression of CDK4 and CDK6. Finally, we show that treatment of patients with TKI-resistant CML with HU results in selective suppression or even depletion of *BCR-ABL1*^{T315I}-positive sub-clones. These observations have clinical implications and may lead to the development of new treatment concepts.

Our initial observation was that during therapy with HU, the *BCR-ABL1*^{T315I}+ sub-clone decreased in size in 4 heavily pretreated, TKI-resistant CML patients. Remarkably, in 3 of 4 patients, the *BCR-ABL1*^{T315I}+ sub-clone was no longer detectable after therapy with HU although total *BCR-ABL1* mRNA levels did not decrease. In one patient, HU showed no major response and the leukocyte counts even increased during HU therapy. In this case, the disease rapidly progressed to BP and the patient died within short time. However, even in this patient, the percentage of *BCR-ABL1*^{T315I} (relative to total *BCR-ABL1*) decreased from 94% to 7.3%. Three out of these four patients received only HU without any other cytoreductive or targeted drugs at the time when *BCR-ABL1*^{T315I} decreased. However, one patient received HU and 3 + 7 chemotherapy before HSCT. In this patient, it remains unclear whether the decrease in *BCR-ABL1*^{T315I} was only caused by HU or the combination of HU and 3 + 7. Our *in vivo* observations suggest, that the *BCR-ABL1*^{T315I}-bearing sub-clone is particularly sensitive against HU. Similar observations were made by Hanfstein et al. [55]. In their study, 4/5 patients showed a decrease in *BCR-ABL1*^{T315I} under HU, but only one patient became *BCR-ABL1*^{T315I}-negative [55]. It has also been hypothesized that the decrease in mutated *BCR-ABL1* is due to a simple deselection of clonal cells. However, in most patients who did not receive HU in their study a decrease in *BCR-ABL1* was not observed, and in the few other patients where *BCR-ABL1*^{T315I} also decreased, other cytoreductive drugs were applied [55], arguing against a simple deselection scenario. In this regard it is also worth noting that the growth disadvantage of *BCR-ABL1*^{T315I}+ cells over wt *BCR-ABL1*+ cells is only seen *in vitro* when no additional pro-oncogenic pathways are activated [56]. However, in the clinical setting, *BCR-ABL1*^{T315I}-bearing sub-clones, once detected, did already undergo clonal selection by further acquisition of such additional pro-oncogenic pathways. Therefore, one would not expect that a fully established *BCR-ABL1*^{T315I}-bearing sub-clone can be deselected by just discontinuing the TKI.

Our in *vitro data* confirmed the assumption that HU is particularly effective in BCR-ABL1 T315I-mutated cells. In fact, HU was found to inhibit proliferation and viability of human and Ba/F3 cells expressing *BCR-ABL1*^{T315I} or T315I-inclusive compound mutatios. Most significantly, leukemic cells harboring *BCR-ABL1*-mutations involving T315I were more sensitive against HU than cells expressing *BCR-ABL1*^{WT} suggesting, that HU may be particularly effective patients in whom TKI-resistant sub-clones express *BCR-ABL1*^{T315I} or *BCR-ABL1*^{T315I}-involving compound mutations.

The biochemical mechanisms underlying the particular, strong effect of HU on CML sub-clones exhibiting *BCR-ABL1*^{T315I} remain unknown. A direct effect of HU on BCR-ABL1 mutants seems unlikely. Rather, other molecular targets may explain responses to HU. Indeed, HU is well known to suppress proliferation in neoplastic cells by interfering with cell-cycle progression [31,35,57,58]. Bruchova et al. described that HU, when applied *in vivo* to patients with CML, inhibits the expression of CDK6 in leukemic cells [36]. In the present study, we were able to confirm this effect of HU *in vitro* using various CML cell lines. In addition, HU was found to counteract cell cycle progression in CML cells. Since CDK6 has recently been identified as a major drug target in applied oncology [53,59,60] we were also interested to learn whether specific blockage of CDK6 would lead to cell cycle arrest and growth inhibition in CML cells. Indeed, we found that simultaneous knockdown of CDK4 and CDK6 with shRNA or application of palbociclib, a potent CDK4/CDK6 inhibitor, counteract cell cycle progression and proliferation in CML. These data point at the possible role of CDK4 and CDK6 as potential drug targets in CML which confirms previous observations [61,62]. Concerning the effects of palbociclib, our results are also in line with recent data demonstrating high anti-leukemic activity of this drug against cell lines reflecting CML in lymphatic BP or BCR-ABL1+ acute lymphoblastic leukemia [60]. Whether this concept can be translated to application in TKI-resistant CML remains at present unknown. The advantage of palbociclib over HU would be that palbociclib is a stronger CDK inhibitor compared to HU. In addition, palbociclib is a selective inhibitor that may have a more favorable side effect profile than HU, at least in long-term treated patients.

A particular problem in the treatment of TKI-resistant CML is the occurrence of sub-clones expressing T315I-involving compound mutants of *BCR-ABL1* [17–20]. Our data show, that sub-clones expressing highly-resistant *BCR-ABL1* compound mutants respond to HU and palbociclib, suggesting, that application of these drugs in the context of complex *BCR-ABL1* mutations may be a reasonable approach.

Despite the remarkable effects HU exerts on *BCR-ABL1*^{T315I}-mutated CML cells, no hematologic or molecular response was observed in our patients, suggesting that other sub-clones are less sensitive to HU. In addition, HU was not able to eliminate the *BCR-ABL1*^{T315I}-mutated sub-clone in all advanced CML patients which is in line with previous observations [55]. Therefore, drug-combinations including HU or palbociclib and BCR-ABL1 TKI may be a preferable approach. We found that HU synergizes with ponatinib in producing growth inhibition in all primary CML cells and all CML cell lines tested, including cells harboring *BCR-ABL1*^{WT}, *BCR-ABL1*^{T315I} or *BCR-ABL1* compound mutations. In addition, palbociclib also induced synergistic growth-inhibitory effects on CML cells when combined with BCR-ABL1 TKI. These results may have clinical implications and point at a new concept in which drug combinations are applied to increase anti-CML effects and to reduce ponatinib doses to avoid side effects, at the same time. Finally, synergistic anti-neoplastic effects on CML cells were seen when HU was combined with ABL001, a new BCR-ABL1^{T315I}-targeting drug that binds the myristate-binding pocket domain of BCR-ABL1 [51]. This combination may be applied in patients failing or not tolerating ponatinib in future studies.

It has previously been described, that some drugs applied in advanced CML, including cytarabine, homoharringtonine, and IFNα, are able to suppress *BCR-ABL1*^{T315I}+ cells *in vivo* [48–50]. However, these drugs have not been investigated in detail in the context of *BCR-ABL1* mutant-expressing sub-clones so far. We examined whether these drugs would produce similar effects in CML clones harboring *BCR-ABL1*^{T315I} compared to HU. We here show that despite strong anti-proliferative effects of cytarabine and homoharringtonine alone or in combination with ponatinib in all *BCR-ABL1*+ cell lines tested, none of the 3 compounds exerted more potent anti-neoplastic effects in cells harboring *BCR-ABL1*^{T315I} than in cells lacking *BCR-ABL1*^{T315I}. Furthermore, none of these drugs led to a decrease in CDK4/CDK6 expression in CML cells. These data point at a unique effect of HU in TKI-resistant CML cells expressing *BCR-ABL1*^{T315I} and related mutations.

The observation that HU exerts particularly strong effects on CML sub-clones exhibiting *BCR-ABL1*^{T315I} and cooperates with ponatinib and other BCR-ABL1 TKI in suppressing growth of CML cells, even in the context of compound mutations, may have clinical implications. First, these results are in favor of using HU alone or in combination with other drugs in patients with multi-mutated CML exhibiting *BCR-ABL1*^{T315I} as palliative therapy or as preparation (bridging) for HSCT, as exemplified in this study. Second, HU may be useful to suppress the outgrowth of new sub-clones exhibiting *BCR-ABL1*^{T315I} or *BCR-ABL1*^{T315I}-containing compound mutations. Especially for TKI-resistant, non-transplantable patients who are at high risk of developing *BCR-ABL1*^{T315I}-expressing sub-clones (which is often fatal), addition of HU to TKI therapy as T315-prophylaxis´might be considered.

Together, we show that targeting of CDK4/CDK6 may be a potent approach to overcome TKI resistance in CML sub-clones exhibiting *BCR-ABL1*^{T315I}. We also show that HU, a ´palliative´antineoplastic drug, suppresses CDK4/CDK6 expression and exerts potent effects on Ph+ cells harboring *BCR-ABL1*^{T315I} or T315I-inclusive compound mutations. HU effects were seen *in vitro* as well as *in vivo* in patients with TKI-resistant CML. We also show that HU synergizes with ponatinib, ABL001 and palbociclib in inhibiting the growth of TKI-resistant and TKI-sensitive CML cells *in vitro*. Whether these drug combinations are effective in all patients with TKI-resistant (*BCR-ABL1*^{T315I}+) CML, remains to be determined in forthcoming studies.

Declaration of Competing Interest

G.H.: research funding from Novartis and honoraria from Novartis, BMS and Pfizer. W.R.S.: honoraria from Novartis, Celgene, Jazz, Pfizer, Abbvie, Daiichi Sankyo and Teva. T.L.: honoraria from Incyte, Pfizer, Angelini, Novartis, Amgen, and a research grant from Novartis. M.D.: Paid consultant for Novartis, Pfizer, Blueprint, Takeda; Research funding: SPARC, Gilead, BMS. K.G.: honoraria from Novartis, Ariad, Roche, BMS and Pfizer. P.V.: research funding and honoraria from Novartis and Incyte, and honoraria from BMS, Pfizer and Ariad. The other authors (M.S., K.B., G.S., D.B., C.B.L., S.H., S.P., N.W., G.G., K.O., P. P. and G.E.) have no conflicts of interest to disclose.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.11.004.

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