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MEK and PI3K-AKT inhibitors synergistically block activated IL7 receptor signaling in T-cell acute lymphoblastic leukemia

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We identified mutations in the *IL7Ra* gene or in genes encoding the downstream signaling molecules JAK1, JAK3, STAT5B, N-RAS, K-RAS, NF1, AKT and PTEN in 49% of patients with pediatric T-cell acute lymphoblastic leukemia (T-ALL). Strikingly, these mutations (except RAS/NF1) were mutually exclusive, suggesting that they each cause the aberrant activation of a common downstream target. Expressing these mutant signaling molecules—but not their wild-type counterparts—rendered Ba/F3 cells independent of IL3 by activating the RAS-MEK-ERK and PI3K-AKT pathways. Interestingly, cells expressing either IL7Ra or JAK mutants are sensitive to JAK inhibitors, but respond less robustly to inhibitors of the downstream RAS-MEK-ERK and PI3K-AKT-mTOR pathways, indicating that inhibiting only one downstream pathway is not sufficient. Here, we show that inhibiting both the MEK and PI3K-AKT pathways synergistically prevents the proliferation of BaF3 cells expressing mutant IL7Ra, JAK and RAS. Furthermore, combined inhibition of MEK and PI3K/AKT was cytotoxic to samples obtained from 6 out of 11 primary T-ALL patients, including 1 patient who had no mutations in the IL7R signaling pathway. Taken together, these results suggest that the potent cytotoxic effects of inhibiting both MEK and PI3K/AKT should be investigated further as a therapeutic option using leukemia xenograft models.

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INTRODUCTION

In the past two decades, T-cell acute lymphoblastic leukemia (T-ALL) has been investigated extensively at the genetic level, revealing several distinct T-ALL subtypes, each of which is characterized by specific oncogenic lesions.[1–5] Because these lesions are generally considered to be the driving oncogenic event, we call these aberrations type A mutations.[5,6] Type A mutations facilitate a differentiation arrest and are accompanied by type B mutations,[6,7] which can contribute to leukemogenesis by disrupting a plethora of cellular processes (including the cell cycle, epigenetic gene regulation and apoptosis), ultimately resulting in the ectopic activation of several signaling pathways, including the NOTCH1, JAK-STAT and PI3K-AKT pathways.[5,8–14]

Activating mutations in the *IL7Ra* gene, which encodes the interleukin-7 receptor alpha chain, have been identified in approximately 6% of pediatric ALL patients, with a slightly higher prevalence reported in pediatric T-ALL patients (9%).[15,16] The majority of mutations in *IL7Ra* introduce a cysteine residue in the juxta-membrane-transmembrane domain; this cysteine residue in the mutant protein facilitates the formation of intermolecular disulfide bonds, protein homodimerization and IL7-independent signaling.[15,16] The *IL7Ra* gene is one of many transcriptional targets of NOTCH1; specifically, NOTCH1 binds to the distal *IL7Ra* enhancer region.[17] Under normal conditions, signaling through the heterodimeric IL7 receptor (IL7Ra-common/γ-chain) is essential for the growth and survival of developing T cells.[18,19] IL7R activation leads to the recruitment, phosphorylation and activation of the Janus kinases JAK1 and JAK3, and to the activation of the STAT5 and PI3K-AKT pathways.[20] Ectopic expression of IL7 in mice is oncogenic and results in the development of gamma-delta T-cell lymphomas, which infiltrate the skin.[21,22] In mice, the development of IL7-induced T-cell lymphomas requires STAT5;[23] in contrast, in human T-cell leukemias, IL7-dependent survival and cell cycle progression require PI3K-AKT signaling.[24,25] Thus, in contrast to normal T cells, the role of IL7R-driven modulation of JAK-STAT signaling in human T-ALL remains to be dissected.

Mutations in the *JAK1* gene have been found in 4–27% of primary T-ALL patients,[13,14,26,27] as well as in acute myeloid leukemia, pre-B-ALL and solid tumors.[13,26–29] Mutant JAK1 molecules transform Ba/F3 pro-B cells and activate downstream AKT and ERK signaling.[13,14,29] Similar to the JAK2^{V617F} mutation in myeloid disorders,[30–33] mutant JAK1 molecules must interact with the IL7Ra chain to drive the ligand-independent activation of STAT molecules.[34,35] The *JAK3* gene can also be mutated in T-cell leukemias, as well as in acute megakaryoblastic leukemia;[14,36–38] the majority of *JAK3* mutations affect the protein's pseudokinase domain.[38] JAK3 normally binds to the common γ-chain in the IL7R[39] and requires JAK1 to transform Ba/F3 cells.[38] Mutations in other IL7R signaling molecules have been identified in T-ALL, including PTPN2,[40] N/K-RAS,[10] NF1,[8] PTEN, PI3K and AKT.[9,11,41,42]

Here, we investigated the prevalence of mutations in the *IL7Ra* gene and its downstream signaling molecules in a pediatric T-ALL cohort. After identifying several mutations, we examined their ability to transform Ba/F3 cells and their potential to activate downstream JAK-STAT, RAS-MEK-ERK and PI3K-AKT-mTOR pathways. To find improved treatment for T-ALL patients, we tested

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the cytotoxic therapeutic effects of inhibiting these pathways, and we investigated the added value of using combined inhibitor therapies. Our results show that blocking two major signaling pathways downstream of the IL7R is synergistic and may be beneficial for patients with IL7R signaling mutations.

MATERIALS AND METHODS

Patient samples

Written informed consent was obtained from the parents or legal guardians of each patient to use excess diagnostic material for research purposes. The study was performed in accordance with the Institutional Review Board of the Erasmus MC Rotterdam and in accordance with the Declaration of Helsinki. Leukemic cells were harvested from blood or bone marrow samples and were enriched to $\geqslant 90\%$ purity.

Mutation screen

We screened 146 patients for mutations in the FERM (4.1 protein, ezrin, radixin and moesin), pseudokinase and kinase domains in all four Janus kinase family members (encoded by the *JAK1*, *JAK2*, *JAK3* and *TYK2* genes), for *STAT5B*^{N642H}, and for the mutation hotspots in the *N-RAS* and *K-RAS* genes. In this cohort, mutations in *IL7Ra*, *NF1*, *PTEN* and *AKT* had been identified previously.[8,16,41,42] Detailed information can be found in the Supplementary Materials and Methods.

Ba/F3 transfectants

The Gateway multi-site recombination system (Life Technologies, Carlsbad, CA, USA) was used to simultaneously clone multiple DNA fragments into our Gateway-adapted pcDNA3.1 destination vector, which contains either an SV40-driven neomycin resistance cassette or an SV40-driven puromycin resistance cassette. Ba/F3 cells were transfected by electroporation, and bulk-transfected cells were enriched to $>95\%$ purity using the CD271 (that is, the low-affinity nerve growth factor receptor or LNGFR) MicroBead kit and magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany).

Doxycycline-inducible expression in Ba/F3 cells

We developed a doxycycline-inducible system using murine Ba/F3 cells (DSMZ, Braunschweig, Germany), a cell line that normally requires IL3 for survival and proliferation (Supplementary Figure S1). Cell line identity was confirmed by DNA fingerprinting and cells were regularly tested for mycoplasma contamination. IL3 was withdrawn from the medium at regular intervals to ensure that all selected Ba/F3 lines remained IL3-dependent. Each transfected line was exposed to doxycycline for 24 h, after which IL3-independent proliferation and activation of signaling molecules were measured. All growth curves ($\pm$ s.d., $n=3$) and IC_{50} determination of the inhibitors ($\pm$ s.d., $n=3$) have been performed in at least three independent experiments and are representative.

Cell survival assay

Patient cells were thawed and immediately cultured in duplicate in 384-well plates (10 000 cells/well) in RPMI medium containing 10% heat-inactivated fetal calf serum. Plated cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Inhibitors were added 2 h after plating the cells. Survival of patient cells was determined 72 h after addition of the inhibitor(s) by the intracellular ATP content as an indirect measure of the number of viable cells using ATPlite 1 Step solution (Perkin Elmer, Waltham, MA, USA). Detailed information can be found in the Supplementary Materials and Methods.

Inhibitors

The following inhibitors were obtained from Selleck Chem (Munich, Germany, unless otherwise indicated): JAK inh 1 (Merck Millipore, Billerica, MA, USA; #420099), ruxolitinib (#S1378), pimozide (Sigma-Aldrich, Zwijndrecht, The Netherlands; #P1793), Ly294002 (Cell Signaling Technology, Leiden, The Netherlands; #9901), MK-2206 (#S1078), rapamycin (#S1039), CI-1040 (Axon Medchem, Groningen, The Netherlands; #1368), AZD6244 (#S1008) and GDC-0941 (#S1065).

Antibodies

The following antibodies used for western blot analyses were obtained from Cell Signaling Technology (unless otherwise indicated): phospho-AKT^{S473} (#9271), phospho-ERK1/2 (#4370), phospho-JAK1 (#3331), phospho-JAK2 (#3771), phospho-MEK1/2 (#9154), phospho-mTOR (#2971), phospho-p70S6Kinase (#9204), phospho-STAT1 (#9167), phospho-STAT3 (#9145), phospho-STAT5 (#9351), phospho-TYK2 (#9321), DYKDDDDK (#2368), CD127 (anti-IL7Ra; R&D Systems, Minneapolis, MN, USA; #MAB306), RAS (Merck Millipore; #05-516) and β-actin (Sigma-Aldrich; #2547). The following antibodies used for flow cytometry were obtained from Miltenyi Biotec: CD127-FITC (#130-094-888) and CD271-APC (#130-091-884).

Statistics

Statistical analyses were performed using SPSS version 15.0. The Pearson's chi-square test was used to test for differences in normally distributed data. If the number of patients in the individual groups was fewer than five, the Fisher's exact test was used. Statistical significance for continuously distributed data was tested using the Mann–Whitney *U* test. Differences were considered to be significant at $P<0.05$ (two-sided).

RESULTS

IL7R signaling mutations in T-ALL are mutually exclusive

To measure the prevalence of mutations in IL7Ra—and/or its downstream signaling molecules—in pediatric T-ALL, we compared the mutations identified in *JAK* family kinase genes, *STAT5B* and *RAS* genes with mutations that we previously identified in the *IL7Ra*, *NF1*, *PTEN* and *AKT* genes[8,16,41,42] (Table 1 and Supplementary Figure S1a). We found no mutations in either *JAK2* or *TYK2*. In contrast, mutations were identified in the *JAK1* and *JAK3* genes of 10 patients; 2 of these patients had mutations in both *JAK1* and *JAK3*. The JAK1^{R724H}, JAK3^{M511I} and JAK3^{R657Q} mutations have been reported by others;[13,14,26,36,38] the remaining five *JAK1* mutations were identified by us as part of this study and recently modeled in the putative JAK1 protein structure.[43] Mutations in either *N-RAS* or *K-RAS* were identified in 15 patients, and inactivating deletions and mutations in *NF1* had been previously detected in three additional patients.[8] NF1 is a RAS-GTPase-activating protein that catalyzes the hydrolysis of active RAS-GTP into inactive RAS-GDP. Three patients with a mutation in *N-RAS*, *K-RAS* or *NF1* also had a *JAK1* and/or *JAK3* mutation, three patients had a mutation in *IL7Ra*, and four patients had a *PTEN*-inactivating event. Aside from *RAS*, the overlap of mutations in *IL7Ra, JAK*, *STAT5B*, *PTEN* and *AKT* is rare in T-ALL patients. Thus, in our cohort, mutations in *IL7Ra*, *JAK*, *STAT5B*, *PTEN* and *AKT* were mutually exclusive from each other, and occurred rarely with mutations in either *N-RAS* or *K-RAS*. We found that patients with the TLX subtype of T-ALL had a high prevalence of *IL7Ra* mutations ($P=0.001$), whereas patients with the proliferative or ETP-ALL subtype had no mutations in *IL7Ra* (Table 2). Only one *JAK* mutation was identified in the TALLMO patient group ($P=0.039$), and mutations in *RAS* or *NF1* were most prevalent among ETP-ALL[14] and TLX patients ($P=0.016$ and $P=0.044$, respectively; Table 2). Overall, activating mutations in the IL7Ra, JAK-STAT, RAS-MEK-ERK or PI3K-PTEN-AKT-mTOR pathways were identified in 49% of our pediatric T-ALL patients and were nearly mutually exclusive, suggesting that these mutations are functionally redundant in T-ALL.

Mutations in IL7R signaling molecules can transform cells

Next, we asked whether expressing the identified IL7R signaling mutations could transform Ba/F3 cells, rendering the cells IL3-independent. We generated doxycycline-inducible expression constructs for C-terminal DDK-tagged mutant JAK1, JAK3 and AKT, as well as non-tagged mutant IL7Ra and N-RAS proteins; for each mutant protein, a construct encoding the corresponding wild-type protein was also generated (Supplementary Figures S1b and S1c). Stably transfected bulk cell lines were sorted based on

Table 1. Mutations in the IL7R and its downstream activated molecules in 71 out of 146 pediatric T-ALL patients

Pt #	*Oncogene*	*Subtype*	*IL7R*	*JAK1*	*JAK3*	*STAT5B*	*N-RAS*	*K-RAS*	*NF1*	*PTEN*	*AKT*	*NOTCH/ FBXW7*
			Non-cysteine mutations									
1	MLL	nd	V253WN	—	—	—	—	—		—	—	—
2		ETP-ALL	V253GPSL	—	—	—	—	—		—	—	PEST
			Cysteine mutations									
3	TLX3	nd	IL241-242CLEG	—	—	—	—	—		—	—	HD/PEST
4	TLX3	TLX	PILLT240-244RFCPH	—	—	—	A146T	—		—	—	HD
5	TLX3	TLX	PILLTIS240-246LKC	—	—	—	—	A146T		—	—	—
6	TLX3	TLX	LLT242-244FHPFNCGP	—	—	—	G12D	—		—	—	HD
7	HOXA	TLX	L243RLECV	—	—	—	—	—		—	—	PEST
8	HOXA	nd	LL242-L243FPHQHC	—	—	—	—	—		—	—	FBXW7
9	SET-NUP214	TLX	LLTIS242-246PQGGC	—	—	—	—	—		—	—	HD/FBXW7
10		nd	PIL240-242QSPSC	—	—	—	—	—		—	—	HD
11		TLX	LT243-244LMCPT	—	—	—	—	—		—	—	JM
12		TALLMO	PILLTIS240-S246LQSC	—	—	—	—	—		—	—	—
13	TLX3	TLX	—	E668Q	—	—	nd	nd	Del/333dupA	—	—	HD/FBXW7
14	LYL1/LMO2	TALLMO	—	L624YPILKV	—	—	—	—		—	—	JM/PEST
15		Proliferative	—	V427M	—	—	—	—		—	—	HD/PEST
16	CALM-AF10	nd	—	P815S	—	—	—	—		—	—	HD/PEST
17	RUNX1-AFF3	ETP-ALL	—	T901G	M511I	—	—	—		—	—	PEST
18		ETP-ALL	—	R724H	M511I	—	G12D	—		—	—	PEST
19		ETP-ALL	—	—	M511I	—	nd	nd	Del/-	—	—	PEST
20	TLX3	TLX	—	—	M511I	—	—	—		—	—	JM
21	CALM-AF10	TLX	—	—	R657Q	—	—	—		—	—	HD/PEST
22	TLX3	TLX	—	—	R657Q	—	nd	nd		—	—	ND
23	TLX1	TLX	—	—	—	N642H	—	—		—	—	—
24	TLX1	TLX	—	—	—	N642H	—	—		nd	—	nd
25	LMO2	nd	—	—	—	N642H	—	—		—	—	PEST
26	SIL-TAL1	TALLMO	—	—	—	N642H	—	—		—	—	HD
27	TLX3	TLX	—	—	—	—	G12D	—		—	—	—
28	TLX3	TLX	—	—	—	—	G12V	—		—	—	HD
29	TLX3	nd	—	—	—	—	G12C	—		—	—	HD/FBXW7
30	TLX3	nd	—	—	—	—	G13V	—		—	—	HD/FBXW7
31	CALM-AF10	ETP-ALL	—	—	—	N642H	G12D	—		—	—	—
32	SIL-TAL1	nd	—	—	—	—	G13C	—		Mut/Del	—	—
33	NKX2-5	ETP-ALL	—	—	—	—	G13D	—		Mut/Mut/Subcl. Del	—	HD
34		TALLMO	—	—	—	—	G12R	—		—	—	FBXW7
35	TAL2	TALLMO	—	—	—	—	—	G12D		no protein	—	FBXW7
36	LMO2	TALLMO	—	—	—	—	—	G12D		—	—	—
37	TLX3	TLX	—	—	—	—	—	Q61R		—	—	HD/FBXW7
38	MEF2C	ETP-ALL	—	—	—	—	—	Q61L		—	—	HD/PEST
39	CALM-AF10	ETP-ALL	—	—	—	—	nd	nd	Del/3734delCinsGGTTTATGGTTT	—	—	—
40		nd	—	—	—	—	—	G12D		—	—	HD
41	TLX3	TLX	—	—	—	—	—	A146T		—	—	PEST
42	TLX3	TLX	—	—	—	—	—	A146T		—	—	HD
43	LMO2	TALLMO	—	—	—	—	—	A146T		—	—	HD/FBXW7
44	SIL-TAL1	TALLMO	—	—	—	—	—	—		Subcl. Del/—	—	HD/PEST
45	SIL-TAL1	TALLMO	—	—	—	—	—	—		Mut/Mut	—	—
46	SIL-TAL1	TALLMO	—	—	—	—	—	—		Del/—	—	HD
47	SIL-TAL1	TALLMO	—	—	—	—	—	—		Subcl. Del/—	—	—
48	SIL-TAL1	TALLMO	—	—	—	—	—	—		Subcl. Mut/—	—	—
49	SIL-TAL1	TALLMO	—	—	—	—	—	A146T		Del/Del	—	—
50	SIL-TAL1	TALLMO	—	—	—	—	—	—		Del/Subcl. Del	—	—
51	SIL-TAL1	TALLMO	—	—	—	—	—	—		Del/Subcl. Del	—	—

Table 1. (Continued)

Pt #	*Oncogene*	*Subtype*	*IL7R*	*JAK1*	*JAK3*	*STAT5B*	*N-RAS*	*K-RAS*	*NF1*	*PTEN*	*AKT*	*NOTCH/ FBXW7*
52	SIL-TAL1	TALLMO	—	—	—	—	—	—		Mut/Mut	—	—
53	LMO3	TALLMO	—	—	—	—	—	—		Mut/Mut	—	—
54	LMO2	TALLMO	—	—	—	—	—	—		Mut/Mut	—	FBXW7
55		TALLMO	—	—	—	—	—	—		Mut/Mut	—	HD
56		TALLMO	—	—	—	—	—	—		Mut/Mut	—	—
57		TALLMO	—	—	—	—	—	—		Subcl. Del/—	—	—
58		TALLMO	—	—	—	—	—	—		Mut/Subcl. Del	—	—
59		TALLMO	—	—	—	—	—	—		Mut/Subcl. Del	—	—
60	NKX2—1	Proliferative	—	—	—	—	—	—		Mut/—	—	HD/FBXW7
61	TAL1	Proliferative	—	—	—	—	—	—		no protein	—	HD
62	SIL-TAL1	Proliferative	—	—	—	—	—	—		Mut/Mut	—	—
63	SIL-TAL1	Proliferative	—	—	—	—	nd	—		Mut/Mut	—	PEST
64		ETP-ALL	—	—	—	—	—	nd		Del/—	—	PEST
65	MYC	nd	—	—	—	—	—	—		Mut/Del	—	—
66	LMO2	nd	—	—	—	—	—	—		Mut/Mut/Subcl. Del	—	PEST
67		nd	—	—	—	—	—	—		Del/—	—	—
68		nd	—	—	—	—	—	—		Mut/Del	—	—
69	TLX1	Proliferative	—	—	—	—	—	—		—	E17K	—
70	TLX3	Proliferative	—	—	—	—	—	—		—	E17K	FBXW7
71	LMO1	TALLMO	—	—	—	—	—	—		—	E17K	—

Abbreviations: nd, not done; T-ALL, T-cell acute lymphoblastic leukemia; —, tested negative. From a cohort of 146 screened pediatric T-ALL patients, 71 are listed with mutations in at least one of the following genes: *IL7Ra, JAK1, JAK3, STAT5B, N-RAS, K-RAS, NF1, PTEN* and *AKT*. The *NOTCH/FBXW7* mutational status is listed in the last column.

the constitutive expression of truncated NGFR (Supplementary Figure S1d); none of these bulk cell lines were able to proliferate in the absence of doxycycline and IL3 (data not shown). Treating the cells with doxycycline induced the expression of wild-type or mutant IL7Ra (Supplementary Figure S1d), DDK-tagged JAK (Supplementary Figure S1e), DDK-tagged AKT (Supplementary Figure S1e) and RAS (Supplementary Figure S1f). The doxycycline-induced expression of the mutant molecules IL7Ra^{RFCPH} (as a representative for all cysteine-containing IL7Ra mutants), JAK1^{R724H}, JAK1^{T901G}, JAK3^{M511I}, JAK3^{R657Q}, N-RAS^{G12D} and AKT^{E17K} transformed Ba/F3 cells, rendering them IL3-independent. In contrast, inducing the expression of the respective wild-type counterparts failed to transform any of the bulk cell lines (Figures 1a and b). Importantly, all bulk lines grew equally well in the presence of IL3 (Supplementary Figure S1g), indicating that the observed differences in growth rates in the absence of IL3 reflect differences in the transforming potential of the mutant molecules.

Interestingly, the cysteine-containing mutant IL7Ra^{RFCPH} was able to form homodimers (Supplementary Figure S2), which is required for interleukin-independent signaling and transformation.[15,16] In contrast, the non-cysteine-containing IL7Ra^{V253GPSL} mutant did not transform Ba/F3 cells (Figure 1a), nor did it form homodimers (Supplementary Figure S2). The cell lines expressing either wild-type IL7Ra or the IL7Ra^{GPSL} mutant grew in the presence of IL7 (data not shown), suggesting that IL7Ra^{GPSL} forms a signaling-competent receptor in the presence of IL7. The AKT^{E17K} cell line proliferated at a slower rate than the mutant JAK and IL7Ra lines. Lastly, although expressing N-RAS^{G12D} did not provide an immediate proliferation advantage, within 7 days of doxycycline induction, these cells reached a rate of proliferation that was on par with the mutant JAK and IL7Ra cell lines (Figure 1b). The activating STAT5B^{N642H} mutation transforms Ba/F3 cells[44] but was not functionally investigated as part of this study.

Mutations in the IL7R pathway induce ligand-independent downstream signaling

Next, we investigated the ability of the mutant and wild-type molecules to activate downstream IL7R signaling (Figures 1c-e). First, we confirmed that the STAT5, RAS-MEK-ERK and AKT-mTOR pathways were activated in all bulk cell lines upon the addition of IL3 following overnight IL3 starvation. Upon doxycycline induction, each cell line expressed its wild-type or mutant protein within 2–4 h (Figures 1c-e, Supplementary Figure S3). In the presence of IL7, the cell lines expressing wild-type IL7Ra, IL7Ra^{GPSL} and IL7Ra^{RFCPH} activated downstream signaling pathways; in the absence of IL7, only the cysteine mutant IL7Ra^{RFCPH} activated downstream signaling. In addition to activating the JAK-STAT and PI3K-AKT-mTOR pathways,[16,45] IL7Ra^{RFCPH} can also activate MEK-ERK signaling (Supplementary Figure S3). Expressing the JAK1^{R724H}, JAK1^{T901G}, JAK3^{M511I} or JAK3^{R657Q} mutants—but not wild-type JAK1 or JAK3—activated the MEK-ERK and PI3K-AKT-mTOR pathways, as well as the downstream kinase S6K (Figures 1c-e and Supplementary Figure S3). N-RAS^{G12D} robustly activated downstream MEK-ERK signaling, as well as AKT-mTOR and the downstream target S6K (Figure 1e). Interestingly, inducing the expression of wild-type N-RAS also activated the same downstream molecules, albeit with a slower time course than the N-RAS^{G12D} mutant (Supplementary Figure S3), even though wild-type N-RAS did not transform Ba/F3 cells (Figure 1b). Similar effects were observed with respect to the AKT^{E17K} mutant and its wild-type counterpart: induction of both proteins led to their self-activation and the downstream activation of mTOR and S6K (Supplementary Figure S3), whereas only the AKT^{E17K} line was able to transform Ba/F3 cells (Figure 1b).

Table 2. Co-occurrence of *IL7Ra*, *JAK1 and/or JAK3,* and *N-RAS, K-RAS and/or NF1* mutations with unsupervised gene expression profile clusters or T-ALL subtypes

T-ALL subtype	*IL7Ra cysteine mutations* (n = *7*)		*JAK1 and/or JAK3 mutations* (n = *9*)		*N-RAS, K-RAS and/or NF1 mutations* (n = *20*)	
		P-*value*		P-*value*		P-*value*
ETP-ALL ($n = 15$)	0 (0%)	0.59	3 (20.0%)	0.09	**6 out of 13 (46.2%)**	**0.016**
TLX ($n = 30$)	**6 (20.0%)**	**0.001**	4 (13.3%)	0.23	**9 out of 27 (33.3%)**	**0.044**
Proliferative ($n = 19$)	0 (0%)	0.60	1 (5.3%)	1	**0 out of 17 (0%)**	**0.038**
TALLMO ($n = 53$)	1 (1.9%)	0.13	**1 (1.9%)**	**0.039**	**5 out of 48 (10.4%)**	**0.047**
Total ($n = 117$)	7 (6.0%)		9 (7.7%)		20 (17.1%)	

Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia. The bold is used for significant values. Note: we reported previously that *PTEN* and *AKT* mutations predominantly fall in the TALLMO subgroup.[41,42]

A close examination of signaling strength revealed that the JAK1 mutant molecules (that is, $JAK1^{R724H}$ and $JAK1^{T901G}$) activated downstream signaling more robustly than the $IL7Ra^{RFCPH}$, $JAK3^{M511I}$ and $JAK3^{R657Q}$ mutants (Figure 1e). Indeed, the JAK3 mutants only weakly activated downstream signaling (Supplementary Figure S3), even though the transforming efficiency of the JAK3 mutants was similar to—if not higher than—the JAK1 mutants (Figure 1a). Thus, the signaling strength of these molecules does not appear to be correlated with their transforming potential.

Pharmacological inhibition of the IL7R pathway

Next, we tested a variety of pharmacological inhibitors for their ability to block signaling and cell proliferation, as well as their ability to induce cell death in cell lines expressing mutant signaling molecules (Figures 2 and 3). Importantly, in the absence of doxycycline (but in the presence of IL3), all cell lines were equally responsive to the inhibitors. Upon addition of doxycycline (and in the absence of IL3), the mutant IL7Ra, JAK1 and JAK3 lines became sensitive to the selective JAK1/2 inhibitor ruxolitinib; in contrast—and as expected—inducing the expression of the mutant N-RAS and AKT molecules induced ruxolitinib resistance (Figure 2a). Consistent with these results, ruxolitinib blocked the activation of STAT5, MEK, ERK, AKT and mTOR in the $IL7Ra^{RFCPH}$ and $JAK1^{T901G}$ mutant lines, but not in the $N\text{-}RAS^{G12D}$ or AKT^{E17K} mutant lines (Supplementary Figure S4).

Inhibiting STAT5 with pimozide had no effect on any of the mutant lines, with the exception of a moderate effect on the JAK1 mutant lines (data not shown); thus, signaling molecules other than STAT5 are important for maintaining cell viability and proliferation. Although the $N\text{-}RAS^{G12D}$ mutant line was resistant to the PI3K inhibitor Ly294002 and the AKT inhibitor MK-2206 (Figures 2c and d), this line was sensitive to the RAS inhibitor tipifarnib (data not shown) and the MEK inhibitor CI-1040 (Figure 2b). In this cell line, tipifarnib reduced the levels of phosphorylated MEK and phosphorylated ERK, whereas CI-1040 increased the levels of phosphorylated MEK but decreased the levels of phosphorylated ERK (Supplementary Figure S4). Both JAK mutant lines were also sensitive (to varying degrees) to the MEK inhibitor CI-1040, whereas the $IL7Ra^{RFCPH}$ and AKT^{E17K} lines were completely resistant to CI-1040 (Figure 2b). Moreover, inhibiting MEK increased AKT phosphorylation in the $IL7Ra^{RFCPH}$ and $JAK1^{T901G}$ lines (Supplementary Figure S4); this effect is likely a cellular escape mechanism used to activate an alternative survival pathway. The JAK1 and JAK3 mutant lines were also sensitive to inhibitors of PI3K and AKT (Ly294002 and MK-2206, respectively); in contrast, the $IL7Ra^{RFCPH}$ line was completely resistant to these inhibitors (Figures 2c and d). Because most JAK mutants are very sensitive to PI3K inhibition and respond to a lesser degree to MEK inhibition, JAK mutants may preferentially signal via PI3K-AKT. As expected, the AKT^{E17K} line was highly sensitive to both Ly294002 and MK-2206 (Figures 2c and d, Supplementary Figure S4). Finally, the $N\text{-}RAS^{G12D}$ line retained S6K activity in the presence of PI3K, AKT and mTOR inhibitors (Supplementary Figure S4), providing further evidence that S6K is a common target downstream of PI3K-AKT-mTOR and RAS-MEK-ERK pathways and can be used as a measure of activity for both pathways (Figure 3a).

As shown above, the IL7Ra and JAK mutant proteins activated both the RAS-MEK-ERK and the PI3K-AKT-mTOR pathways; moreover, inhibiting MEK led to the activation of AKT. Therefore, we tested the effect of treating cells with various combinations of MEK, PI3K and AKT inhibitors, using phosphorylated S6K levels as a measure of signaling activity (Figure 3b). Individually, none of these inhibitors completely silenced downstream signaling in either the $IL7Ra^{RFCPH}$ or the $JAK1^{T901G}$ cell line. In contrast, combining CI-1040 with Ly294002 or MK-2206 completely blocked the activation of ERK, AKT, mTOR and S6K (Figure 3b). Thus, we hypothesized that these combinations of inhibitors may exert synergistic cytotoxic effects in cells carrying mutations in IL7 signaling molecules.

Synergistic inhibition using combinations of MEK and PI3K/AKT inhibitors

To test the hypothesis that applying combinations of inhibitors has a synergistic effect on cytotoxicity, we first exposed each mutant cell line to serial dilutions of the MEK inhibitor AZD6244, the PI3K inhibitor GDC-0941 and the AKT inhibitor MK-2206, each of which is used clinically. After we obtained IC_{50} values for each inhibitor, cytotoxicity was then measured using serial dilutions of inhibitor combinations that were prepared at three different fixed ratios (1:1, 4:1 and 1:4); for an example of this approach, see Supplementary Figure S5. Synergy was then tested by calculating the combination index for each inhibitor combination's dose–response curve relative to the respective single inhibitors' dose–response curves. For each mutant line (with the exception of AKT^{E17K}), the MEK+PI3K and/or MEK+AKT inhibitor combinations were synergistic (Table 3). Interestingly, these two combinations had a synergistic effect in the $N\text{-}RAS^{G12D}$ line, in which AKT is activated (Figure 1e, Supplementary Figure S3).

Lastly, to investigate the potential clinical relevance of these findings, we tested the synergistic effects of MEK+PI3K and MEK +AKT inhibitor combinations using primary leukemic cells obtained from 11 T-ALL patients. Specifically, we tested the efficacy of the MEK inhibitors AZD6244 and trametinib, the PI3K inhibitor GDC-0941, the PI3K/mTOR inhibitor GDC-0980 and the AKT inhibitor MK-2206, as well as various combinations of these inhibitors (Table 4). Six of the 11 patient samples had a measurable synergistic response to the inhibitor combinations tested. Five cases were relatively resistant, because the IC50 of one or both of the inhibitors could not be determined and/or the maximum efficacy was lower than is necessary to determine the effective dose for 50 or 75% of responding cells. Therefore, no synergy could be determined in these five cases. Interestingly, one (patient #3821) of the five samples in which we could not measure

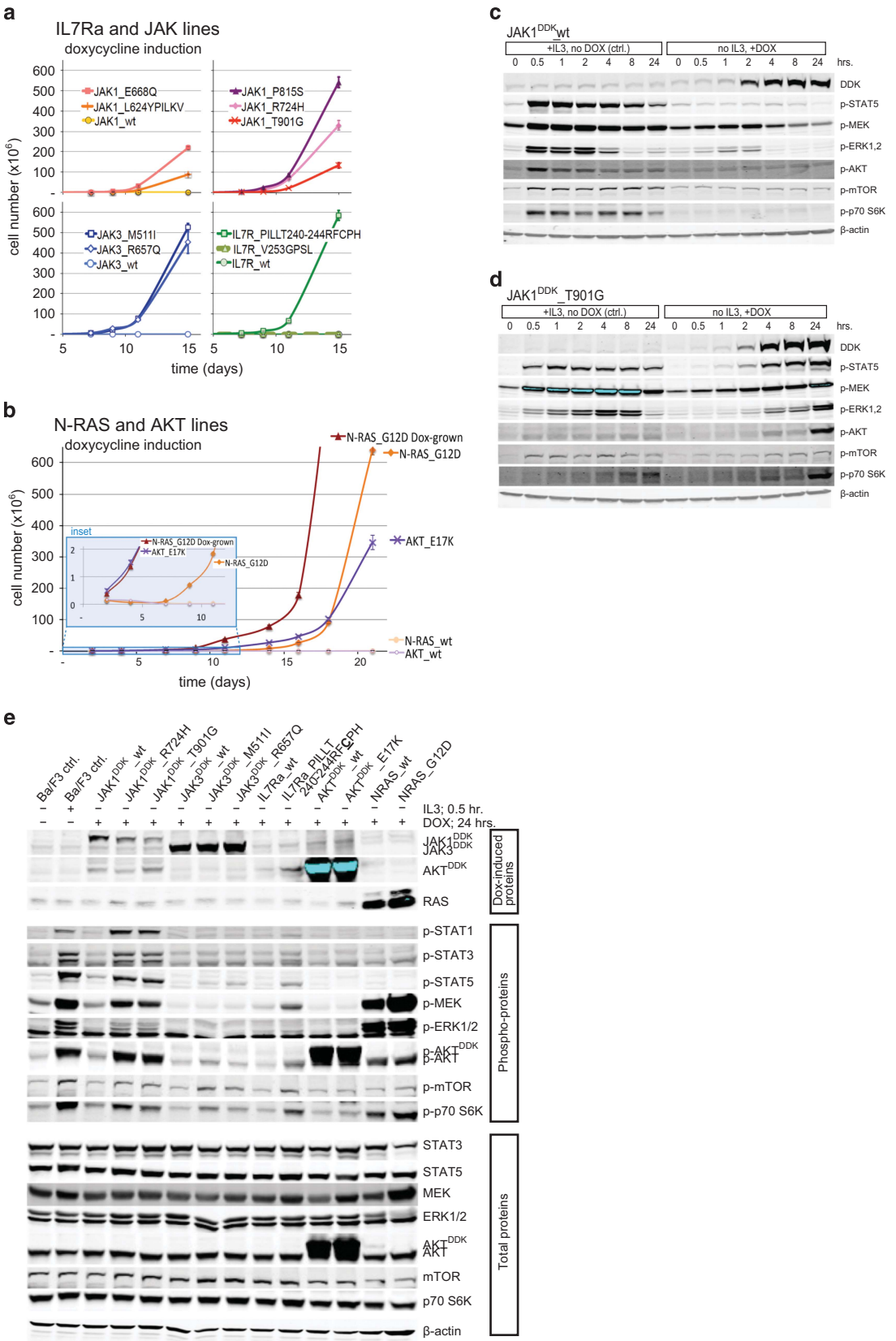

Figure 1. Transforming potential of activating mutations in IL7R signaling molecules in Ba/F3 cells. (**a**, **b**) Growth curves of Ba/F3 cell lines expressing the indicated IL7Ra, JAK1, JAK3, N-RAS and AKT constructs ($n = 3$ experiments per group) following induction with doxycycline. N-RAS^{G12D} 'Dox-grown' represent the growth curve of N-RAS^{G12D} mutant Ba/F3 cells after full adaptation to the mutant molecule. Each culture was started with 2×10^5 cells on day 0 after extensive washing to remove IL3. The inset in panel **b** shows an expanded view of the first 12 days of culture. (**c**–**e**) Western blot analysis of DDK-tagged and phosphorylated/total proteins from Ba/F3 cells treated in the presence or absence of IL3 or doxycycline (DOX). β-Actin was used as a loading control.

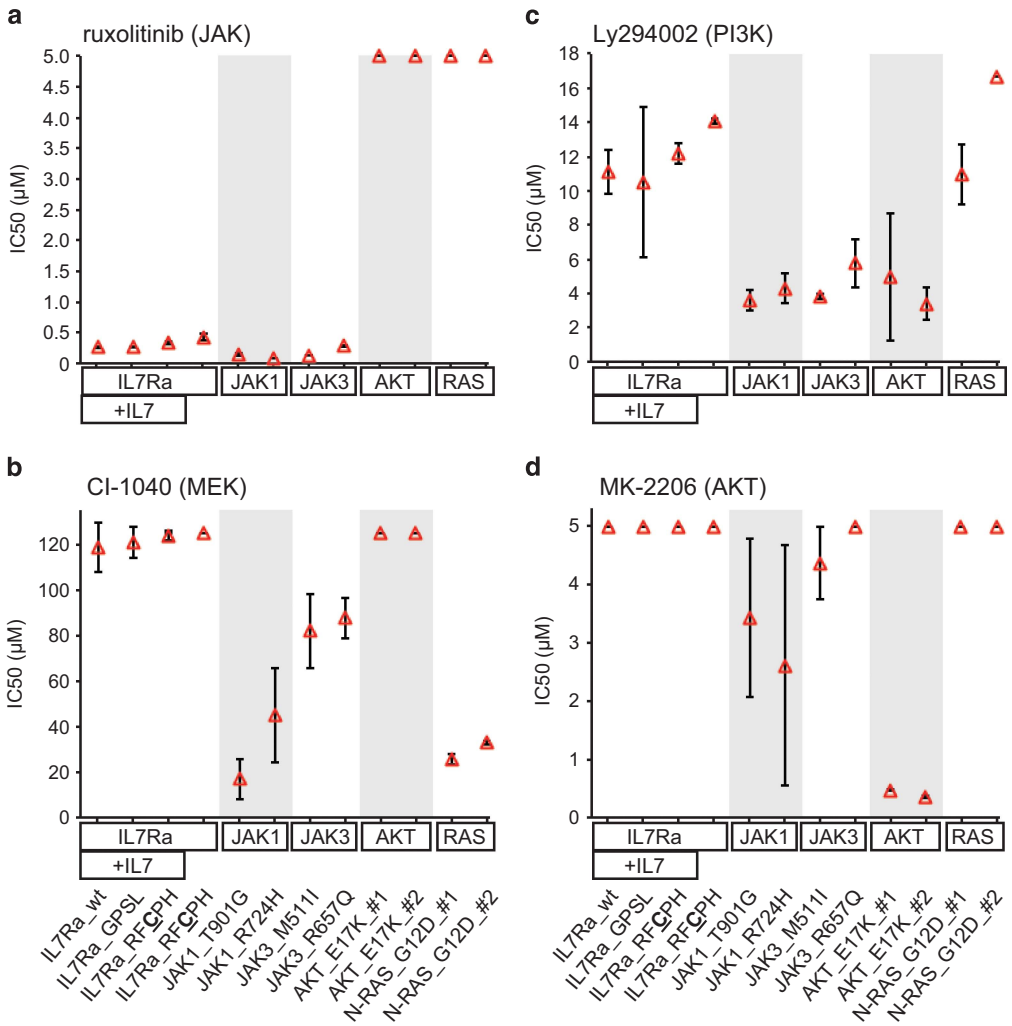

Figure 2. Summary of the effects of various inhibitors of IL7R signaling molecules in Ba/F3 cell lines expressing wild-type or mutant IL7Ra, JAK, AKT and RAS. In each plot, the mean ± s.d. IC_{50} values are shown for each Ba/F3 line ($n = 3$) after doxycycline induction in the absence of IL3. For mutant AKT and N-RAS, two independent lines were tested. The maximum concentrations used were 5 μM for ruxolitinib (**a**), 125 μM for CI-1040 (**b**), 16.7 μM for Ly294002 (**c**) and 5 μM for MK-2206 (**d**). Cell lines that showed no effect at the maximum inhibitor concentration were considered to be completely resistant to that inhibitor, and thus no IC_{50} was obtained.

a synergistic response had no mutations in *NOTCH1*, *IL7Ra*, *JAK1*, *JAK3* or *RAS* and was resistant to all inhibitors tested, suggesting that the survival and proliferation of these leukemic cells do not require these signaling pathways.

DISCUSSION

Here, we report that activating mutations in the signaling molecules IL7Ra, JAK1/3,STAT5B, PTEN and AKT were mutually exclusive in a cohort of 146T-ALL patients, indicating that these mutations have shared mechanisms for cell survival and/or proliferation. Ten out of 24 N-RAS/K-RAS/NF1 mutations occurred in combination with other IL7R pathway mutations. In Down Syndrome ALL, *RAS* and *JAK2* mutations are mutually exclusive.[46] Some of our mutations—for example, mutations in the *IL7Ra*, *JAK* and *RAS* genes—were more prevalent in patients with the TLX and ETP-ALL subtypes of T-ALL. These cases frequently also carry mutations in *NOTCH1* and/or *FBXW7*; in particular, most patients with the TLX subtype carry strongly activating mutations in *NOTCH1*.[47] A similar association with *Notch1* mutations was also reported in mouse models of T-ALL induced by mutations in K-Ras and RasGRP1.[48–50]

The majority of mutations in the *IL7Ra* gene introduce a cysteine residue, which facilitates receptor homodimerization and IL7-independent signaling.[15,16] Although the cysteine mutant $IL7Ra^{RFCPH}$ confers IL7-independent growth and signaling, the function of non-cysteine mutations (for example, $IL7Ra^{GPSL}$) is not currently understood. We found that the non-cysteine $IL7Ra^{GPSL}$ mutant supports the growth of Ba/F3 cells better than the wild-type IL7Ra; thus, non-cysteine IL7Ra mutations may promote leukemogenesis by increasing the IL7 response.

Activating mutations in the IL7R signaling pathway can act at different levels and to varying degrees. For example, the JAK3 mutant Ba/F3 lines grew robustly in the absence of IL3, but their potential to activate downstream signaling molecules was relatively weak compared with the JAK1 and IL7Ra mutants. The strongest level of activation was conferred by the *JAK1* mutations, conferring even stronger activation than the $IL7Ra^{RFCPH}$ mutant. These differences in activation strength may be due to different properties of the mutant molecules. JAK3 mutant Ba/F3 cells

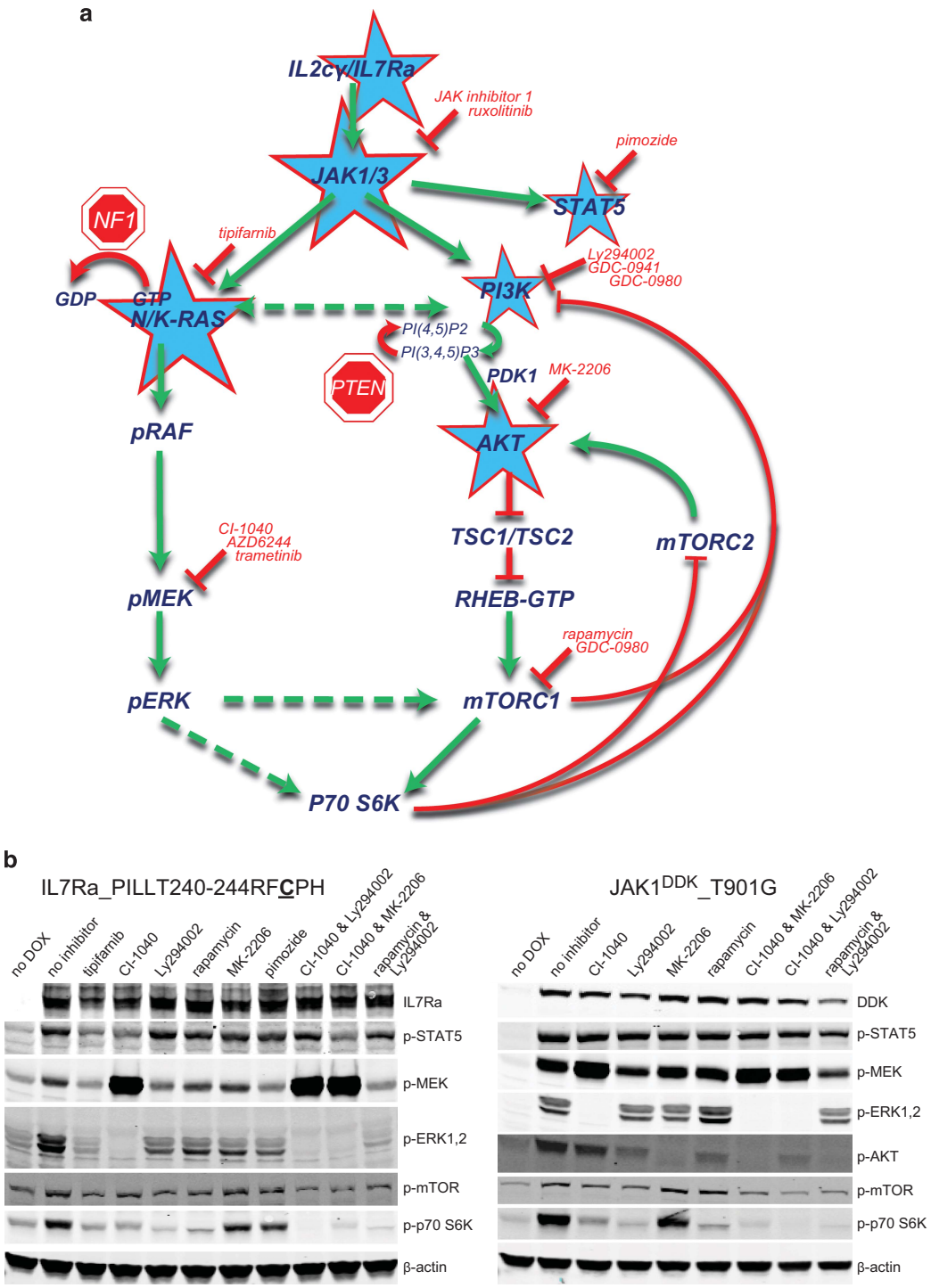

Figure 3. The effect of using combinations of inhibitors on downstream signaling in cell lines expressing mutant IL7Ra and JAK molecules. (**a**) Schematic representation of the IL7R signaling pathways. The molecules with activating and inactivating mutations found in T-ALL patients are indicated with stars and 'stop' signs, respectively. The inhibitors used in this study are listed in red. The green arrows indicate activation, the dashed arrows indicate putative activation, the red arrows mediate inactivating processes, and the red lines indicate inhibition. (**b**) Western blot analysis of phosphorylated proteins in the indicated doxycycline-treated Ba/F3 lines in the presence of the indicated inhibitors (24 h). The following concentrations were used: tipifarnib, 10 μM; CI-1040, 10 μM; Ly294002, 10 μM; MK-2206, 2 μM; rapamycin 1 μM. β-Actin was used as a loading control.

signal to STAT5 and ERK, but this signaling was generally weaker than that in the JAK1 mutant.[38] Furthermore, *IL7Ra* mutations that facilitate the formation of IL7Ra homodimers primarily recruit JAK1 molecules rather than JAK3 molecules, activate JAK1 but not JAK3 and also require JAK1 (but not JAK3) to activate STAT5.[16,51] Conversely, mutant JAK3 molecules require a functional cytokine receptor complex, likely via binding to the common γ-chain;[39,52] however, they also require JAK1 for ligand-independent signaling.[38] This may explain why two of our patients with *JAK3* mutations also have a *JAK1* mutation. Weak signaling by JAK3

Table 3. MEK and PI3K/AKT pathway inhibitors display synergy in most Ba/F3 mutant lines

Ba/F3 line	*Inhibitor 1*		*Inhibitor 2*		*Inhibitor 1+2*	
	MEK1/2 inhibitor	*IC50 (μM)*	*PI3K or AKT inhibitor*	*IC50 (μM)*	*CI at ED50 (mean ± s.d.)*	*Synergy*
IL7Ra_RFCPH	AZD6244	20.9	GDC-0941	9.2	0.33 ± 0.15	+
	AZD6244	20.1	MK-2206	4.4	0.68 ± 0.12	+
JAK1_R724Q	AZD6244	24.2	GDC-0941	3.9	0.28 ± 0.08	+ +
	AZD6244	20.6	MK-2206	7	0.57 ± 0.18	+
JAK1_P815S	AZD6244	65.6	GDC-0941	1.6	0.56 ± 0.08	+
	AZD6244	41.8	MK-2206	1.9	0.46 ± 0.06	+
JAK1_T901G	AZD6244	4.1	GDC-0941	1.4	0.79 ± 0.06	+
	AZD6244	3	MK-2206	1.1	1.32 ± 0.79	−
JAK3_M511I	AZD6244	17.8	GDC-0941	1.7	0.45 ± 0.15	+
	AZD6244	12.2	MK-2206	3.2	0.46 ± 0.17	+
JAK3_R657Q	AZD6244	22.9	GDC-0941	4	0.27 ± 0.02	+ +
	AZD6244	20.1	MK-2206	7.9	0.56 ± 0.08	+
N-RAS_G12D	AZD6244	2.7	GDC-0941	1.1	0.70 ± 0.05	+
	AZD6244	2.2	MK-2206	1.3	0.68 ± 0.08	+
AKT_E17K	AZD6244	ND (< 20%)	GDC-0941	0.4	0.71 ± 0.17	+
	AZD6244	ND (< 20%)	MK-2206	0.05	0.80 ± 0.58	−

IC50 of each inhibitor in μM, with ~100% efficacy (except where indicated) ND (< 20%): IC50 could not be determined and the inhibitor had low maximum efficacy (< 20%) CI: mean Combination Index of the 1:1, 1:4 and 4:1 combinations at the 50% effective dose (ED50) −: CI > 1.0 (no synergy); +: CI < 1.0 (synergy); ++: CI < 0.3 (strong synergy).

Table 4. MEK and PI3K/AKT pathway inhibitors display synergy in approximately half of the tested primary T-ALL samples

Mutations AND ID T-ALL patients	*Inhibitor 1*		*Inhibitor 2*		*Inhibitor 1+2*		*Synergy*
	MEK1/2 inhibitor	*IC50 (nM) (% efficacy)*	*PI3K, mTOR, or AKT inhibitor*	*IC50 (nM) (% efficacy)*	*CI at ED50 (mean ± s.d.)*	*CI at ED75 (mean ± s.d.)*	
TLX3^{tr}, mutations in IL7Ra, NRAS, NOTCH1 (HD domain), WT1, BCL11B, CDH9, STIL							
6	AZD6244	79 (73%)	GDC-0941	346 (63%)	0.39 ± 0.02	N/A	+
	trametinib	2 (80%)	GDC-0980	210 (64%)	0.79 ± 0.18	0.36 ± 0.17	+
HOXA (Inv(7)), mutations in IL7Ra, NOTCH1 (PEST domain), WT1, ZNF717							
7	AZD6244	214 (51%)	GDC-0941	848 (87%)	0.29 ± 0.15	0.11 ± 0.05	+ +
	trametinib	132 (79%)	GDC-0980	156 (86%)	0.76 ± 0.06	0.47 ± 017	+
TLX3^{tr}, TCRyd^{+}, mutation in WT1, no mutations in NOTCH1, IL7Ra, JAK1, JAK3, N/K-RAS							
3976	AZD6244	143 (56%)	GDC-0941	540 (62%)	0.33 ± 0.01	0.39	+
	AZD6244	143 (56%)	MK-2206	2594 (73%)	0.49 ± 0.23	0.61 ± 0.18	+
3543	AZD6244	ND (< 20%)	GDC-0941	925 (84%)	0.79 ± 0.07	0.81 ± 0.09	+
	trametinib	ND (< 20%)	GDC-0980	322 (93%)	0.82 ± 0.09	0.79 ± 0.07	+
9175	AZD6244	101 (44%)	GDC-0941	351 (85%)	0.65 ± 0.02	0.39 ± 0.07	+
	AZD6244	101 (44%)	MK-2206	105 (68%)	0.49 ± 0.08	N/A	+
9791	AZD6244	95 (31%)	GDC-0941	195 (87%)	0.52 ± 0.23	0.28 ± 0.09	+
	AZD6244	64 (34%)	MK-2206	72 (60%)	0.54 ± 0.10	N/A	+
	trametinib	53 (64%)	GDC-0980	54 (78%)	0.34 ± 0.08	0.07 ± 0.04	+ +
HOXA, mutations in IL7Ra, NOTCH1 (JM domain), FREM2, RUNX1							
11	AZD6244	32 (22%)	GDC-0941	164 (< 20%)	N/A	N/A	N/A
	trametinib	175 (45%)	GDC-0980	404 (52%)	N/A	N/A	N/A
LMO3-TCRB^{tr}, mutations in PTEN, BCL11B, no mutations in NOTCH1, IL7Ra, JAK1, JAK3, N/K-RAS							
53	AZD6244	ND (< 20%)	GDC-0941	473 (60%)	N/A	N/A	N/A
	AZD6244	ND (< 20%)	MK-2206	125 (49%)	N/A	N/A	N/A
TCRyd^{+}, no mutations in NOTCH1, IL7Ra, JAK1, JAK3, N/K-RAS							
3821	AZD6244	ND (< 20%)	GDC-0941	2005 (20%)	N/A	N/A	N/A
	AZD6244	ND (< 20%)	MK-2206	ND (33%)	N/A	N/A	N/A
7267	AZD6244	ND (< 20%)	GDC-0941	278 (75%)	0.87	N/A	−
	AZD6244	ND (< 20%)	MK-2206	72 (51%)	1.17	N/A	−
10880	AZD6244	ND (< 20%)	GDC-0941	537 (56%)	1.04 ± 0.08	N/A	−
	trametinib	ND (66%)	GDC-0980	226 (65%)	1.09 ± 0.32	0.29 ± 0.08	−/+

Note that patients # 6, 7, 11 and 46 correspond to those in Table 1. IC50 of each inhibitor in nM, with % efficacy indicated ND (< 20%): IC50 could not be determined and the inhibitor had low maximum efficacy (< 20%). CI: Mean Combination Index of the 1:1, 1:4 and 4:1 combinations at the 50% and 75% effective doses (ED50 and ED75) −: CI > 1.0 (no synergy); +: CI < 1.0 (synergy); ++: CI < 0.3 (strong synergy); N/A, not applicable.

mutants cannot be explained by low endogenous expression of the IL7Ra/IL2cγ heterodimeric receptor in Ba/F3 cells, as these *JAK3* mutations also result in weaker downstream signaling compared with *JAK1* and *IL7Ra* mutations when measured in SUPT1 and P12 Ichikawa T-ALL cell lines (both of which express IL7Ra/IL2cγ receptors; data not shown).

Mutations in the IL7R signaling pathway may provide a therapeutic window of opportunity. We used the Ba/F3 model system to measure cellular responses to a variety of signaling inhibitors in the context of specific individual mutations. Strikingly, the IL7Ra, JAK and RAS mutant lines had different responses to MEK, PI3K and AKT inhibitors. For example, JAK mutants seem to be more sensitive to PI3K inhibition and less to MEK inhibition, suggesting JAK mutants preferentially signal through PI3K-AKT. Moreover, inhibiting MEK increased the activation of AKT in some cell lines, possibly because of a cellular escape mechanism. Combining a MEK inhibitor with either a PI3K inhibitor or an AKT inhibitor robustly blocked downstream signaling and had a synergistic cytotoxic effect in nearly all Ba/F3 lines tested. This synergy underscores the importance of both MEK-ERK and PI3K-AKT-mTOR downstream pathways, as well as the need for combined inhibition of these pathways. Interestingly, none of the lines responded to the STAT5 inhibitor pimozide, suggesting that activation of STAT5 may not be a common survival pathway downstream of mutant IL7Ra or JAK molecules. Ba/F3 cells are transformed by the activating $STAT5B^{N642H}$ mutation,[44] but this mutation was not functionally investigated as part of this study; nonetheless, it would be interesting to assess the sensitivity of $STAT5B^{N642H}$ mutant cells to the inhibitors tested here.

Combined therapy using MEK and PI3K inhibitors has been suggested as a viable treatment option for several solid tumors.[53–56] With respect to acute myeloid leukemia, MEK and AKT inhibitors have been combined in a current phase II study (trial NCT01907815). This combination of inhibitors may prevent the cross-activation of one pathway upon inhibition of the other,[57,58] for example, as suggested for the activation of AKT by RAS (this study) and for the activation of ERK by the PI3K-dependent feedback loop involving mTORC1.[59] More than half of the primary T-ALL patient samples that we tested had a synergistic response to inhibitors of the RAS-MEK and PI3K-AKT pathways, suggesting that these patients will benefit from compounds that inhibit downstream IL7R signaling. Historically, our laboratory and others have correlated *in vitro* cytotoxicity to *in vivo* response for many chemotherapeutics and many patients, so it seems that the *in vitro* data are a good indicator of *in vivo* response,[60–64] especially in T-ALL.[65] Patient cells only survive in culture for several days, but do not proliferate. The *in vitro* cytotoxicity assay measures cell survival over the course of 3 days in the absence of cytokines and stromal cell support. This assay was chosen because cytokines and stromal support conditions may induce cell growth, which would lead to results in which the potential effects of inhibitors on cell survival versus cell growth could not be separated. The efficacy of inhibitors should not reflect the efficacy of cell cycle inhibition but should reflect the potential to kill leukemia cells. Moreover, cytokine addition or stromal support does not consistently support growth of all patient samples, adding an extra variable in the results. Interestingly, one primary T-ALL patient (case #3976) lacks mutations in IL7R signaling, but leukemic cells from this patient had a synergistic response to MEK +PI3K and MEK+AKT inhibitor combinations, suggesting the presence of additional mutations and/or oncogenic mechanisms that apparently depend on the MEK-ERK and PI3K-AKT-mTOR signaling pathways. This finding also underscores the importance of performing *in vitro* inhibitor testing in addition to screening for mutations; this dual diagnostic strategy can be used to stratify patients in specific treatment groups.

Finally, our results may also be relevant to precursor B-ALL patients with mutations in IL7R signaling molecules.[14,15] Furthermore, the incidence of RAS mutations is higher in relapsed patients with ALL, and this is likely due to selection of RAS mutant subclones during therapy.[66]

In conclusion, we report that combining MEK inhibitors with PI3K or AKT inhibitors has synergistic cytotoxic effects in leukemic cells carrying mutations at various levels in the IL7R signaling pathway. In addition, this combination of inhibitors may also be toxic to cells without apparent IL7R pathway mutations. Therefore, the cytotoxic effects of combining MEK inhibitors with PI3K and/or AKT inhibitors warrant further study of *in vitro* and *in vivo* models using leukemic cells from primary and relapsed ALL patients.

CONFLICT OF INTEREST

GJRZ and RCB are founders and shareholders of The Netherlands Translational Research Center B.V. The remaining authors declare no conflict of interests.

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AUTHOR CONTRIBUTIONS

KC-B, JAPS-H, JGCAMB-G, JCMU, WKS, JvdZ, RCB and GJRZ performed the experiments and/or analyzed the data; KC-B and JPPM wrote the manuscript; RP and JPPM designed and supervised the study.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)