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ORIGINAL ARTICLE

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Checkpoint kinase 1 inhibitor + low-dose hydroxyurea efficiently kills BRAF inhibitor- and immune checkpoint inhibitor-resistant melanomas

Zhen Zeng[1] | Hung Long Ngo[1] | Martina Proctor[1] | Helen Rizos[2] | Riccardo Dolcetti[3] | Jazmina Gonzalez Cruz[4] | James W. Wells[4] | Brian Gabrielli[1]

[1]Mater Research Institute, The University of Queensland, Brisbane, Queensland, Australia

[2]Faculty of Medicine, Health and Human Sciences, Macquarie University, Sydney, New South Wales, Australia

[3]Sir Peter MacCallum Department of Oncology and Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Victoria, Australia

[4]Faculty of Medicine, Frazer Institute, The University of Queensland, Brisbane, Queensland, Australia

Correspondence
Brian Gabrielli, Mater Research Institute, The University of Queensland, Brisbane, QLD 4102, Australia.
Email: briang@uq.edu.au

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Abstract

Treatment of melanomas with targeted and immunotherapies has proven effective, but resistance to both treatments is a common outcome leaving a high proportion of patients without effective alternative treatment options. Replication stress is a common feature of melanomas, and this is effectively targeted using a combination of checkpoint kinase 1 (CHK1) inhibitor and low-dose hydroxyurea (LDHU). This combination also promotes inflammatory and anti-tumour immune responses in vivo. Melanoma cell lines resistant to BRAF inhibitor (BRAFi) or immune checkpoint inhibitors (ICI) retain their sensitivity to CHK1i + LDHU, with sensitivity similar to that of parental tumours. In vivo, BRAFi-resistant and BRAFi-sensitive parental tumours produce an identical immune response with treatment.

KEYWORDS
CHK1 inhibitor, immune response, replication stress, treatment resistance

1 | INTRODUCTION

Targeting the MAPK pathway using BRAF and MEK inhibitors or targeting immune checkpoints with antibodies directed against PD-1 or its ligand PD-L1 have significantly improved melanoma patient survival (Christofyllakis et al., 2021; Luke, 2019). However, acquired resistance to these treatments is a major obstacle to long-term survival of late-stage melanoma patients. Resistance to BRAF inhibitors can occur through many pathways (Johnson et al., 2015; Lim et al., 2017), whereas combination with MEK inhibitors delays the onset of resistance, and resistance to BRAF inhibitors is correlated with resistance to MEK inhibitors (Kim et al., 2013). We have previously reported a novel treatment approach that effectively controlled melanomas in vitro and in vivo by combining subclinical doses of the replication stress inducer hydroxyurea (HU) with an inhibitor of the cell cycle checkpoint regulator checkpoint kinase 1 (CHK1; Oo et al., 2019). Notably, the sensitivity to this combination treatment was not affected by BRAF mutation status. This combination also triggered an immunogenic form of cell death, pro-inflammatory cytokine expression and increased immune cell infiltration into the tumours (Proctor et al., 2021). Here, we have investigated the efficacy of this combination treatment on BRAF mutant melanomas with acquired resistance to MAPK inhibitors and acquired resistance to immune checkpoint inhibitors (ICIs).

We show that resistance to either targeted therapy or immunotherapy had a little effect on sensitivity to our CHK1 inhibitor + low-dose HU treatment (CHK1i + LDHU) in vitro and in vivo and had no negative effect on the immune response promoted by this treatment in vivo.

Significance

The lack of effective alternative treatments for patients who develop resistance to targeted and/or immunotherapies is a significant problem for a large proportion of melanoma patients. CHK1i + LDHU is an effective treatment in a high proportion of melanomas and retains this efficacy in current treatment-resistant tumours. This suggests that CHK1i + LDHU could be an effective alternative therapy in otherwise treatment-resistant melanoma patients.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Immune-edited mouse melanoma cell line YUMMUV1.7 (*BRAFV600E:CDKN2A−/−:PTEN−/−*; Proctor et al., 2021), human melanoma cell lines A2058, WM164 and its BRAFi-resistant version (Menon et al., 2015), SKMEL28 and BRAFi-resistant clones, patient tumour-derived SMU-030 parental and two resistant clones, patient-derived BRAFi-resistant lines WMD-009, SMU-027 and ICI-resistant lines SMU-011, SMU-013, SMU-015 and SCC-16 were derived as described in Table 1. Cells were cultured in RPMI (Gibco) with 10% heat-treated foetal bovine serum (FBS; Bovogen), 1 mM sodium pyruvate (Gibco), GlutaMAX (Gibco), 20 mM HEPES (Sigma-Aldrich) and antibiotic–antimycotic. Cell cultures were maintained in a Binder low-oxygen incubator at 37°C, 5% CO_2 and 2% O_2.

To generate BRAFi-resistant cells, YUMMUV1.7 and A2058 cells were grown continuously in increasing concentrations of dabrafenib up to 4 μM. Once cells were proliferating normally in 4 μM dabrafenib, they were grown on drug for another 2 weeks, and then a dose response of dabrafenib was performed to confirm resistance status.

2.2 | Dose response of sensitive/resistant lines

All cell lines were seeded in 96-well plates and incubated in a 37°C incubator overnight, then treated with increasing concentrations of the dabrafenib or CHK1i (SRA737, Sierra Oncology) in combination with 0.2 mM HU (Sigma). Cells were assessed for viability after treatment for 3 days using resazurin (Sigma). Assays were performed in quadruplicate.

TABLE 1 Characteristic of the melanoma cell lines used.

Cell line	Genotype	Details
YUMMUV1.7 parent	BRAFV600E pre-treatment	
YUMMUV1.7 Dab-R	BRAFV600E, unknown	Progressed on dabrafenib
A2058 parent	BRAFV600E pre-treatment	
A2058 Dab-R	BRAFV600E, unknown	Progressed on dabrafenib
WM164 parent	BRAFV600E pre-treatment	
WM164 Dab-R	BRAFV600E, unknown	Progressed on vemurafenib (Menon et al., 2015)
SMU-030 Pre	BRAFV600K pre-treatment	(Ming et al., 2023)
SMU-030 R1	BRAFV600K, BRAF amplification	Progressed on CombiDT (Ming et al., 2023)
SMU-030 R2	BRAFV600K, unknown	Progressed on CombiDT (Ming et al., 2023)
SKMEL28 parent	BRAFV660E pre-treatment	
SKMEL28 BR2	BRAFV600E, NRASQ61H	Progressed on dabrafenib (Ming et al., 2023)
SKMEL28 BR4	BRAFV600E splice (exon4-10)	Progressed on dabrafenib (Ming et al., 2023)
SKMEL28 BR9	BRAFV600E dedifferentiated	Progressed on dabrafenib (Ming et al., 2023)
WMD-009	BRAFV600E, BRAF exon 2-10Δ	Progressed on dabrafenib (Ming et al., 2023)
SMU-027	BRAFV600E, unknown	Progressed on vemurafenib (Ming et al., 2023)
SCC16-0016	NRASQ61E/K, JAK2 mutation	Progressed on pembrolizumab, previously on Ipilimumab + Pembrolizumab (Lee et al., 2020)
SMU11-0376 M2	BRAFV600E, PTEN loss	Progressed on Nivolumab (Lee et al., 2020)
SMU13-0183 M3	BRAFV600E, dedifferentiated	Progressed on Nivolumab (Lee et al., 2020)
SMU15-0404	BRAF G469R/S, dedifferentiated	Progressed on pembrolizumab (Lee et al., 2020)

Abbreviation: CombiDT, combination dabrafenib and trametinib.

2.3 | Mouse tumour assays

Experiments were performed with approval from The University of Queensland Animal Ethics Committee (2017/AE000211). Syngeneic mouse YUMMUV1.7 tumours were established in C57BL/6J mice and treated with CHK1i (SRA737)+LDHU as described previously (Proctor et al., 2021). Tumours were excised on Day 10 after treatment and processed for staining with Live/Dead Aqua (Thermo Fisher Scientific) and a panel of lymphoid cell markers (CD45.2-PE dazzle, CD3-FITC, TCRβ-PercpCy5.5, CD8α-BV605, CD4-AF700, NK1.1-PE-Cy7, NKp46- BV785). Stained cells were analysed using LSR-Fortessa X20 Flow Cytometer (BD BioSciences) with FACSDiva software (Becton Dickinson). Flow-Count™ Fluorospheres (Beckman Coulter) were used for assessment of total cell counts. Data analyses were performed using FlowJo software (Treestar Inc.).

Human tumours were xenografted onto nude mice, and tumour growth was followed as described previously (Oo et al., 2019).

2.4 | Time-lapse cell viability killing assay

YUMMUV1.7 (Parent and DabR Tx, a BRAFi-resistant line grown from the remaining tumour material after CHK1i+LDHU treatment) and A2058 (Parent and DabR, a BRAFi-resistant line generated in-house) cells were seeded into 96-well plates and treated with or without 1 μM BRAFi (dabrafenib). Sytox Green (250 nM; Molecular Probes) to mark dead cells was also added, and cells were imaged every 4 h for up to 5 days using an IncuCyte S3 live cell imaging system. Analysis was performed using IncuCyte software to identify total number of cells and percentage of cells positive for Sytox Green staining.

2.5 | Statistical analysis

All statistical analyses were done using GraphPad Prism 9. Bar graphs display mean values and standard deviation. For pairs of sensitive/resistant lines, unpaired Student's *t*-test were performed with p value $< .05$.

3 | RESULTS AND DISCUSSION

We assessed the efficacy of CHK1i+LDHU treatment in a panel of melanoma cell lines with resistance to BRAF inhibitor (BRAFi) acquired either in vitro or from patients who have developed resistance to indicated treatment (Table 1). These represent different mechanisms of acquisition and resistance. This assessment included four sets of cell lines with pre-treatment and resistant pairs (or multiple resistant clones for SKMEL28). The parental cell lines were shown to be sensitive to BRAFi (dabrafenib) with $IC_{50} < 0.1$ μM, whereas the resistant lines were >10 fold less sensitive (Figure 1). When sensitivity to CHK1i (SRA737)+LDHU was assessed in these BRAFi-sensitive and BRAFi-resistant pairs, both lines were inhibited similarly (Figures 1, 2a). To further define whether tumour cells from patients that had acquired resistance to BRAFi or ICIs are sensitive to CHK1i+LDHU, six more cell lines were assessed, including two melanoma cell lines derived from patients with acquired BRAFi resistance and four cell lines from patients who had acquired resistance to different ICIs (Table 1). All of these cell lines displayed similar sensitivity to CHK1i+LDHU (Figure 2b,c), and five out of six displayed IC_{50} values around 2 μM for CHK1i (Figure 2d–f; Data S1) which is below the 6 μM Cmax found in patients (Plummer et al., 2019). One cell line, WMD-009, did not achieve 50% growth inhibition (Figure 2b).

To assess whether patient-derived BRAFi-sensitive and BRAFi-resistant melanomas were sensitive to CHK1i+LDHU treatment in vivo, SUM-030 parental and R2 resistant lines were grown as

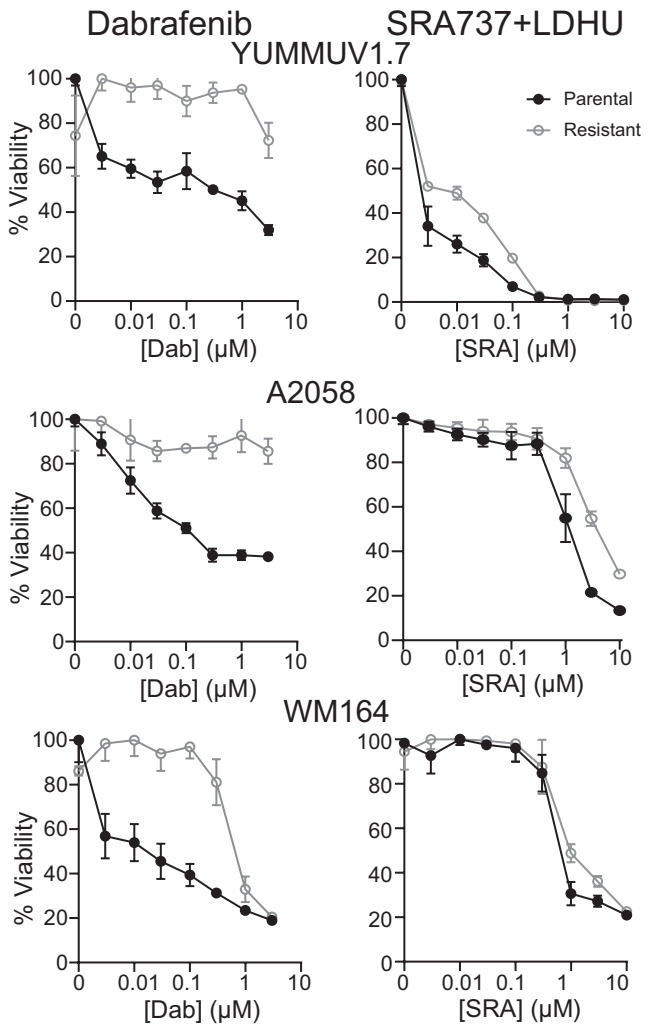

FIGURE 1 Dose response of the indicated melanoma cell lines—dabrafenib-sensitive parental and acquired resistance pairs—to dabrafenib or CHK1i (SRA737) with a constant 0.2 mM HU: (a) YUMMUV1.7 (mouse), (b) A2058 (human), (c) WM164 (human). Cell viability was measured by resazurin assay, and the data are the mean and SD of triplicate determinations. These are representative of duplicate experiments.

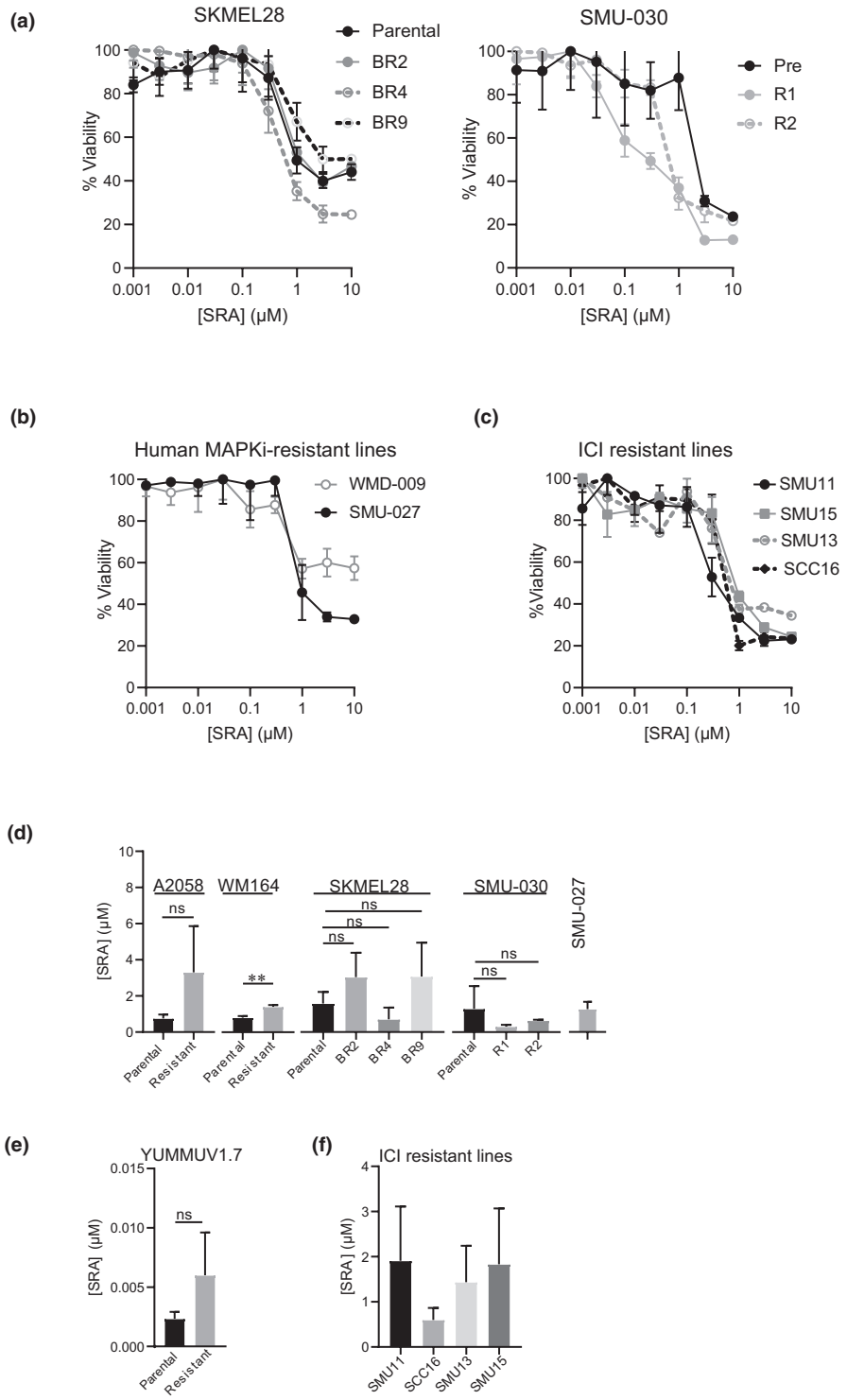

FIGURE 2 Dose response of the indicated cell lines—(a) SKMEL28 human melanoma cell lines, either parental or three acquired BRAFi-resistant clones, SMU-030 patient-derived cell lines pre-treatment (Pre) and two clones from progression on combination BRAFi + MEKi (Table 1), (b) patient-derived BRAFi-resistant melanoma cell lines, (c) patient-derived ICI-resistant lines—to CHK1i (SRA737) + 0.2 mM HU. Cell viability was measured by resazurin assay, and the data are the mean and SD of triplicate determinations. These are representative of at least three separate experiments. (d–f): IC50 values from the experiments shown in Figures 1, 2a–c.

xenografts in immunocompromised mice. Interestingly, only the BRAFi-resistant line grew as a tumour, and the parental line only produced a barely palpable tumour by 60 days after implanting. When the R2 BRAFi-resistant tumours were treated with the normal regimen of CHK1i + LDHU, it was found to have sensitivity to the treatment (Figure 3).

Finally, we have previously reported that CHK1i + LDHU triggers an immune response in vivo characterised by increased natural killer (NK) cells and NK-T cell (NKT) infiltration into syngeneic mouse melanomas with treatment (Proctor et al., 2021). Using the BRAFi-sensitive and BRAFi-resistant YUMMUV1.7 pairs developed in-house, CHK1i + LDHU treatment inhibited tumour growth in an identical manner regardless of their sensitivity to BRAFi (Figure 3a,b) and promoted a very similar pattern of immune cell infiltration (Figure 3c). As observed previously, the major immune response to CHK1i + LDHU was the high level of NKT cells infiltrating into tumours, identified as $CD45^+$ $CD3^+$ $TCR\beta^+$ $NK1.1^+$ $CD4^-$ $CD8^-$ $NKp46^-$. This population increased significantly after CHK1i + LDHU

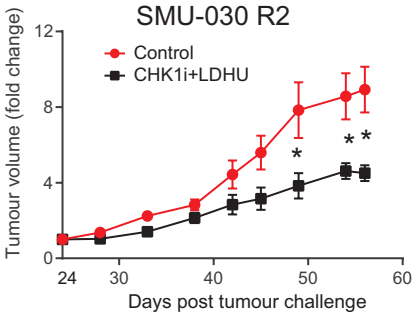

FIGURE 3 SMU-030 R2 human BRAFi-resistant melanoma was established in immune compromised mice. When a tumour had reached >100 mm^3, treatment as in A was commenced (Day 24). Tumour growth is presented as fold increase over Day 24. The * indicated p value < .05 at individual time points.

treatment in both BRAFi-sensitive and BRAFi-resistant models (Figure 3c). The NK cell population (CD45$^+$ CD3$^-$ TCRβ$^-$ NK1.1$^+$ CD4$^-$ CD8$^-$ NKp46$^+$) also increased to a similar degree in both models, whereas there was no effect on the recruitment of CD4$^+$ or CD8$^+$ T cell populations to the tumour sites, as reported previously (Proctor et al., 2021). The BRAFi-resistant YUMMUV1.7 line retained its resistance to BRAFi during CHK1i+LDHU treatment, as a line grown from the remaining tumour material after treatment showed reduced sensitivity to killing by BRAFi (dabrafenib), similar to BRAFi-resistant A2058 line (Data S1; Figure 4).

Together, these data indicated that the majority of melanoma cell lines tested are sensitive to the CHK1i+LDHU combination at concentrations of CHK1i that are readily achievable in patients. Sensitivity to the combination is not significantly affected by acquired resistance to either BRAFi, BRAFi+MEKi combinations, or resistance to immunotherapy. Resistance to BRAFi has been acquired through a variety of mechanisms that result in reactivation of the MAPK signalling pathway (Czarnecka et al., 2020) and increased replication stress (Yang et al., 2021). This suggests that BRAFi resistance may increase sensitivity to treatments targeting the replication stress such as the CHK1i+LDHU combination, although that was not observed here. Several mechanisms of resistance to immunotherapy have been identified including reduced MHC I expression (Lee et al., 2020). The cell lines from ICI-resistant patients used here have reduced MHC I expression and were associated with increased AXL expression and dedifferentiation. However, inhibition of AXL increased DNA damage and replication stress (Ramkumar et al., 2021). Thus, the mechanism by which MHC I expression is reduced is more likely to be involved in tolerance of replication stress which could reduce sensitivity to treatments targeting replication such as WEE1, ATR and CHK1 inhibitors, although it did not reduce sensitivity to CHK1i+LDHU. The majority of BRAFi and ICI treatment-resistant tumours demonstrated to be sensitive to the CHK1i+LDHU combination in this study mirrors our previous studies that reported >70% of melanomas were sensitive to this combination (Oo et al., 2019). This high proportion of sensitive melanomas to CHK1i+LDHU and their remained sensitivity after

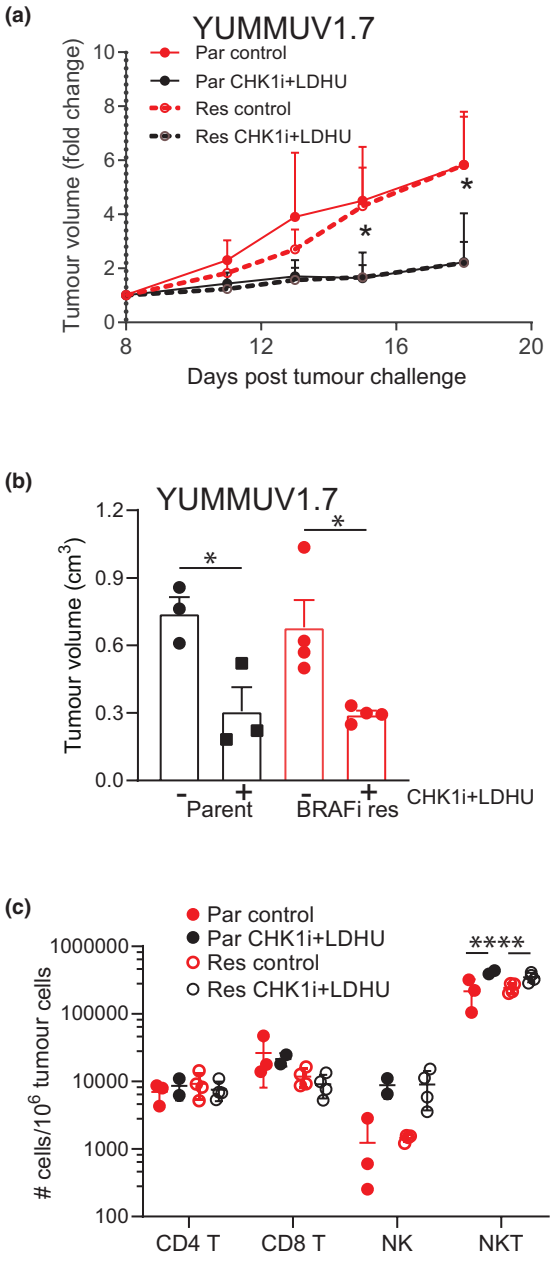

FIGURE 4 (a): Syngeneic mouse melanoma YUMMUV1.7 was established in immune competent mice, either BRAFi-sensitive parental (Par) or resistant (Res). Once a tumour had reached >100 mm^3, mice were treated with vehicle control or CHK1i (SRA737)+LDHU, as previously described (Proctor et al., 2021). Tumour growth was measured and reported, and the fold increases over Day 8 (first day of treatment). The * indicated p value < .05 at individual time points. (b): The tumour volume at the end of treatment. (c): The immune cell profile in the tumour microenvironment was assessed 10 days after drug treatment started. The abundance of the indicated major immune cell types assessed, CD4$^+$ and CD8$^+$ T cells, NK and NKT cells, was shown as number of immune cells/10^6 tumour cells for each mouse.

acquiring resistance to current clinical treatments suggests that CHK1i+LDHU combination could be a useful treatment in patients that have exhausted current therapy options.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Zhen Zeng https://orcid.org/0000-0002-8120-0973
Helen Rizos https://orcid.org/0000-0002-2094-9198
Brian Gabrielli https://orcid.org/0000-0003-3933-1651

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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