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EPICYCLE: A confirmatory preclinical study of the anti-rhabdomyosarcoma efficacy of BET bromodomain and cyclin-dependent kinase 9 inhibitors

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ABSTRACT

Hypothesis-driven academic research identifies interventions with likely disease-specific effects. Yet, many candidate drugs fail upon further development, emphasizing the need for acquisition of more robust preclinical data. We demonstrate that planning and executing multicentre confirmatory preclinical studies in an academic setting by applying the quality standards of early phase clinical trials is feasible. Randomization, blinding, stratification by sex of and quality control measures were carried out successfully. The primary objective of our specific study – to confirm synergistic effects of BET bromodomain protein 4 (BRD4) and cyclin-dependent kinase 9 (CDK9) inhibitors against PAX3::FOXO1 (P3F)-positive rhabdomyosarcoma (RMS) – was not met. Post-hoc analyses support that single-agent BRD4 inhibition by JQ1 effectively reduced the growth and viability of P3F+ RMS cells *ex vivo* with adequate on-target activity as evidenced by reduced expression of P3F, MYCN, MYOG, and MYOD. The antiproliferative effects of JQ1 and vincristine were comparable, and there was trend towards reduced and delayed xenograft growth in JQ1-treated mice. Yet, *in vivo* assays were flawed by lower xenograft penetrance, variable xenograft latency, gastrointestinal toxicity, and inadequate on-target activity of drugs. We conclude that confirmatory preclinical trials allow for robust assessment of the efficacy of candidate interventions and reduce bias in academic research. The study platform established here provides a framework that may be of particular benefit for the development of new drugs for rare cancers.

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1. Introduction

Cancer in children is a rare disease. The motivation to invest in early phase clinical trials for refractory paediatric cancers is limited by economic considerations. Lack of transferability of basic research data into the clinic adds to the challenge [1,2]. High failure rates of candidate interventions in hypothesis-driven academic studies [1–4] have been attributed to inadequate sample sizes, improper allocation of treatment groups, lack of quality control measures and failure to report negative results [5,6]. Immense publication pressure further enhances bias. Implementation of clinical development based on rigorously controlled experiments is more likely to result in successful clinical trial outcomes, which should translate into a much-needed acceleration of the development of new drugs [4]. Thus, this consortium implemented a multicentre, randomised, blinded preclinical study framework by applying the quality standards of early phase clinical trials.

The preclinical study platform was established and tested by planning and conducting a trial aimed at evaluating the efficacy of new drugs for refractory PAX3::FOXO1 (P3F)-positive rhabdomyosarcoma (RMS). P3F+ RMS is an aggressive soft-tissue sarcoma in children, adolescents, and young adults. Advanced disease stages continue to be essentially incurable [7]. The disease-defining fusion oncogene P3F cooperates with the master transcription factors MYOG, MYOD, and MYCN to recruit super-enhancers and establish autoregulatory loops that enforce the disease's oncogenic transcriptional program [8]. P3F activity at super-enhancers depends on BET bromodomain protein 4 (BRD4). BRD4 inhibition results in P3F degradation and growth arrest [8]. Cyclin-dependent kinase 9 (CDK9) serves as the catalytic subunit in the positive transcription elongation factor (p-TEFb) and is recruited by BRD4 [9]. CDK9 inhibitors may therefore exert synergistic effects with BRD4 inhibitors (Fig. 1A, Supplemental Tables S1-S2). Preliminary observations indicated anti-tumour efficacy of CDK9 and BRD4 inhibition in Ewing sarcomas [10] and P3F+ RMS (unpublished data). Our primary goal was to assess the feasibility of confirmatory preclinical studies within an academic research setting (Fig. 1B) by establishing a trial to evaluate the synergistic effects of the BRD4 inhibitor JQ1 and the CDK9 inhibitor CDKI73 against P3F+ RMS.

2. Methods

2.1. Study management

The preclinical study protocol (Supplementary Text S1) was established before any experiments were initiated. It was designed to meet the quality criteria of an early phase clinical trial protocol. A preclinical study coordinator supervised all experiments at all study sites (Supplemental Tables S1–2). Adverse events were defined as complications in any experiment. Associated events (presumed to be in a causal relationship with the study drugs) and non-associated events (presumed to be independent of drug effects) were reported using standardised adverse event forms. The protocol was registered with the Animal Study Registry (https://www.animalstudyregistry.org/; 101.7590/

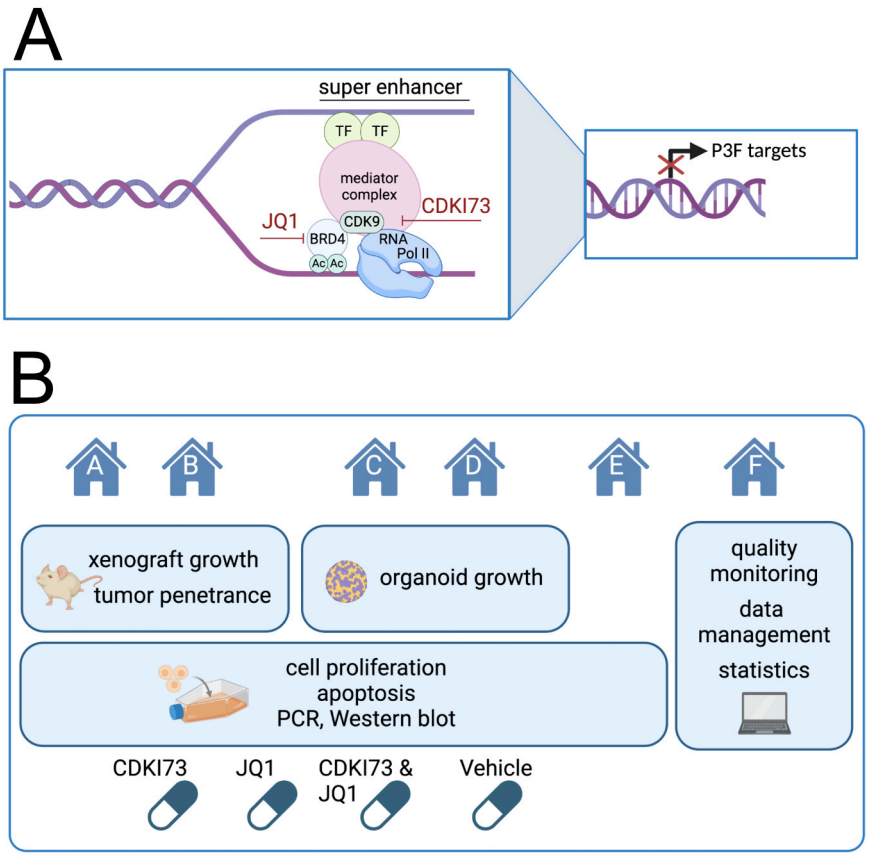

Fig. 1. Study design. (A) This preclinical study was designed to evaluate the hypothesis that inhibitors of BRD4 (i.e. JQ1) and inhibitors of CDK9 (i.e. CDKI73) act synergistically against refractory PAX3::FOXO1 (P3F)-positive rhabdomyosarcoma (RMS) by interrupting autoregulatory loops of transcription factors that maintain expression of the fusion oncogene and enforce its oncogenic program. (B) The multicentre, randomised, and blinded preclinical study platform was established by applying the rigorous quality standards of early phase clinical trials. Experiments were executed at five independent study centres (A-E). There was one coordinating study centre (F). Four treatment groups (CDKI73, JQ1, CDKI73 & JQ1, vehicle) were considered. Experimental endpoints were cell proliferation (determined at five centres in parallel), organoid growth (determined at one of two centres) and apoptosis (determined at five centres in parallel) as well as penetrance of P3F+ mouse RMS xenografts (determined at two centres in parallel), and growth of P3F+ PDX tumours (determined at two centres in parallel) in mice. All experiments were carried out using P3F+ patient derived xenograft (PDX)-derived RMS cell cultures.

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2.2. Randomization and blinding

The study included four treatment groups: BRD4 inhibitor (JQ1) alone, CDK9 inhibitor (CDKI73) alone, JQ1 and CDKI73 in combination and vehicle only. For *in vitro* proliferation assays, cells were also treated with vincristine (positive control). For all experiments, chemicals were allocated in a randomised fashion using computer-generated lists (software R version 4.1.2). Randomization of animal experiments was stratified by study site and sex. All experiments at all study sites were conducted by two technicians. One technician handled the chemicals. The second study technician was blinded to treatment allocations, executed the experiments and assessed outcomes.

2.3. Cell culture and organoid induction

Low-passage human P3F+ RMS cell cultures (derived from patient-derived xenografts (PDX) or patient tumours, Supplemental Table S3) and U23674 cells (established from Myf6Cre, Pax3:Foxo1,p53 mouse sarcomas) were cultured as previously described [11,12] and authenticated by short tandem repeat (STR) analyses (Supplemental Table S4). The PDX cell cultures used in this study were established from patient biopsies collected at the University Children's Hospital Zurich, Institut Curie Paris and Charité University Hospital Berlin. Written informed consent was obtained from all patients at the participating institutions, as described in earlier publications [13]. Their gene expression profiles clustered with published datasets obtained from patients with alveolar RMS (Supplemental Fig. S1). Organoids were induced from P3F+ RMS cell cultures as previously described [11]

2.4. Animals

NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) and NOD.CB17-Prkdcscid/J (NOD.SCID) mice were purchased from Janvier labs. All animal experiments were approved by the Regierungspräsidium Freiburg (G21–105) or the Zurich veterinary office (file number 35–9185.81/G-21/105; license ZH013/2021).

2.5. In vitro growth and apoptosis assays

Cell viability was measured by WST-1 assay (10008883, Cayman Chemical) for 2D cultures and by CellTiter-Glo® 3D Cell Viability assay (G9681, Promega, Walldorf, Germany) for organoid cultures. Apoptosis was determined by Annexin V-APC (550474, BD Biosciences)/ 7AAD staining (559925, BD Biosciences). Outcome assessment took place 72 h after chemical exposure. 2D assays were repeated at 5 study centers. 3D assays were performed once at one of two study centers (Supplemental Tables S1–2).

2.6. In vivo tumour growth assays

Xenografts were derived from low-passage human RMS cultures by subcutaneous injection of 2,5–5 $\times$ 10^6 tumour cells into the flanks of 6–10 weeks-old NSG mice (14–16 mice per cell culture and study site) as previously described [11] (Supplemental Table S12). Sample size calculation required ten distinct RMS cell cultures (Supplemental Table S3–4) and six animals per group. Experiments were shared between two sites (goal 3 mice per RMS culture and treatment group at each site) (Supplemental Table S1–2).

2.7. In vivo tumour penetrance assays

Xenografts were derived from mouse U23674 cells [14,15] by intramuscular injection into the gastrocnemius muscles of NOD.SCID mice as previously described [15] (Supplemental Table S12). Chemical administration was started 3 days after tumour cell implantation, continued for 14 days, and stratified by gender. Sample size calculation required 22 animals per group. The assay was shared between two sites (goal 11 mice per RMS culture and treatment group at each site) (Supplemental Table S1–2).

2.8. Statistics

The primary objective was to assess the synergistic effects of CDKI73 and JQ1. Consequently, statistical planning focused on testing the interaction between CDKI73 and JQ1. For all experiments, four treatment groups (vehicle only, CDKI73 alone, JQ1 alone, CDKI73 and JQ1) were considered and analyzed following a 2 $\times$ 2 design by estimating the main effects of CDKI73 and JQ1 as well as their interaction. Mean values were calculated for observations from the same cell culture under the same treatment obtained in the different centres. Linear mixed effects regression models with treatment indicators and their interaction as independent variables were fitted to the data for assessment of synergy using standardised outcome measures (relative difference to the mean over all measurements from the vehicle group). A random intercept for cell line was considered to account for the assessment of the same cell line under different treatment options. The Kenward-Roger approximation for denominator degrees of freedom was applied. For tumour penetrance (time-to-event outcome), a Cox regression model with the two treatment indicators (JQ1, CDKI73), their interaction, and centre as fixed effects was fitted to the data. Kaplan-Meier curves were calculated per centre and overall. For all statistical tests, a significance level of 5 % was used.

Please see Supplemental Text S2 for further details regarding experimental procedures.

3. Results

3.1. JQ1 inhibits proliferation of RMS cells grown in monolayers

The growth of seven low-passage *ex vivo* P3F+ RMS models (Supplemental Tables S1–4, Fig. S1) was examined after exposure with JQ1 and/ or CDKI73 (Fig. 2A). Based on dose-finding experiments using three different concentrations of each chemical alone and in combination (Supplemental Fig. S2-S4, Supplemental Table S5), all *in vitro* assays in the confirmatory study were carried out by administering 2000 nM JQ1 once, 100 nM CDKI73 once, 2000 nM JQ1 and 100 nM CDKI73 in combination once, or vehicle once. Inhibition of growth (compared to the vehicle control) was observed for both drugs, with a stronger effect for JQ1 (average relative reduction 60 %, $p < 0.01$) than for CDKI73 (average relative reduction 24 %, $p = 0.15$). Indeed, supplemental Fig. S5 demonstrates that the observed inhibitory effects of JQ1 and CDKI73 on 2D cell growth were markedly stronger than anticipated. Combined JQ1 and CDKI73 led to a stronger growth inhibition than each chemical alone. Yet, the effect of the combination did not exceed additivity of the individual chemicals' effects and thus did not meet the predefined statistical criteria for synergism ($p = 0.96$, Fig. 2B-C upper panel, Supplemental Table S6). The original statistical approach, which was not intended to evaluate JQ1 or CDKI73 alone, suggested additive effects of the two chemicals, as no relevant interaction was observed. Consequently, a model without an interaction term (including JQ1, CDKI73, and the combination) was fitted to the data post-hoc. Significant effects were confirmed for JQ1 (average reduction 59 %, $p < 0.0001$), while a small, uncertain effect was observed for CDKI73 (average reduction 24 %, $p = 0.05$, Fig. 2C lower panel). Exposure to vincristine – a drug with long-established anti-RMS efficacy – was included as a positive control and administered at 50 nM based on dose-finding experiments (Supplemental Fig. S6). Post-hoc analyses demonstrated that JQ1 and vincristine had comparable antiproliferative effects (average reduction 68 %, $p < 0.001$, Fig. 2C lower panel).

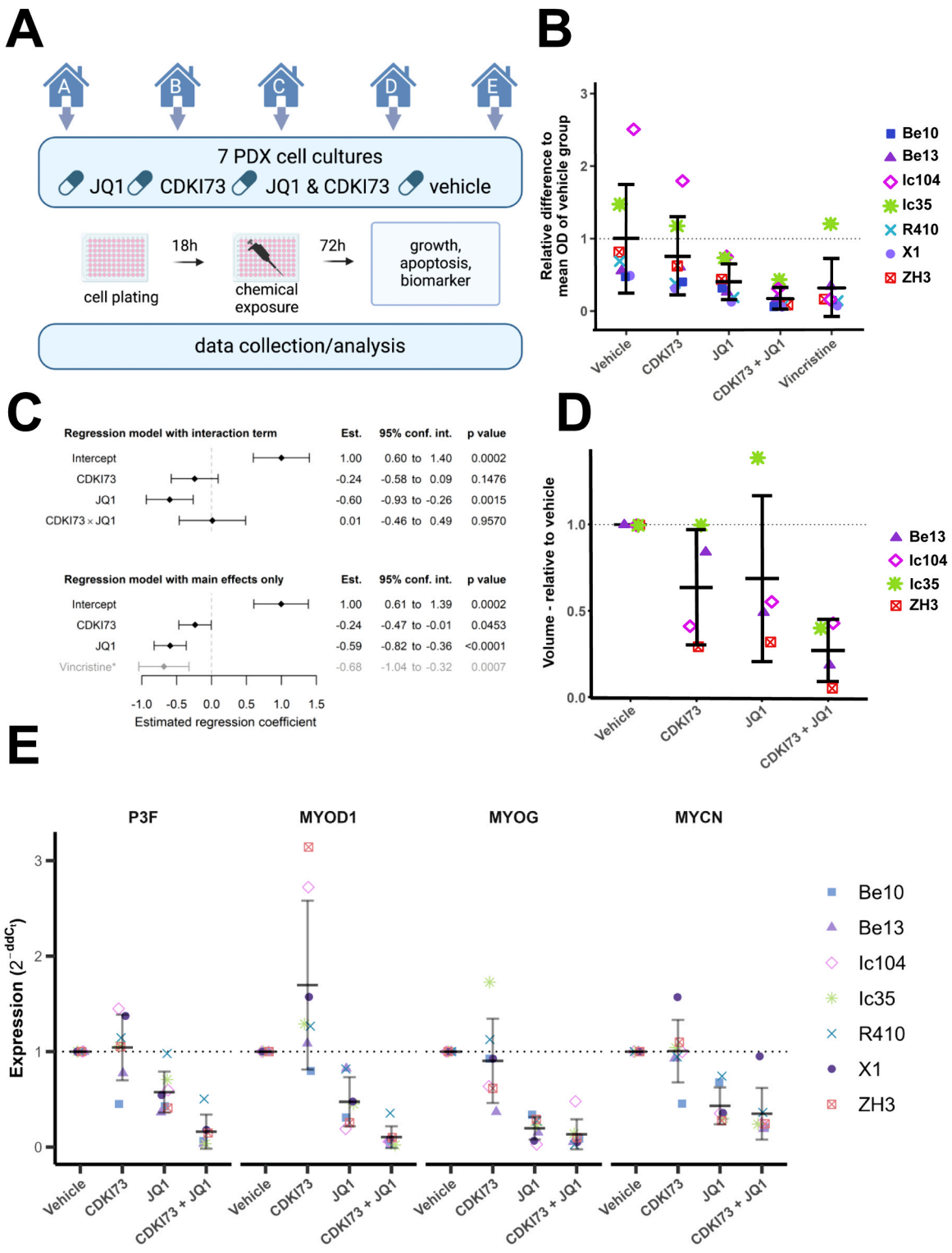

Fig. 2. *In vitro* assays using P3F+ RMS cells grown in monolayers. (A) P3F+ RMS cell cultures were seeded in 96-well plates and exposed to chemicals (four main treatment groups) approximately 18 h after plating. All assays were randomised, blinded, and repeated at all five study centres. All experiments were carried out using seven distinct low-passage human RMS cell cultures. Experimental endpoints were proliferation, apoptosis and expression of P3F and key transcription factors. (B-C) Inhibition of cell growth (compared to the vehicle control) was observed for both drugs, with a stronger effect for JQ1 than for CDKI73. The combination of JQ1 and CDKI73 led to a stronger inhibition of cell growth than each chemical alone, but did not exceed additivity of the effects of the individual chemicals. (C) Combinatorial effects were evaluated using statistical models with (C, upper panel) and without (C, lower panel) assessment of interaction effects between the experimental treatments. The effect of vincristine was estimated in a model considering all five treatment groups as separate groups. (D) On a protein level, there was a trend towards lower expression of P3F after treatment with JQ1 and CDKI73 alone. The JQ1 and CDKI73 combination lowered P3F expression substantially. (E) On a transcript level, JQ1 alone and in combination with CDKI73 reduced *P3F*, *MYCN*, *MYOG* and *MYOD1* expression, thereby confirming on-target activity of the drugs. Error bars indicate standard deviation (B, D, E) or 95 % confidence intervals (C).

3.2. JQ1 decreases P3F, MYCN, MYOG, and MYOD1 expression in RMS cells

As BRD4 inhibitors induce degradation of the fusion oncogene and impede expression of master transcription factors, changes in the expression of P3F, MYCN, MYOD1, and MYOG should reflect on-target activity of JQ1 and CDKI73. In keeping with the study design, blinded samples - obtained at 4 different study sites - were allocated to PCRs and Western blots by central randomizations. Although low protein yields and/or poor protein quality compromised P3F Western blots, they were run successfully using protein from 4 of 7 RMS cultures grown in monolayer with a trend towards lower and substantially reduced P3F expression after single drug treatments and combined JQ1 and CDKI73, respectively (Fig. 2D, Supplemental Fig. S7, Supplemental Table S7). P3F, MYCN, MYOD1, and MYOG levels were measured successfully by PCR in all 7 RMS cultures grown in monolayers. While CDKI73 alone had no effect, JQ1 alone and combined with CDKI73 reduced *P3F*, *MYCN*, *MYOG* and *MYOD1* levels (Fig. 2E, Supplemental Table S8). These observations confirm on-target efficacy of JQ1 in P3F+ RMS cells. We note that samples were obtained from blinded assays and analyzed centrally. For each target and RMS culture, RNA from one of 4 centres was assigned randomly to PCR runs. In vehicle-treated samples, concordance between blinded PCR-based and unblinded RNA-seq-based target gene quantification supported the reliability of sample logistics (Supplemental Fig. S8).

3.3. JQ1 has pro-apoptotic effects on RMS cells

The influence JQ1 and CDKI73 on cell viability (% AnV-/7AAD-), apoptosis (% AnV+/7AAD-) and necrosis/late stage apoptosis (% AnV+/7AAD+) of 7 RMS cultures (Supplemental Tables S1–4) was evaluated via AnV/7AAD-staining (Fig. 2A). JQ1 and CDKI73 hampered viability (average reduction 40 %, $p < 0.0001$ for JQ1 and average reduction 8 %, $p = 0.22$ for CDKI73, Fig. 3A-B, Supplemental Table S9) and increased apoptosis (average increase 81 %, $p < 0.0001$ for JQ1 and 15 %, $p = 0.32$ for CDKI73, Fig. 3C-D, Supplemental Table S9) and necrosis (average increase 86 %, $p < 0.001$ for JQ1 and average increase 15 %, $p = 0.43$ for CDKI73, Fig. 3E-F, Supplemental Table S9). There were no super-additive effects of JQ1 and CDKI73 on viability, apoptosis and necrosis, and no interaction was observed (Fig. 3B, D, F upper panels). A post-hoc model without an interaction term showed significant effects of both chemicals on viability (average reduction 44 %, $p < 0.001$ for JQ1 and 12 %, $p = 0.016$ for CDKI73, Fig. 3B lower panel) and necrosis (average increase 103 % for JQ1, $p < 0.0001$ and 31 %, $p = 0.028$, Fig. 3F lower panel). However, CDKI73 effects may be overestimated for minor interaction due to individual drug effects under the assumption of additivity.

3.4. JQ1 inhibits 3D growth of RMS organoids

Organoids derived from seven RMS cultures (Supplemental Tables S1–4) were exposed to chemicals by administering the same JQ1 and CDKI73 concentrations as in 2D assays (Fig. 4A). Organoid-specific dose-finding experiments were not performed. Reduced organoid growth was observed for JQ1 (average reduction 70 %, $p < 0.01$) and for CDKI73 (average reduction 44 %, $p = 0.06$, Fig. 4B-C, Supplemental Table S10) without evidence of synergy (Fig. 4C upper panel). A post-hoc model without an interaction term confirmed significant inhibitory effects of JQ1 (average reduction 52 %, $p < 0.01$), but not CDKI73 (average reduction 26 %, $p = 0.12$, Fig. 4C lower panel). JQ1 alone and combined with CDKI73 (but not CDKI73 alone) reduced *P3F*, *MYCN*, *MYOG* and *MYOD1* expression (Fig. 4E, Supplemental Table S11). Thus, JQ1 and CDKI73 effects on RMS cells appear to be independent of the *ex vivo* model system used.

3.5. JQ1 has mild effects on human RMS xenograft growth

NSG mice bearing subcutaneous xenografts derived from human RMS cultures (Supplemental Tables S1–4) were exposed to chemicals alone and in combination (Fig. 5A). *In vivo* drug doses were chosen based on previously published studies [10]. It was not possible to execute all assays as planned in the preclinical study protocol. X16 cells were replaced by ZH4 cells because of slow *in vitro* expansion. Mice implanted with two RMS cultures at one study site (BE14, X1) and one RMS culture at the other site (X1) did not develop tumours. Tumour latency was 18–86 days, and tumour penetrance was 0–86 % across xenografts derived from ten RMS cultures (Supplemental Fig. S9–S10, Supplemental Tables S12–13). Ultimately, the number of animals required to achieve a power of 80 % was not met. Xenograft volume at treatment start varied widely (Supplemental Table S14); xenografts smaller than 10 mm^3 at treatment start were excluded from analyses. Tumour growth and treatment effects were overall similar in the two centers (Supplemental Fig. S11), demonstrating that the results are reproducible at two sites. There was a trend towards tumour regression upon JQ1 treatment (average reduction 36 %, $p = 0.04$) and CDKI73 (average reduction 28 %, $p = 0.12$, Fig. 5B-C, Supplemental Fig. S10, Supplemental Table S14). Drug interaction analysis did not demonstrate synergy. In fact, the observed combination effect was less than additive (Fig. 5C, upper panel). A post-hoc model without an interaction term supported the mild decrease in xenograft growth in mice treated with JQ1 (average reduction 26 %, $p = 0.04$, Fig. 5C lower panel). Only minor CDKI73 effects on tumour growth were estimated in the model assuming additive effects (average reduction 17 %, $p = 0.15$, Fig. 5C, lower panel). No differences in *P3F, MYCN*, *MYOG,* and *MYOD1* levels were detected in RNA isolated from xenografts exposed to JQ1 and/ or CDKI73 (Fig. 5D, Supplemental Table S15). We note that randomization of treatment groups was successfully stratified by gender: each group comprised similar numbers of male and female mice (Supplemental Fig. S9A). Tumour latency and penetrance were similar in male and female mice (Supplemental Fig. S9B, S10) and for tumour cells from male and female patients (Supplemental Fig. S9C).

3.6. JQ1 delays engraftment of mouse Myf6Cre, Pax3:Foxo1, p53 RMS cells

Penetrance assays aimed at determining if chemicals prolong the time to xenograft growth (Fig. 6A). On day 40 after cell injection, 11 of 22 (50 %) vehicle-treated mice were tumour-free compared to 12 of 19 (63 %) in the JQ1 group, seven of 21 (33 %) in the CDKI73 group, and 5 of 10 (50 %) mice in the combination group (Fig. 6B). There was no interaction between JQ1 and CDKI73; no synergy was observed ($p = 0.9$, Fig. 6C, Supplemental Table S16). Post-hoc analysis using a model without an interaction term indicated significant inhibition of xenograft induction by JQ1 ($p < 0.01$, Fig. 6C lower panel), while CDKI73 alone decreased time to xenograft growth ($p < 0.05$, Fig. 6C lower panel). Frequent adverse events reduced the number of mice followed until tumour occurrence (Fig. 6D).

3.7. JQ1 and CDKI73 have relevant gastrointestinal toxicity in mice

Many adverse events were reported for *in vivo* assays, including associated (e.g., diarrhea) and non-associated events (various injuries) (Fig. 5D, Fig. 6D, Supplemental Table S17–18). Considerable toxicity observed during penetrance assays (Fig. 6D, Supplemental Table S18) resulted in adjustments in chemical reconstitution. Subsequently, drug tolerance was improved during growth assays (Fig. 5D, Supplemental Table S17). In total, 28 associated events and eight non-associated events were observed in 154 animals (Supplemental Tables S17–18). 22 of 28 (79 %) associated events occurred in animals treated with the combination. Gastrointestinal toxicity accounted for 21 of 28 (75 %) of associated events.

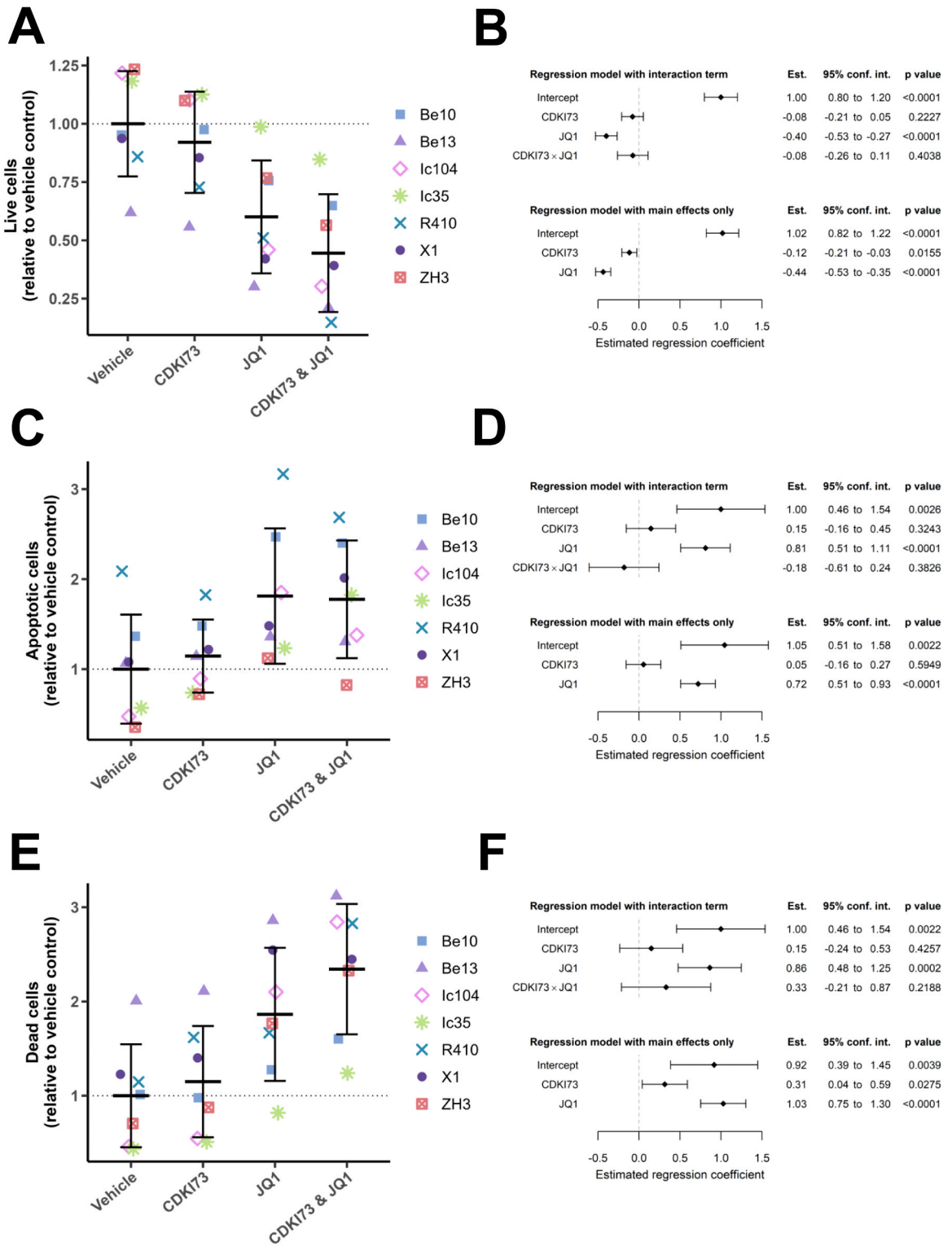

Fig. 3. Survival, apoptosis and death of P3F+ RMS cells after exposure to chemicals *in vitro*. The influence of treatment with JQ1 and CDKI73 on (A-B) cell viability (% AnV-/7AAD-), (C-D) apoptosis (% AnV+/7AAD-) and (E-F) necrosis/late stage apoptosis (% AnV+/7AAD+) of 7 P3F+ RMS tumour cell cultures was evaluated. Treatment with JQ1 and CDKI73 (A-B) hampered cell viability and (C-D) increased tumour cell apoptosis and (E-F) necrosis. (B, F) Combined JQ1 and CDKI73 had a stronger effect on cell survival and cell death than either chemical alone, but the combination had similar effects on apoptosis compared to JQ1 alone. (B, D, F) The effects of the combination again did not exceed additivity of the effects of the individual chemicals on cell survival, apoptosis and death. Combinatorial effects were evaluated using statistical models with (B, D, F, upper panels) and without (B, D, F, lower panels) assessment of interaction effects between the experimental treatments. Error bars indicate standard deviation (A, C, E) or 95 % confidence intervals (B, D, F).

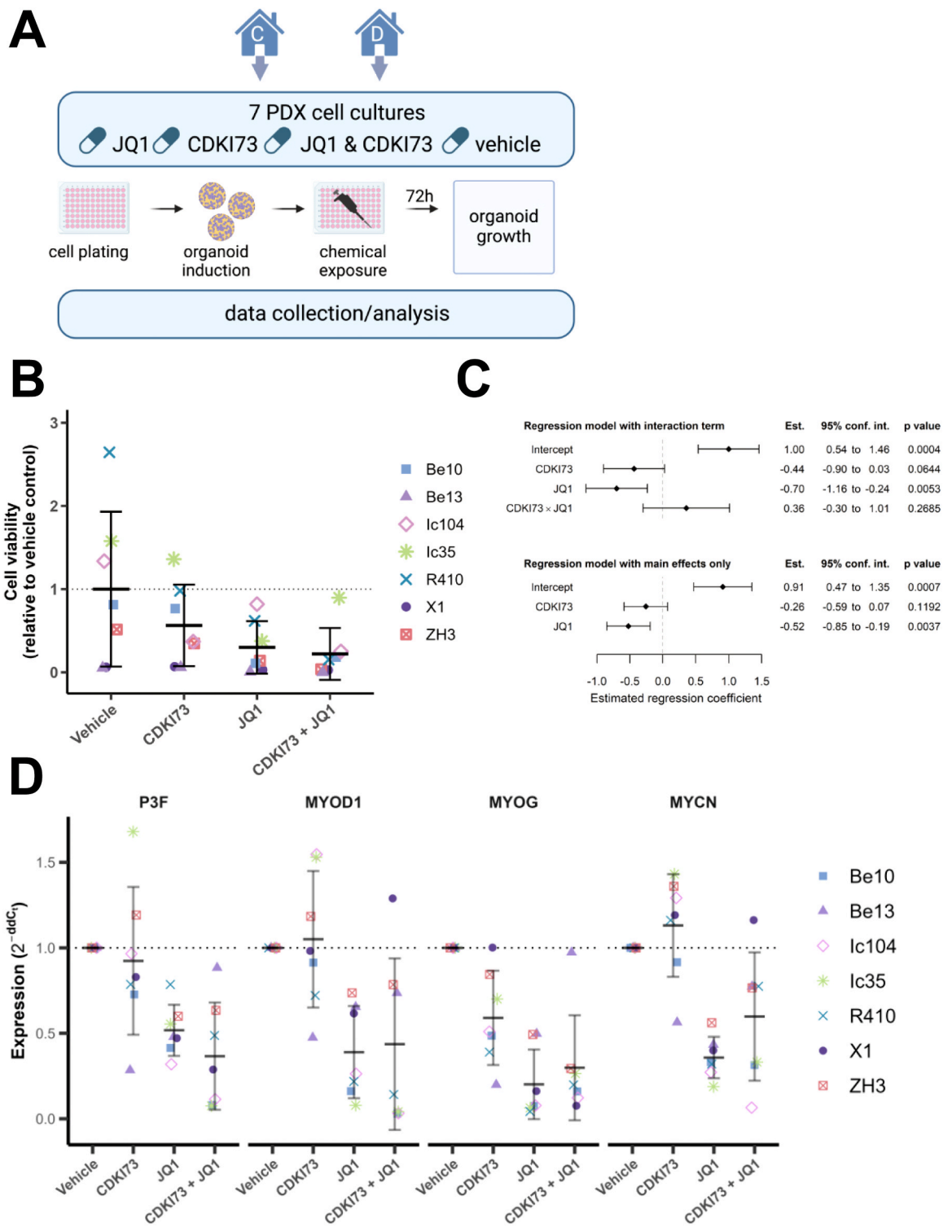

Fig. 4. *In vitro* assays using P3F+ RMS cells grown as organoids (A) Organoids were induced from P3F+ RMS cell cultures and exposed to chemicals (4 treatment groups) 7 days after the start of organoid induction. All assays were randomised, blinded, and carried out at one of two study centres. Seven distinct low-passage human RMS cell cultures were used. Experimental endpoint was cell viability 72 h after start of treatment. (B-C) Reduced organoid growth was observed for JQ1 and for CDKI73. (C) The combination of JQ1 and CDKI73 led to a stronger inhibition of organoid growth than each chemical alone, but the combined effects of both drugs did not exceed additivity of the effects of the individual chemicals. (C) Combinatorial effects were evaluated using statistical models with (C, upper panels) and without (C, lower panels) assessment of interaction effects between the experimental treatments. (D) On a transcript level, JQ1 alone and in combination with CDKI73 reduced *P3F*, *MYCN*, *MYOG* and *MYOD1* expression, thereby confirming on-target activity of the drugs. Error bars indicate standard deviation (B, D) or 95 % confidence intervals (C).

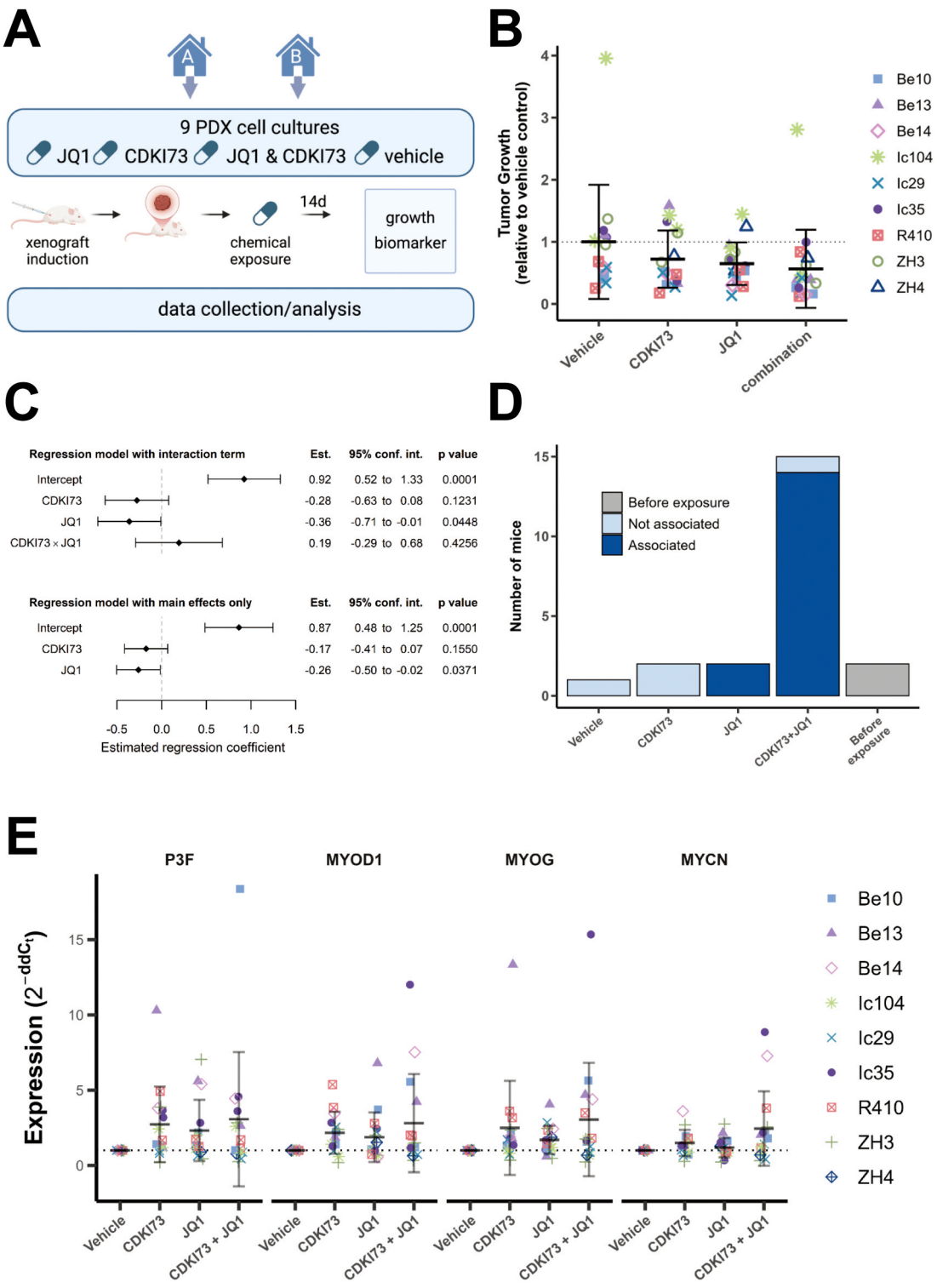

Fig. 5. *In vivo* tumour growth assays using xenografts derived from P3F+ RMS cells. (A) Xenografts were derived from P3F+ RMS cell cultures by subcutaneous injection into the flanks of NSG mice. Randomised and blinded administration of chemicals took place in 4 treatment groups. Experiments were shared between two study sites. Experimental endpoint was relative increase in tumour size determined by three-dimensional tumour measurements. Initially, 181 mice were included in the experiment. Data of 141 mice were considered for estimation of treatment effects. Data from 13 mice with baseline tumour volumes of less than 10 mm^3, 18 mice with AEs before final assessment of tumour volumes and 9 mice with no valid assessment of tumour volume between day 13 and 15 were excluded. (B) There was a trend towards tumour regression upon treatment with JQ1 and CDKI73. (C) Drug interaction analysis (C, upper panel) did not demonstrate synergistic effects of the two compounds. A model without an interaction term (C, lower panel) was fitted to the data post-hoc and confirmed these observations. (D) 16 associated events and 6 non-associated events were observed; 2 of 6 non-associated events occurred before any study drugs were given. 15 of 22 (68 %) adverse events occurred in animals treated with both chemicals in combination. (E) No relevant differences in *P3F*, *MYCN*, *MYOG,* and *MYOD1* transcript levels were detected in RNA isolated from xenograft tissue exposed to JQ1 and/ or CDKI73, suggesting that the drugs did not hit their presumed targets in tumour tissue. Error bars indicate standard deviation (B, E) or 95 % confidence intervals (C).

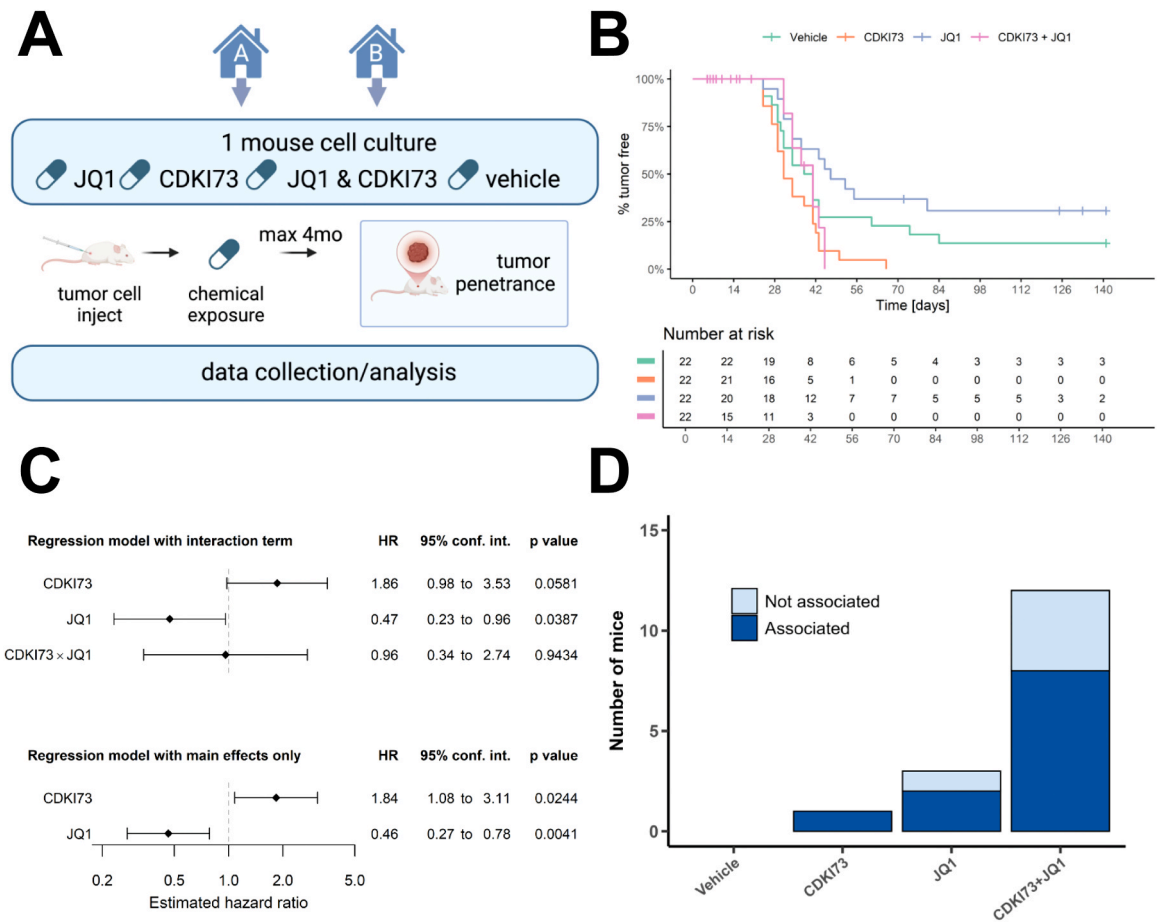

Fig. 6. *In vivo* tumour penetrance assays. (A) Mouse U23674 RMS cells were injected into the gastrocnemius muscles of NOD.SCID mice. Randomised and blinded administration of chemicals in four treatment groups was started 3 days after tumour cell implantation and continued for14 days. The assay was shared between two study sites. Experimental endpoint was detection of a palpable tumour at the injection sites. (B-C) JQ1 and CDKI73 did not exert any synergistic effects on time to tumour induction. (C) Combinatorial effects were evaluated using statistical models with (C, upper panel) and without (C, lower panel) interaction terms. Post-hoc analysis using a model without an interaction term indicated that JQ1 as a single drug increased and CDKI73 as a single drug decreased time to xenograft growth. Error bars indicate 95 % confidence intervals. (D) Frequent adverse events reduced the number of mice, which were followed until the occurrence of a tumour. 12 of 16 (75 %) adverse events occurred in animals treated with both chemicals in combination.

3.8. Differences in drug response among distinct RMS cells may be evaluated

Differences in underlying biology cause profound interindividual differences in treatment response and outcome. Multicentre data from seven RMS 2D cultures allowed for post-hoc analyses directed at determining differences in response between RMS cells obtained from individual patients. Variable anti-tumour efficacy of vincristine was noted: 50 nM Vincristine did not reduce the proliferation of one RMS cultures (IC35, Supplemental Fig. S12), and 20 nM vincristine had no effect on two RMS cultures (IC35 and BE13, Supplemental Fig. S4). By contrast, there were no relevant differences concerning JQ1- and CDKI7-induced changes in cell growth and apoptosis (Supplemental Fig. S13).

4. Discussion

This study demonstrates that planning and executing confirmatory preclinical studies in an academic setting by applying the quality standards of early phase clinical trials is feasible. Broad implementation of such efforts will maximize the quality of preclinical data and reduce the risk of transferring flawed data from the bench to the bedside [6,16]. Investigator-initiated preclinical trials may provide particular advantages for rare diseases, including all paediatric cancers.

Findings from this preclinical study show that the BRD4 inhibitor JQ1 effectively reduced growth and viability of P3F+ human RMS cells. The *in vitro* antiproliferative effects of JQ1 and vincristine were comparable. Reduction of P3F, MYCN, MYOG, and MYOD1 expression by JQ1 confirmed on-target activity. Observations were independent of the type of model system used.

The primary objective of this study was to evaluate BRD4 and CDK9 synergy. While the combination of JQ1 and CDKI73 frequently appeared to produce stronger inhibitory effects than either agent alone, the predefined criteria for synergy—requiring that the combination effects exceed the sum of the individual effects—were not met. This lack of synergy may, in part, be attributable to suboptimal drug concentrations and/ or potent single-agent activity of JQ1 or limited efficacy of CDKI73 (Fig. S5), which may have masked a potential synergistic interaction.

The randomised, blinded *in vivo* growth assays were flawed by lower xenograft penetrance and more variable xenograft latency than expected. To assess the potential impact of the slightly reduced sample size on statistical power, the simulations originally used for sample size calculation were rerun using the final number of animals (n = 64 instead of 72). All original assumptions were retained. Statistical power remained above 75 %, suggesting that the study was still adequately powered to detect the anticipated effects.

The *in vivo* studies were further compromised by gastrointestinal toxicity of the drug combination reminiscent of side effects reported in recent clinical studies investigating the BRD4 inhibitor AZD5153 and

the CDK inhibitor dinaciclib [17,18]. Still, the animal experiments revealed a trend towards tumour regression upon JQ1 treatment. No differences in *P3F*, *MYCN*, *MYOG,* and *MYOD1* transcript levels were detected in RNA from xenograft tissue exposed to chemicals, indicating that – other than in the *ex vivo* experiments – the drugs did not hit their presumed targets optimally. This could be due to insufficient dosing and/ or bioavailability. Several previous studies have reported that JQ1 has low bioavailability and a short half-life, [19,20]. Moreover, *in vivo* doses were determined based on published efficacy and toxicity data in other mouse models of cancer. Dose-finding experiments in animals and choice of inhibitors with optimal bioavailability should be considered in future experiments.

Our study design joins other preclinical study platforms, such as the Paediatric Preclinical Testing Program (PPTP), supported by the National Cancer Institute (NCI), or the European Innovative Therapies for Children with Cancer - Paediatric Preclinical Proof-of-Concept Platform ITCC-P4, which seek to improve the quality of preclinical research [4, 21]. To our best knowledge, our study is the first investigator-initiated multicentre, randomised, blinded *in vitro* and *in vivo* platform in academic paediatric oncology, and it provides a framework that can be adapted to develop future, optimized efforts.

Taken together, we strongly believe that confirmatory preclinical studies offer tremendous potential in sparing resources required for clinical drug development, saving patients with rare diseases from being enrolled in futile trials, preventing their families from nurturing false hopes and improving patient recruitment into early phase clinical trials with the highest possible chance of success.

Data sharing statement

All source code is available at https://github.com/epicycle-rms/. The raw and normalised count matrices of the RNA sequencing project are available via the European Genome Phenome Archive (EGA Accession ID EGAS00001008196). Access may be granted upon request.

CRediT authorship contribution statement

Claudia Winter: Investigation. **Marvin Jens:** Formal analysis, Visualization. **Ulf Tölch:** Formal analysis, Methodology. **Ingrid Bechtold:** Investigation. **Beat W Schäfer:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Yekaterina Gadasheva:** Visualization, Writing – original draft, Writing – review & editing. **Ana Banito:** Data curation, Project administration, Resources, Supervision, Writing – review & editing. **Ebrahem Hamed:** Investigation. **Irene von Lüttichau:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Samanta Kisele:** Investigation. **Simone Hettmer:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Mathilda Knoblauch:** Investigation. **Bernhard Haller:** Conceptualization, Formal analysis, Methodology, Visualization, Writing – review & editing. **Maximilian RA Koch:** Data curation, Project administration. **Günther HS Richter:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. **Marco Wachtel:** Data curation, Methodology, Resources, Supervision, Writing – review & editing. **Dmitry Lupar:** Investigation. **Satoshi Nakano:** Investigation. **Lena Schuler:** Data curation, Project administration, Writing – original draft, Writing – review & editing. **Katharina Pardon:** Investigation. **Carla Regina:** Formal analysis, Methodology, Project administration. **Paul J Pärschke:** Investigation. **Bernhard Renz:** Resources, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare no conflicts of interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118704.

Data availability

The data link is included in the data sharing statement in the manuscript.

References

[1] D.G. Contopoulos-Ioannidis, E. Ntzani, J.P. Ioannidis, Translation of highly promising basic science research into clinical applications, Am. J. Med. 114 (6) (2003) 477–484.
[2] P. Pound, M. Ritskes-Hoitinga, Is it possible to overcome issues of external validity in preclinical animal research? Why most animal models are bound to fail, J. Transl. Med. 16 (1) (2018) 304.
[3] P. Perel, I. Roberts, E. Sena, P. Wheble, C. Briscoe, P. Sandercock, M. Macleod, L. E. Mignini, P. Jayaram, K.S. Khan, Comparison of treatment effects between animal experiments and clinical trials: systematic review, BMJ 334 (7586) (2007) 197.
[4] P.J. Houghton, C.L. Morton, C. Tucker, D. Payne, E. Favours, C. Cole, R. Gorlick, E. A. Kolb, W. Zhang, R. Lock, H. Carol, M. Tajbakhsh, C.P. Reynolds, J.M. Maris, J. Courtright, S.T. Keir, H.S. Friedman, C. Stopford, J. Zeidner, J. Wu, T. Liu, C. A. Billups, J. Khan, S. Ansher, J. Zhang, M.A. Smith, The pediatric preclinical testing program: description of models and early testing results, Pedia Blood Cancer 49 (7) (2007) 928–940.
[5] S.C. Landis, S.G. Amara, K. Asadullah, C.P. Austin, R. Blumenstein, E.W. Bradley, R. G. Crystal, R.B. Darnell, R.J. Ferrante, H. Fillit, R. Finkelstein, M. Fisher, H. E. Gendelman, R.M. Golub, J.L. Goudreau, R.A. Gross, A.K. Gubitz, S.E. Hesterlee, D.W. Howells, J. Huguenard, K. Kelner, W. Koroshetz, D. Krainc, S.E. Lazic, M. S. Levine, M.R. Macleod, J.M. McCall, R.T. Moxley, 3rd, K. Narasimhan, L.J. Noble, S. Perrin, J.D. Porter, O. Steward, E. Unger, U. Utz, S.D. Silberberg, A call for transparent reporting to optimize the predictive value of preclinical research, Nature 490 (7419) (2012) 187–191.
[6] C.R. Hooijmans, M. Ritskes-Hoitinga, Progress in using systematic reviews of animal studies to improve translational research, PLoS Med. 10 (7) (2013) e1001482.
[7] D. Williamson, E. Missiaglia, A. de Reynies, G. Pierron, B. Thuille, G. Palenzuela, K. Thway, D. Orbach, M. Lae, P. Freneaux, K. Pritchard-Jones, O. Oberlin, J. Shipley, O. Delattre, Fusion gene-negative alveolar rhabdomyosarcoma is clinically and molecularly indistinguishable from embryonal rhabdomyosarcoma, J. Clin. Oncol. 28 (13) (2010) 2151–2158.
[8] B.E. Gryder, M.E. Yohe, H.C. Chou, X. Zhang, J. Marques, M. Wachtel, B. Schaefer, N. Sen, Y. Song, A. Gualtieri, S. Pomella, R. Rota, A. Cleveland, X. Wen, S. Sindiri, J.S. Wei, F.G. Barr, S. Das, T. Andresson, R. Guha, M. Lal-Nag, M. Ferrer, J.F. Shern, K. Zhao, C.J. Thomas, J. Khan, PAX3-FOXO1 establishes myogenic super enhancers and confers BET bromodomain vulnerability, Cancer Discov. 7 (8) (2017) 884–899.
[9] S. Eyvazi, M.S. Hejazi, H. Kahroba, M. Abasi, R.E. Zamiri, V. Tarhriz, CDK9 as an appealing target for therapeutic interventions, Curr. Drug Targets 20 (4) (2019) 453–464.
[10] G. H.S. Richter, T. Hensel, O. Schmidt, V. Saratov, K. von Heyking, F. Becker-Dettling, C. Prexler, H.Y. Yen, K. Steiger, S. Fulda, U. Dirksen, W. Weichert, S. Wang, S. Burdach, B.W. Schafer, Combined inhibition of epigenetic readers and transcription initiation targets the EWS-ETS transcriptional program in Ewing Sarcoma, Cancers 12 (2) (2020).

[11] G. Manzella, L.D. Schreck, W.B. Breunis, J. Molenaar, H. Merks, F.G. Barr, W. Sun, M. Rommele, L. Zhang, J. Tchinda, Q.A. Ngo, P. Bode, O. Delattre, D. Surdez, B. Rekhi, F.K. Niggli, B.W. Schafer, M. Wachtel, Phenotypic profiling with a living biobank of primary rhabdomyosarcoma unravels disease heterogeneity and AKT sensitivity, Nat. Commun. 11 (1) (2020) 4629.
[12] C. Regina, E. Hamed, G. Andrieux, S. Angenendt, M. Schneider, M. Ku, M. Follo, M. Wachtel, E. Ke, K. Kikuchi, A.G. Henssen, B.W. Schafer, M. Boerries, A. J. Wagers, C. Keller, S. Hettmer, Negative correlation of single-cell PAX3:FOXO1 expression with tumorigenicity in rhabdomyosarcoma, Life Sci. Alliance 4 (9) (2021).
[13] S.G. Danielli, E. Porpiglia, A.J. De Micheli, N. Navarro, M.J. Zellinger, I. Bechtold, S. Kisele, L. Volken, J.G. Marques, S. Kasper, P.K. Bode, A.G. Henssen, D. Gurgen, O. Delattre, D. Surdez, J. Roma, P. Buhlmann, H.M. Blau, M. Wachtel, B. W. Schafer, Single-cell profiling of alveolar rhabdomyosarcoma reveals RAS pathway inhibitors as cell-fate hijackers with therapeutic relevance, Sci. Adv. 9 (6) (2023) eade9238.
[14] C. Keller, B.R. Arenkiel, C.M. Coffin, N. El-Bardeesy, R.A. DePinho, M.R. Capecchi, Alveolar rhabdomyosarcomas in conditional Pax3:Fkhr mice: cooperativity of Ink4a/ARF and Trp53 loss of function, Genes Dev. 18 (21) (2004) 2614–2626.
[15] S. Hettmer, J. Liu, C.M. Miller, M.C. Lindsay, C.A. Sparks, D.A. Guertin, R. T. Bronson, D.M. Langenau, A.J. Wagers, Sarcomas induced in discrete subsets of prospectively isolated skeletal muscle cells, Proc. Natl. Acad. Sci. 108 (50) (2011) 20002–20007.
[16] A. Sertkaya, H.H. Wong, A. Jessup, T. Beleche, Key cost drivers of pharmaceutical clinical trials in the United States, Clin. Trials 13 (2) (2016) 117–126.
[17] E.P. Hamilton, J.S. Wang, A.M. Oza, M.R. Patel, S.V. Ulahannan, T. Bauer, J. L. Karlix, J. Zeron-Medina, G. Fabbri, P. Marco-Casanova, G. Moorthy, M. M. Hattersley, G.M. Littlewood, P. Mitchell, J. Saeh, G.P. Pouliot, K.N. Moore, First-in-human study of AZD5153, a small-molecule inhibitor of Bromodomain Protein 4, in patients with relapsed/refractory malignant solid tumors and lymphoma, Mol. Cancer Ther. 22 (10) (2023) 1154–1165.
[18] S.K. Kumar, B. LaPlant, W.J. Chng, J. Zonder, N. Callander, R. Fonseca, B. Fruth, V. Roy, C. Erlichman, A.K. Stewart, C. Mayo Phase, Dinaciclib, a novel CDK inhibitor, demonstrates encouraging single-agent activity in patients with relapsed multiple myeloma, Blood 125 (3) (2015) 443–448.
[19] S. Nair, A. Davis, O. Campagne, J.D. Schuetz, C.F. Stewart, Development and validation of an LC-MS/MS method to quantify the bromodomain and extra-terminal (BET) inhibitor JQ1 in mouse plasma and brain microdialysate: application to cerebral microdialysis study, J. Pharm. Biomed. Anal. 204 (2021) 114274.
[20] F. Li, K.R. MacKenzie, P. Jain, C. Santini, D.W. Young, M.M. Matzuk, Metabolism of JQ1, an inhibitor of bromodomain and extra terminal bromodomain proteins, in human and mouse liver microsomes, Dagger Biol. Reprod. 103 (2) (2020) 427–436.
[21] A.D.J. Pearson, S.M. Pfister, A. Baruchel, J.P. Bourquin, M. Casanova, L. Chesler, F. Doz, A. Eggert, B. Geoerger, D.T.W. Jones, P.R. Kearns, J.J. Molenaar, B. Morland, G. Schleiermacher, J.H. Schulte, J. Vormoor, L.V. Marshall, C. M. Zwaan, G. Vassal, Executive, C. biology committees of the innovative therapies for children with cancer european, from class waivers to precision medicine in paediatric oncology, Lancet Oncol. 18 (7) (2017) e394–e404.

Glossary

7-AAD: 7-Aminoactinomycin D, fluorescent dye that intercalates into double-stranded DNA
Annexin V: member of the annexin family of intracellular proteins during early apoptosis translocates to the external membrane
CDK9: Cyclin-dependent kinase 9
CDKI73: specific inhibitor of CDK9
FOXO1: Forkhead-Box-Protein O1, transcription factor
GAPDH: Glycerinaldehyd-3-Phosphat-DeHydrogenase
JQ1: Thienotriazolodiazepine, potent inhibitor of the BET family of bromodomain proteins
MYCN: MYeloCytomatosis viral-related Oncogene, neuroblastoma derived, transcription factor
MYOD: Myogenic Differentiation Antigen, transcription factor essential for myogenic differentiation and repair
MYOG: MYOGenin, transcription factor that induces myogenesis
PAX3: Paired-Box 3, transcription factor
p-TEFb: positive transcription elongation factor composed of CDK9 and Cyclin T1
PAX3::FOXO1 (P3F): contains the N-terminal DNA-binding domains of PAX3, the paired box and the homeobox, fused to the C-terminal domain of FOXO1 mediating transactivation