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RESEARCH ARTICLE

# Therapeutic efficacy of the resorcylic acid lactone LL-Z1640-2 for adult T-cell leukaemia/lymphoma

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## Abstract

Adult T-cell leukaemia/lymphoma (ATL) remains incurable. The NF-κB and interferon regulatory factor 4 (IRF4) signalling pathways are among the critical survival pathways for the progression of ATL. TGF-β-activated kinase 1 (TAK1), an IκB kinase-activating kinase, triggers the activation of NF-κB. The resorcylic acid lactone LL-Z1640-2 is a potent irreversible inhibitor of TAK1/extracellular signal-regulated kinase 2 (ERK2). We herein examined the therapeutic efficacy of LL-Z1640-2 against ATL. LL-Z1640-2 effectively suppressed the in vivo growth of ATL cells. It induced in vitro apoptosis and inhibited the nuclear translocation of p65/RelA in ATL cells. The knockdown of *IRF4* strongly induced ATL cell death while downregulating MYC. LL-Z1640-2 as well as the NF-κB inhibitor BAY11-7082 decreased the expression of IRF4 and MYC at the protein and mRNA levels, indicating the suppression of the NF-κB-IRF4-MYC axis. The treatment with LL-Z1640-2 also mitigated the phosphorylation of p38 MAPK along with

the expression of CC chemokine receptor 4. Furthermore, the inhibition of STAT3/5 potentiated the cytotoxic activity of LL-Z1640-2 against IL-2-responsive ATL cells in the presence of IL-2. Therefore, LL-Z1640-2 appears to be an effective treatment for ATL. Further studies are needed to develop more potent compounds that retain the active motifs of LL-Z1640-2.

**KEYWORDS**
ATL, HTLV-1, IRF4, NF-κB, TAK1

## 1 | INTRODUCTION

Adult T-cell leukaemia/lymphoma (ATL) is caused by human T-cell leukaemia virus type 1 (HTLV-1) infection. The acute and lymphoma types of ATL show aggressive progression with a dismal prognosis [1–3]. The NF-κB signalling pathway is vital downstream of the T-cell receptor (TCR) in malignant progression of HTLV-1-infected cells [4]. Genetic alterations have been reported in multiple genes associated with the TCR-NF-κB signalling pathway in ATL cells, including *PLCG1*, *PRKCB* and *CARD11*, indicating the aberrant activation of NF-κB [5]. Gene alterations enriched in TCR and NF-κB signalling pathways are associated with a poor prognosis in patients with ATL [5, 6]. Therefore, NF-κB appears to be an important therapeutic target in ATL cells, and the development of novel agents that effectively preclude the activation of the NF-κB signalling pathway is urgently needed to overcome the aggressiveness of ATL cells with drug resistance.

Transforming growth factor-β activated kinase-1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family that regulates several signalling pathways, including NF-κB signal transduction and p38 mitogen-activated protein kinase (MAPK) activation [7]. The aberrant activation of NF-κB is generally considered to be essential for inflammation and cancer progression. LL-Z1640-2, also known as 5Z-7-oxozeaenol, is a resorcylic acid lactone-related compound that was discovered in fungi as an irreversible inhibitor of TAK1/extracellular signal-regulated kinase 2 (ERK2) [8]. Among a series of structurally related resorcylic acid lactones, LL-Z1640-2 has been identified as the most promising compound based on its inhibitory activity against cell proliferation and the findings of NF-κB activation assays [9]. Furthermore, LL-Z1640-2 was shown to be the most effective inhibitor of the activation of synovial fibroblasts in rheumatoid arthritis [10]. We previously reported that LL-Z1640-2 effectively suppressed inflammation and joint destruction in mice with collagen-induced arthritis as a model of rheumatoid arthritis [11]. Moreover, the therapeutic efficacy of TAK1 inhibition has been examined in preclinical models of various cancers, including mantle cell lymphoma [12], breast cancer [13], and ovarian cancer [14]. We demonstrated the therapeutic impact of LL-Z1640-2 on tumour progression and bone destruction in animal models of the bone-residing tumour multiple myeloma (MM), as well as osteoporotic bone loss in ovariectomised mice [15].

Therefore, the potent and irreversible inhibition of TAK1 by LL-Z1640-2 may be beneficial for suppressing the NF-κB and other survival signalling pathways in ATL cells. In the present study, we examined the therapeutic efficacy of LL-Z1640-2 for ATL. LL-Z1640-2 effectively suppressed ATL growth in vivo, and induced in vitro apoptosis and inhibited the nuclear translocation of p65/RelA in ATL cells. LL-Z1640-2 and the inhibition of NF-κB reduced the expression of IRF4 and MYC at the protein and mRNA levels. Therefore, LL-Z1640-2 appears to suppress the NF-κB-IRF4-MYC axis in ATL cells. Furthermore, the inhibition of STAT3/5 potentiated the cytotoxic activity of LL-Z1640-2 against interleukin-2 (IL-2)-responsive ATL cells in the presence of IL-2. Based on these results, LL-Z1640-2 inhibits critical signalling pathways for ATL cell survival and function and, thus, is an effective treatment for ATL. Further studies are needed to develop potent compounds based on LL-Z1640-2 as a lead compound to inhibit multiple kinases relevant to the drug resistance of ATL cells.

## 2 | MATERIALS AND METHODS

For more information regarding this section, see Supporting Information.

### 2.1 | Cells and culture

The human ATL cell line TL-Om1 and the human HTLV-1-infected cell lines MT-2 and Hut102 were kindly provided by Dr. Fujiwara (Mie University). TL-Om1 cells expressing *luciferase* (TL-Om1-Luc) were generated by retrovirally transducing the MSCV-Luc vector into TL-Om1 cells. The human ATL cell lines Su9T01, KOB, KK1, LMY1 and ST1 were kindly provided as follows: Su9T01 by Dr. Yoshimitsu (Kagoshima University) and KOB, KK1, LMY1 and ST1 by Dr. Hasegawa (Nagasaki University). The 293T cell line was purchased from the American Type Culture Collection (Manassas). Peripheral blood mononuclear cells (PBMCs) were prepared from blood drawn from healthy volunteers or patients with ATL by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) density gradient centrifugation. Primary ATL samples were then isolated from PBMCs using the CD4 MicroBeads separation system (Miltenyi Biotec). All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki using a protocol approved by the Institutional Review Board (3582 in Tokushima University Hospital).

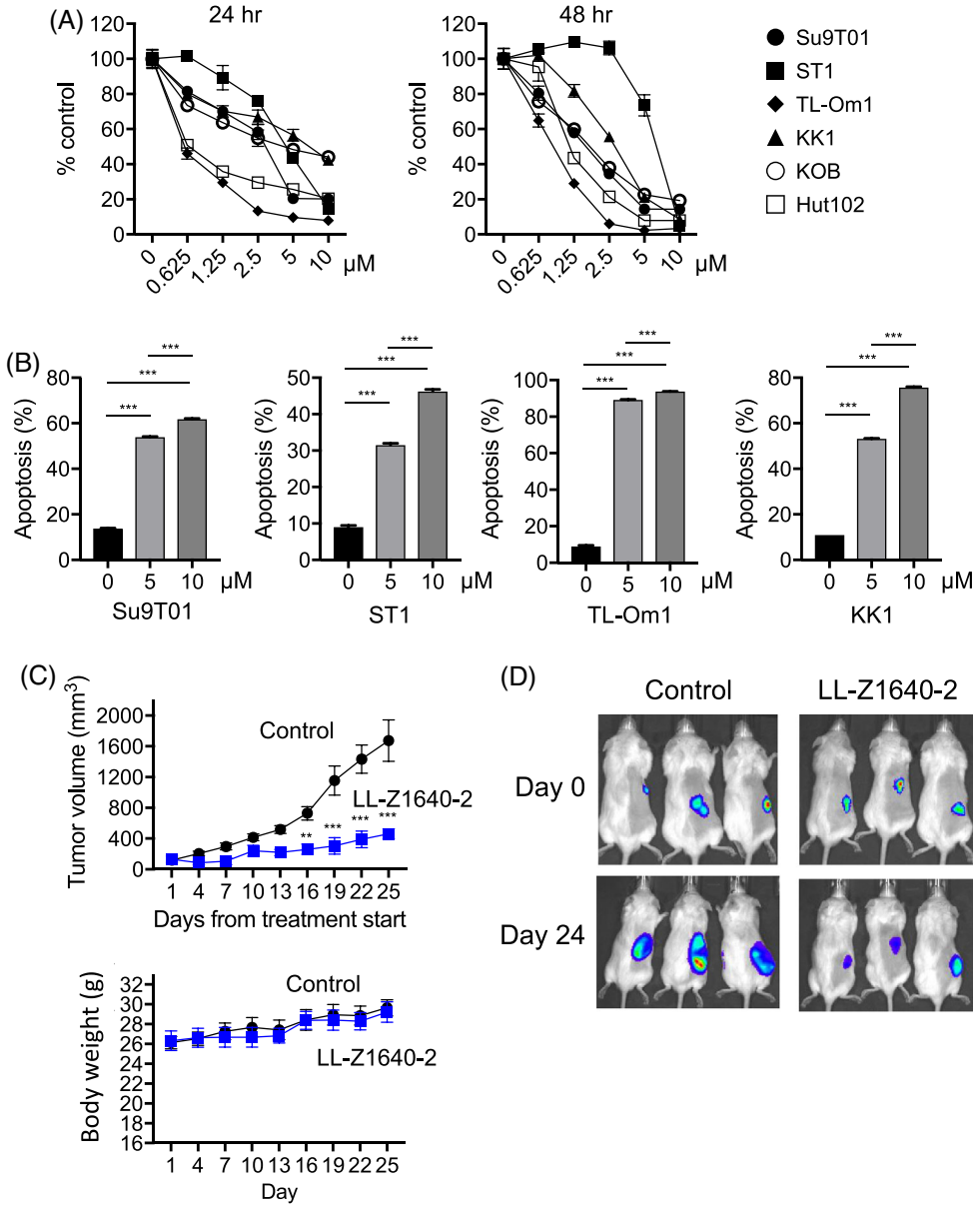


**FIGURE 1** LL-Z1640-2 suppresses ATL cell growth and survival in vitro and in vivo. (A) ATL cell lines were cultured in triplicate in the presence of LL-Z1640-2 at the indicated concentrations for 24 and 48 h. Cell viability was assessed by a CCK-8 assay. Data are expressed as a change from the control without LL-Z1640-2 (means ± SD). (B) The indicated ATL cell lines were treated with LL-Z1640-2 at the indicated concentrations for 24 h. Apoptosis was detected using the annexin V-propidium iodide (PI) assay. The percentage distributions of annexin V-positive apoptotic cells were analysed (means ± SD). ***$p < 0.001$. TL-Om1-Luc cells were subcutaneously inoculated in NOD/SCID mice. After the development of measurable tumours (>50 $mm^3$), mice were treated with LL-Z1640-2 at 20 mg/kg ($n = 8$, blue line) or a vehicle control ($n = 8$, black line) 3 days a week for 3 weeks. (C) Tumour growth was monitored with callipers, and mouse bodyweight was measured every 3 days (means ± SD). **$p < 0.01$, ***$p < 0.001$. (D) Tumour sizes were also assessed on Day 24 via in vivo bioluminescence measurements using the IVIS Imaging System. Regarding luciferase detection imaging, 100 $\mu$L of 15 mg/mL D-luciferin in PBS was injected intraperitoneally before taking images. Representative IVIS images on Days 0 and 24 are shown.

## 2.2 | Transduction

Lentiviral shRNA-containing media were produced according to our previous methods [16, 17]. Briefly, pLKO-based plasmids were transfected into 293T cells in combination with pCMV-dvpr and VSV-G for lentiviral packaging using TransIT-LT1 Transfection Reagent (Mirus Bio). Virus-containing media were then harvested. Cells were transfected with lentivirus-containing media in the presence of 8 mg/mL polybrene. After an incubation for 3–5 h in six-well plates, cells were washed with PBS and then cultured with fresh growth media. Cells were selected with 1 $\mu$g/mL puromycin for 48 h for further experiments.

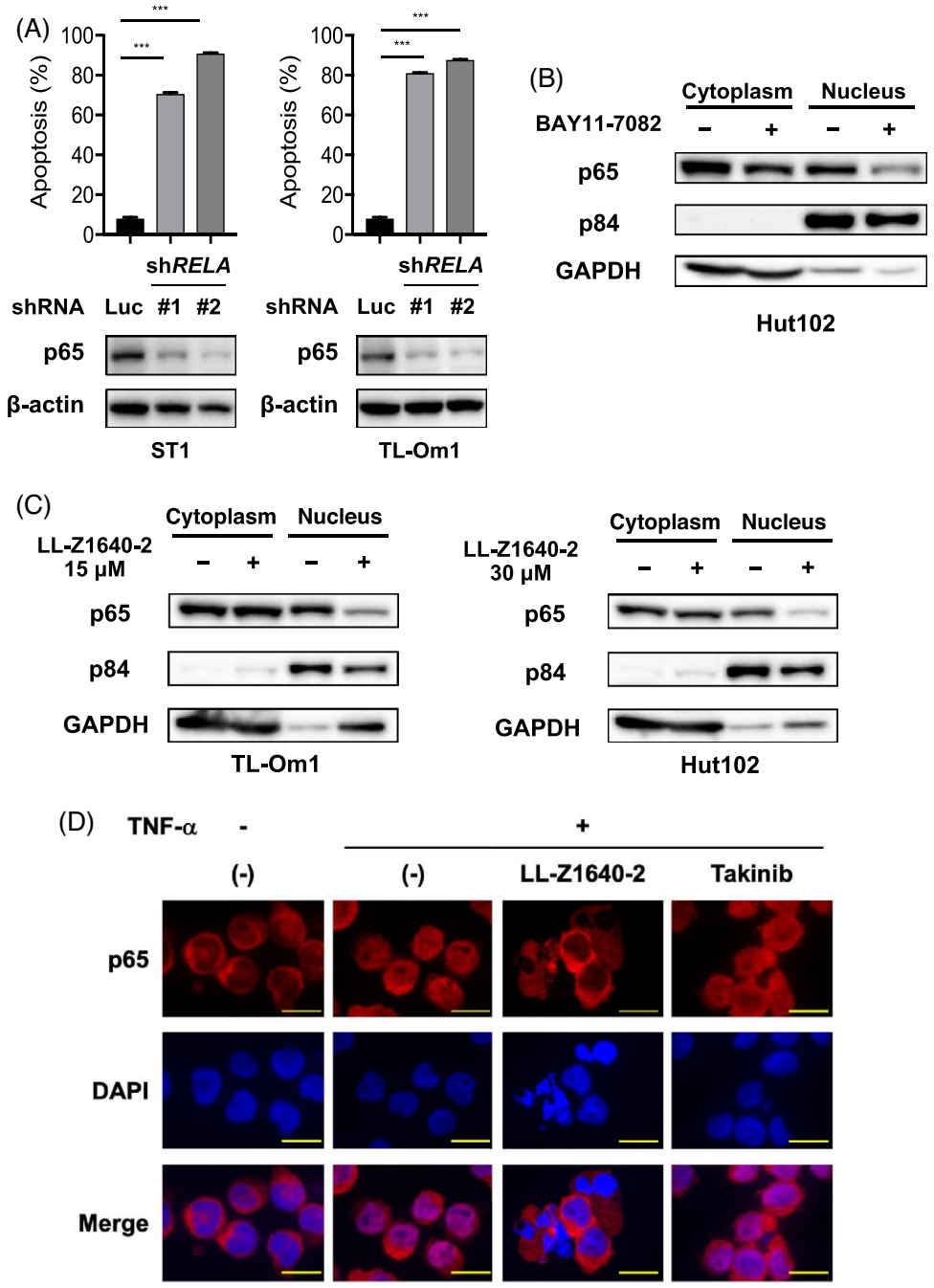


**FIGURE 2** LL-Z1640-2 abrogates NF-κB activation to induce ATL cell death. (A) ST1 and TL-Om1 cells were transduced with human *RELA* or *Luc* shRNA. After puromycin selection, the percentage distributions of annexin V-positive apoptotic cells were analysed using an annexin V-PI assay (means ± SD). ***$p < 0.001$. Extracted cell lysates were subjected to immunoblotting to detect the p65 protein. β-Actin served as a loading control. (B) Hut102 cells were treated with BAY11-7082 at 10 μM for 5 h. The p65 protein levels were analysed in cytoplasmic and nuclear extracts by immunoblotting. (C) TL-Om1 and Hut102 cells were treated with LL-Z1640-2 at the indicated concentrations for 5 h. The p65 protein levels were analysed in cytoplasmic and nuclear extracts by immunoblotting. p84 and GAPDH served as loading controls for the nuclear and cytoplasmic extracts, respectively. (D) Su9T01 cells were cultured in RPMI 1640 with 1% FBS for 12 h, and LL-Z1640-2 or takinib at 10 μM was then added as indicated. Cells were incubated for a further 5 h. TNF-α at 10 ng/mL was added as indicated for the last 30 min of the incubation period. Cells were fixed and stained with the anti-p65 antibody (red), and their nuclei were stained with DAPI (blue). p65 localisation was observed using a fluorescence microscope. Scale bars represent 20 μm. Original magnification ×40. (E) TL-Om1 and Su9T01 cells were treated with 10 μM LL-Z1640-2 for 8 h. Total RNA was isolated and *RELA* mRNA expression was measured using qPCR. *PPIE* served as an internal control. Data represent the means ± SD ($n = 3$). ***$p < 0.001$. (F) TL-Om1 and Hut102 cells were transduced with human *RELA* or *Luc* shRNA. After puromycin selection, cell lysates were extracted. p65, TAK1 and MYC protein levels were analysed by immunoblotting. (G) TL-Om1 and Hut102 cells were treated for 24 h with BAY11-7082 at the indicated concentrations. (F and G) Cell lysates were subjected to immunoblotting to analyse TAK1 and MYC protein levels. β-Actin served as a loading control.

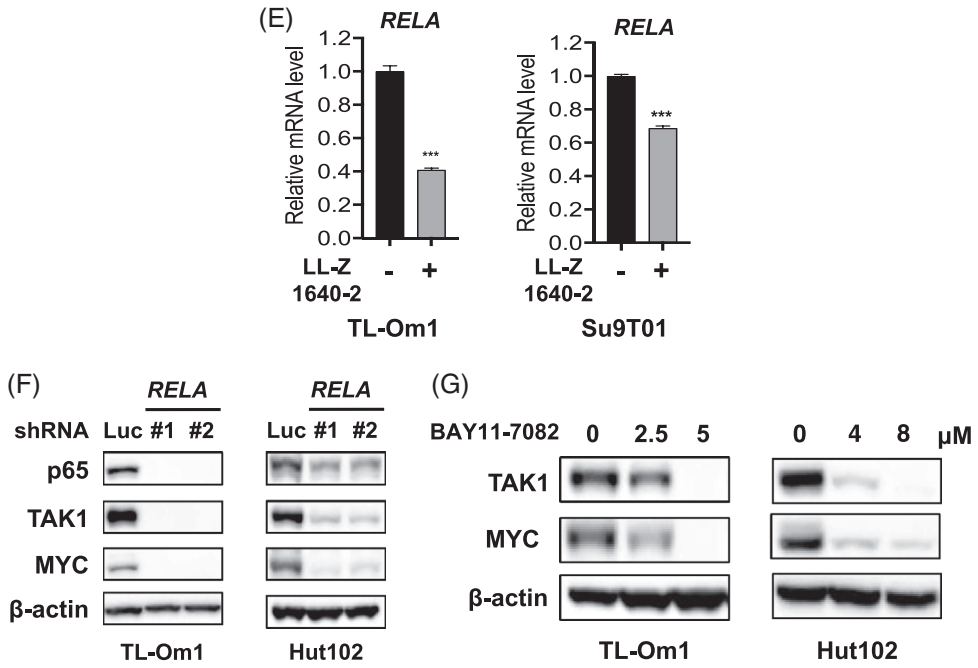


**FIGURE 2** Continued

## 2.3 | Statistical analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, version 7). Data distributions were analysed using the $X^2$ test. Statistical differences were assessed with the Student's *t*-test for two normally distributed groups and an ordinary one-way or two-way analysis of variance (ANOVA) with Tukey's test, Sidak's test or Dunnett's test for multiple normally distributed groups. Differences in survival were evaluated by the log-rank test. *p*-Values less than 0.05 were considered to be significant.

# 3 | RESULTS

## 3.1 | LL-Z1640-2 suppresses ATL cell growth and survival

Publicly available data showed that *MAP3K7*, a gene of TAK1, is upregulated in ATL cells (Figure S1A). The NF-κB signalling pathway is essential for the survival and proliferation of ATL cells. As TAK1, an IκB kinase-activating kinase, triggers the subsequent activation of the NF-κB signalling pathway, we initially examined the cytotoxic effects of TAK1 inhibitors on ATL cells. LL-Z1640-2 is a unique irreversible inhibitor of TAK1 and ERK2 [9]. LL-Z1640-2 dose-dependently induced cell death with apoptosis in all ATL cell lines (Figure 1A) and primary ATL cells (Figure S1B). The treatment with LL-Z1640-2 induced apoptotic cell death, as shown by the results of the annexin V-PI assay (Figure 1B and Figure S1C). However, the reversible TAK1-specific inhibitor takinib at the same range of concentrations as LL-Z1640-2 only weakly induced cell death in the ATL cell lines (Figure S1D). We previously reported the therapeutic efficacy and safety of LL-Z1640-2 using mouse models of MM with the intra-tibial inoculation of MM cells, ovariectomised mice as an osteoporosis model, and mice with collagen-induced arthritis as a rheumatoid arthritis model [11, 15]. Following these experimental procedures, we intraperitoneally injected LL-Z1640-2 in mouse xenograft models bearing TL-Om1 cells. Tumour growth was gradual for the first 2 weeks, and became accelerated thereafter in control mice (Figure 1C, upper). However, the treatment with LL-Z1640-2 suppressed tumour growth (Figure 1C,D). The distribution of bodyweights was similar between the treatment and control groups at each time point (Figure 1C, lower). These results demonstrate the therapeutic efficacy and safety of LL-Z1640-2 as a treatment for ATL.

## 3.2 | LL-Z1640-2 abrogates the activation of NF-κB to induce ATL cell death

We investigated the mechanisms underlying the therapeutic effects of LL-Z1640-2. The NF-κB signalling pathway is among the critical survival pathways for the progression and drug resistance of ATL [18]. In line with the conventional role of TAK1 in NF-κB activation, we examined the involvement of NF-κB activation in ATL cell death by LL-Z1640-2. The knockdown of the *RELA* gene strongly induced apoptosis in ATL cells (Figure 2A). BAY11-7082 is an IκBα inhibitor that suppresses the activation of NF-κB (Figure 2B). The treatment with BAY11-7082 reduced the nuclear localisation of p65 in ATL cells. LL-Z1640-2 also reduced the nuclear localisation of p65, indicating the suppression of NF-κB activity (Figure 2C). To further confirm the effects of LL-Z1640-2, we examined the effects of LL-Z1640-2 on

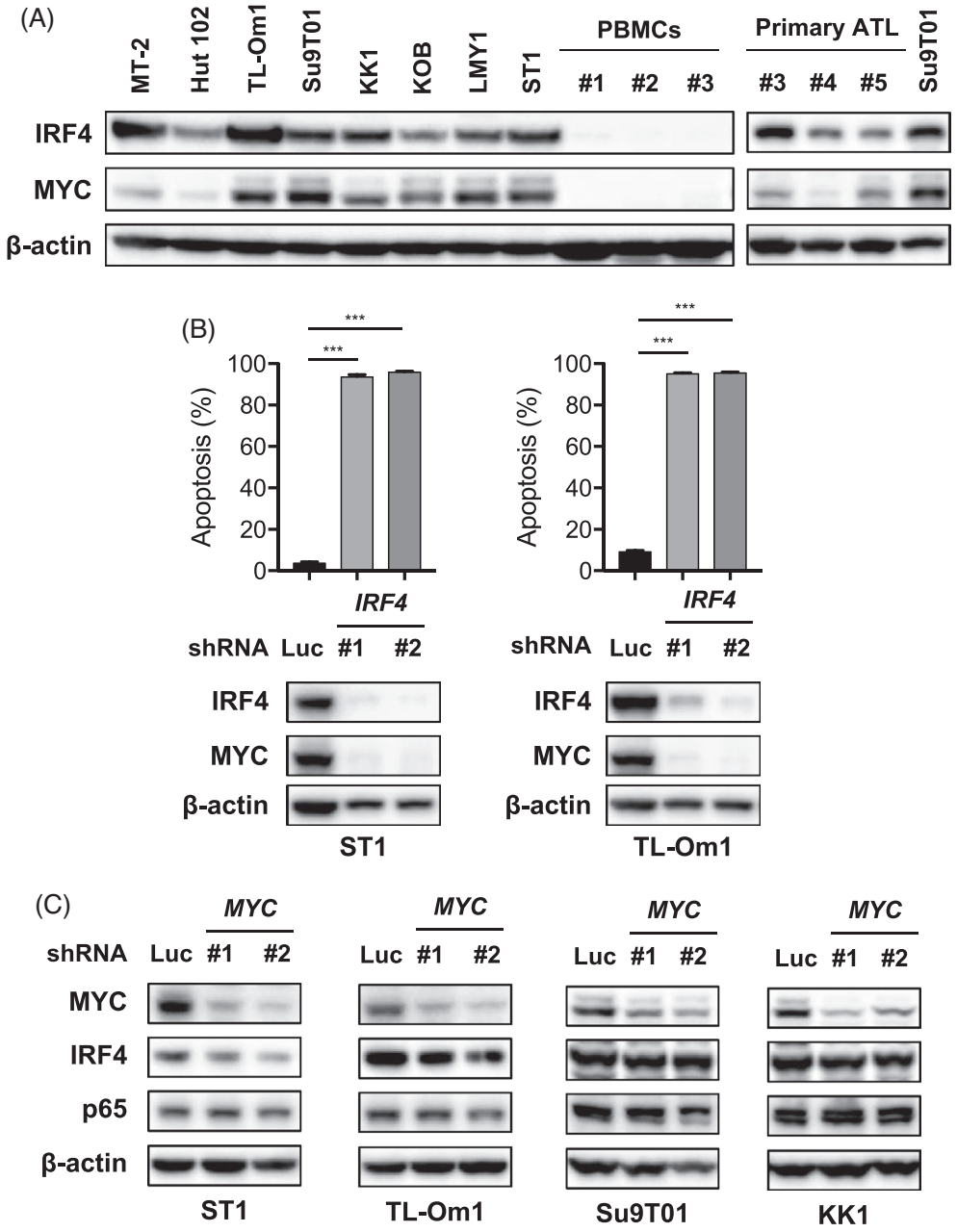


**FIGURE 3** IRF4 is overexpressed as a vital survival factor in ATL cells. (A) Whole-cell lysates, which were extracted from the ATL cell lines, indicated peripheral blood mononuclear cells (PBMCs) from healthy donors, and primary CD4-positive cells derived from patients with ATL were subjected to immunoblotting using the indicated antibodies. $\beta$-Actin served as a loading control. (B) ST1 and TL-Om1 cells were transduced with human *IRF4* or *Luc* shRNA. After puromycin selection, the percentage distributions of annexin V-positive apoptotic cells were analysed using the annexin V-PI assay (means ± SD). $^{***}p < 0.001$. The extracted cell lysates were subjected to immunoblotting to analyse IRF4 and MYC protein levels. $\beta$-Actin served as a loading control. (C) The indicated ATL cell lines were transduced with human *MYC* or *Luc* shRNA. After puromycin selection, extracted cell lysates were subjected to immunoblotting to analyse MYC, IRF4 and p65 protein levels. $\beta$-Actin served as a loading control.

ligand-induced NF-$\kappa$B activation. The addition of TNF-$\alpha$ induced the translocalisation of p65 from the cytoplasm to nuclei; however, LL-Z1640-2 markedly reduced the nuclear localisation of p65 in ATL cells (Figure 2D). Takinib only slightly affected the nuclear localisation of p65 under these experimental conditions. These results demonstrated that LL-Z1640-2 effectively suppressed NF-$\kappa$B activity in ATL cells. We also showed that LL-Z1640-2 suppressed the expression of *RELA* at the mRNA level (Figure 2E), indicating the suppression of NF-$\kappa$B activity by LL-Z1640-2 through the kinase inhibition of TAK1 as an IKK kinase and a reduction in the expression of p65.

We previously reported that the activation of NF-$\kappa$B transcriptionally upregulated TAK1 expression in MM cells, thereby forming an auto-amplification loop between TAK1 activation and its expression levels [15]. Similarly, the knockdown of the *RELA* gene or a treatment

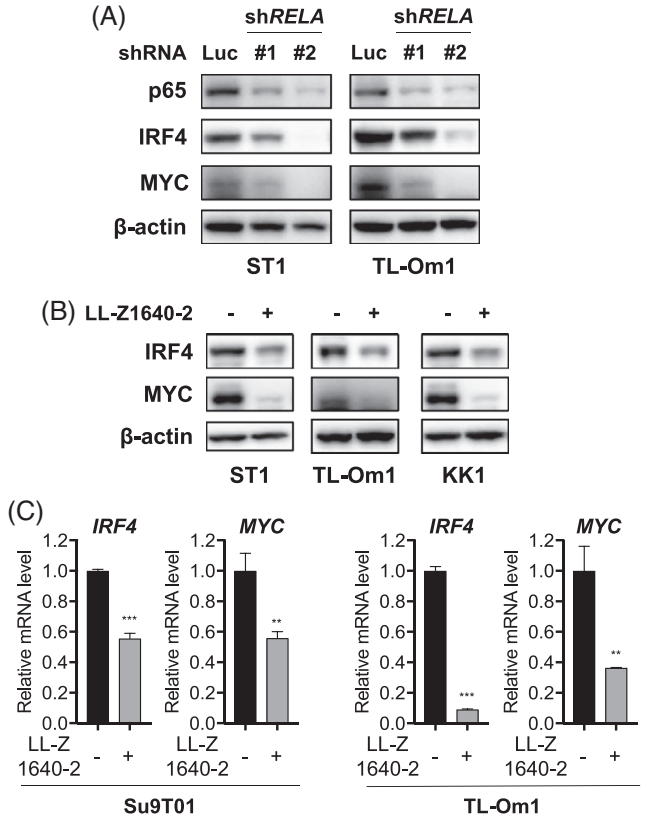


**FIGURE 4** LL-Z1640-2 suppresses the NF-κB-IRF4 axis in ATL cells. (A) ST1 and TL-Om1 cells were transduced with human *RELA* or *Luc* shRNA. After puromycin selection, the extracted cell lysates were subjected to immunoblotting to analyse p65, IRF4 and MYC protein levels. β-Actin served as a loading control. (B) The indicated ATL cell lines were cultured with or without LL-Z1640-2 at 10 μM for 24 h. The extracted cell lysates were subjected to immunoblotting to analyse IRF4 and MYC protein levels. β-Actin served as a loading control. (C) Su9T01 and TL-Om1 cells were treated with 10 μM LL-Z1640-2 for 8 h. Total RNA was isolated, and *IRF4* and *MYC* mRNA expression was assessed using qPCR. *PPIE* served as an internal control. Data represent the means ± SD ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$.

with BAY11-7082 reduced TAK1 and MYC protein levels (Figure 2F,G). ATL cells are also suggested to have the auto-amplification of TAK1 activation and its expression.

## 3.3 | IRF4 is overexpressed as a vital survival factor in ATL cells

Gene expression profiling for primary ATL samples previously demonstrated that the oncogenic transcription factor *IRF4* was highly expressed in ATL cells [5, 19]. IRF4 was constitutively overexpressed in all ATL cell lines and primary ATL cells examined in the present study (Figure 3A). MYC was also overexpressed in these ATL cells at various levels, but was not expressed by normal peripheral blood mononuclear cells. The knockdown of *IRF4* markedly induced apoptosis while downregulating the expression of MYC in ATL cells (Figure 3B). The knockdown of *MYC* did not affect the expression of IRF4 or p65 in ATL cells, suggesting the overexpression of MYC downstream of IRF4 and NF-κB (Figure 3C). There results demonstrate that IRF4 is overexpressed as a vital survival factor in ATL cells.

## 3.4 | LL-Z1640-2 suppresses the NF-κB-IRF4 axis in ATL cells

NF-κB has been shown to enhance IRF4 gene expression [20, 21], and the NF-κB-IRF4 axis has also been identified as a pivotal signalling pathway for the survival and proliferation of ATL cells [19]. The IRF4 and NF-κB binding was found to be enriched in super-enhancers in ATL cells and formed a feed-forward loop to coordinate the regulation of critical oncogenes, including *MYC* [19]. Consistent with these findings, the knockdown of *RELA* reduced both IRF4 and MYC in ATL cells (Figure 4A). As suggested by the suppression of NF-κB activity (Figure 2), LL-Z1640-2 reduced the expression of IRF4 and MYC in ATL cells at the protein and mRNA levels (Figure 4B,C), indicating the transcriptional regulation of *IRF4* and *MYC* gene expression downstream of NF-κB signalling in ATL cells in a manner that is inhibited by LL-Z1640-2.

## 3.5 | LL-Z1640-2 also suppresses signalling pathways other than the NF-κB-IRF4 axis

Although the primary target of LL-Z1640-2 is TAK1, LL-Z1640-2 has been shown to exert off-target effects, including inhibition of MEK1/2 and MKK3/6 [10]. The inhibition of MKK3/6-p38 MAPK signalling together with TAK1 by LL-Z1640-2 has been shown to contribute to the anti-inflammatory effects of LL-Z1640-2 in rheumatoid arthritis [11]. Therefore, we examined the effects of LL-Z1640-2 on p38 MAPK-mediated signalling. The treatment with LL-Z1640-2 reduced the phosphorylation of p38 MAPK in ATL cells (Figure 5A). ATL cells are characterised by the high expression of CC chemokine receptor 4 (CCR4) on their surface. The upregulation of CCR4 in ATL cells was previously shown to be dependent on p38 MAPK [22]. The treatment with LL-Z1640-2 reduced the surface levels of CCR4 on ATL cells (Figure 5B). Migratory activity toward CCL22 was reduced in the LL-Z1640-2-treated KOB and TL-Om1 cells (Figure S2). These results suggest that LL-Z1640-2 inhibits the clinical manifestation and sequelae mediated by p38 MAPK in ATL cells.

## 3.6 | LL-Z1640-2 and the STAT3/5 inhibitor SH-4-54 synergistically induce cytotoxic activity against ATL cells in the presence of IL-2

IL-2 plays a role in the survival and growth of IL-2-responsive ATL cells and HTLV-1-infected T cells. Among STAT family members, STAT3 and STAT5 are implicated in oncogenesis [23], and *STAT3* mutations frequently occur in ATL cells [5, 6]. Nevertheless, exogenous IL-2 has

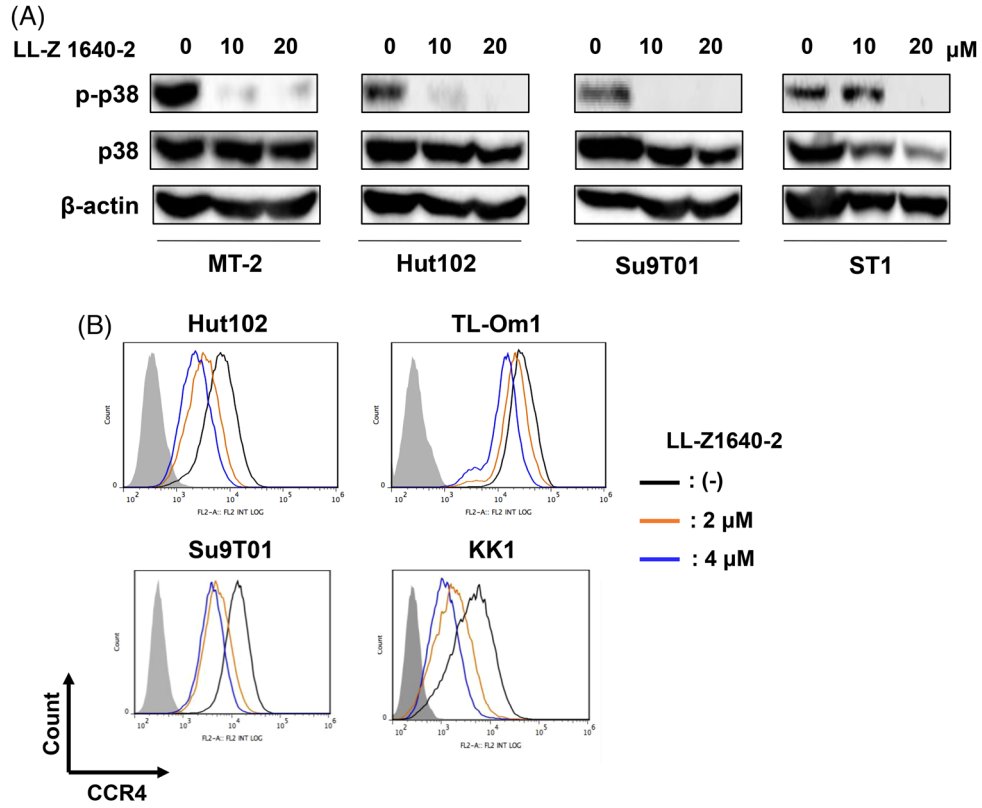


**FIGURE 5** LL-Z1640-2 suppresses signalling pathways other than the NF-κB-IRF4 axis. (A) The ATL cell lines indicated were treated with LL-Z1640-2 at the indicated concentration for 24 h. Whole-cell lysates were then extracted from treated cells and subjected to immunoblotting using the indicated antibodies. β-Actin served as a loading control. (B) The indicated ATL cell lines were cultured in the presence or absence of LL-Z1640-2 at the indicated condition for 24 h. The cell surface expression of CCR4 was analysed by flow cytometry. Grey areas indicate background staining with the isotype control IgG.

been shown to stimulate ATL cell growth and proliferation by activating JAK-STAT3/5 signalling [24, 25]. Therefore, we examined the cytotoxic effects of LL-Z1640-2 on ATL cells in combination with the STAT3/5 inhibitor SH-4-54. The addition of IL-2 blunted the cytotoxic activity of LL-Z1640-2 for IL-2-responsive TL-Om1 and Su9T01 cells (Figure 6A). The phosphorylation of STAT5 was induced in ATL cells in the presence of IL-2 (Figure 6B). STAT3 was constitutively phosphorylated in Su9T01 and Hut102 cells, but only slightly in TL-Om1 cells, and the phosphorylation of STAT3 was upregulated by a stimulation with IL-2 (Figure 6B). The STAT3/5 inhibitor SH-4-54 abolished the inducible and constitutive phosphorylation of both STAT3 and STAT5 in ATL cells (Figure 6C,D). However, LL-Z1640-2 was not able to inhibit the phosphorylation of STAT5 in TL-Om1 cells by IL-2 (Figure S3). We then examined the cytotoxic activity of LL-Z1640-2 in combination with SH-4-54 against ATL cells in the presence or absence of IL-2. The addition of IL-2 blunted the cytotoxic effects of LL-Z1640-2 in IL-2-responsive TL-Om1 cells (Figure 6E). However, SH-4-54 at 10 μM sensitised TL-Om1 cells to LL-Z1640-2, and LL-Z1640-2 and SH-4-54 cooperatively induced cell death in the presence of IL-2. The combination of LL-Z and SH4-54 exhibited synergistic cytotoxicity against TL-Om1 cells in the presence of IL-2 (Bliss synergy score of 10.743) (Figure 6E). The combination of LL-Z1640-2 and SH-4-54 more potently induced cell death in IL-2-dependent ST1 cells in the presence of IL-2 (Bliss synergy score of 36.106) (Figure 6F). The present results suggest that the inhibition of STAT3/5 potentiates the cytotoxic activity of LL-Z1640-2 against ATL cells in the presence of IL-2.

## 4 | DISCUSSION

The present results show that LL-Z1640-2 inhibited NF-κB and IRF4 signalling and induced cell death in ATL cells. TAK1 expression was transcriptionally upregulated in ATL cells by the activation of NF-κB downstream of TAK1, suggesting a feed-forward amplification mechanism of TAK1 by itself. One of the distinct characteristics of LL-Z1640-2 is its irreversibility, which differs from other TAK1 inhibitors, including takinib. Irreversible binding to TAK1 induces the potent and prolonged suppression of kinase activity [9], which may contribute to the stronger cytotoxic activity of LL-Z1640-2 against ATL cells than that of takinib. LL-Z1640-2 may also inhibit MKK3/6 and MEK1/2, which is regarded as an off-target effect of LL-Z1640-2 [10]. LL-Z1640-2 suppressed not only the activation of NF-κB, but also other mediators responsible for activation of growth and survival pathways in ATL cells, including p38 MAPK-mediated pathway (Figure 5). Multiple activated mutations in different factors may result in refractoriness to treatment with molecular targeted agents against ATL. Nonetheless, LL-Z1640-2

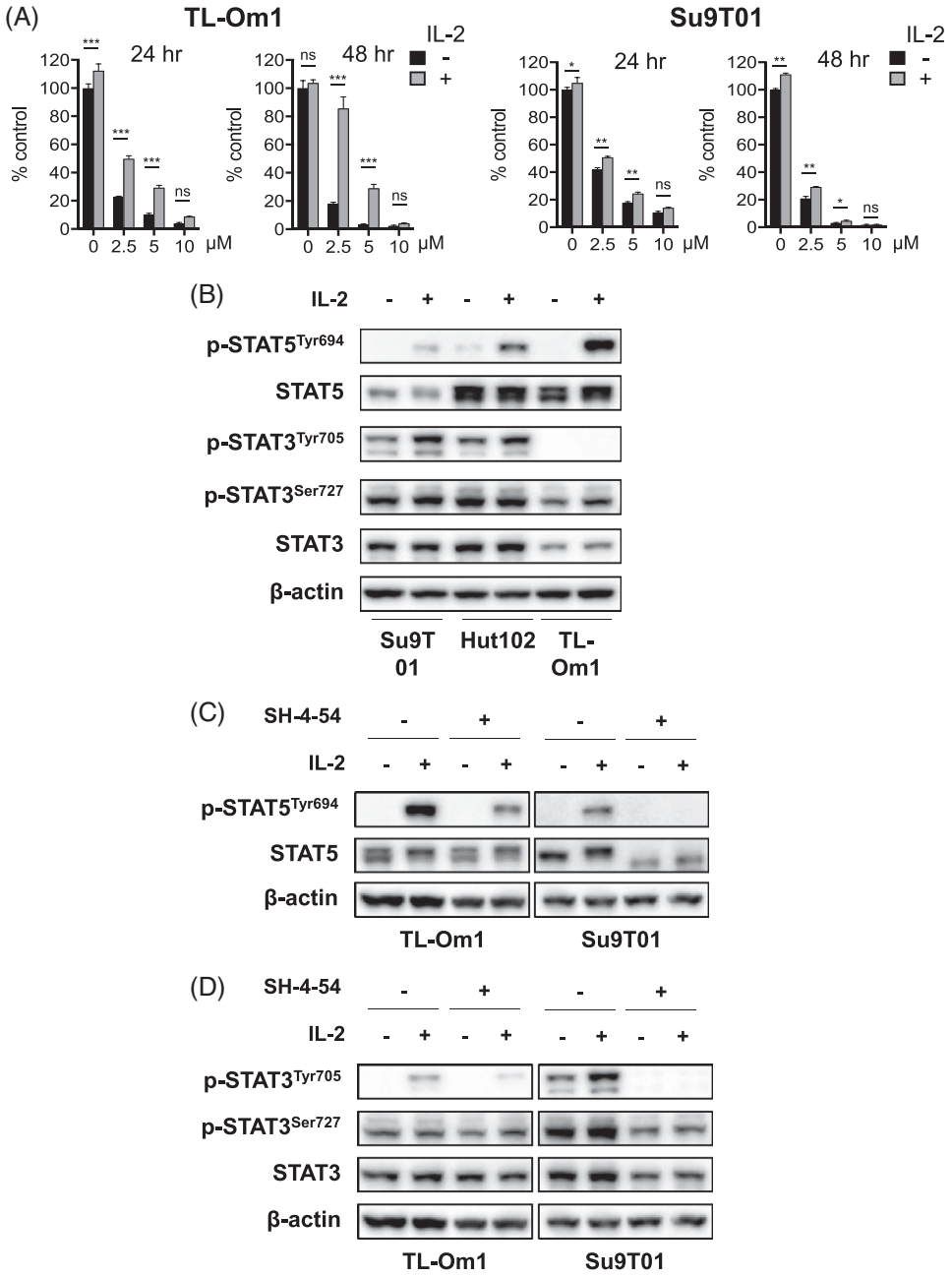


**FIGURE 6** STAT5 inhibition induces synergistic cytotoxic effects of LL-Z1640-2 in ATL cells in the presence of IL-2. (A) TL-Om1 and Su9T01 cells were treated with LL-Z1640-2 at the indicated concentrations in the presence or absence of IL-2 (100 U/mL) for 24 and 48 h. Cell viability was assessed by the CCK-8 assay. Results are expressed as percentage changes from the controls without LL-Z1640-2 in the absence of IL-2 ($n = 3$, means ± SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant. (B) Su9T01, Hut102 and TL-Om1 cells were cultured in the presence or absence of IL-2 (100 U/mL) for 6 h. Whole-cell lysates were subjected to immunoblotting using the indicated antibodies. $\beta$-Actin served as a loading control. (C and D) TL-Om1 and Su9T01 cells were treated with or without 10 $\mu$M SH-4-54 for 3 h, cultured in the presence or absence of IL-2 (100 U/mL) for 1 h, and the lysates extracted were subjected to immunoblotting using the indicated antibodies. $\beta$-Actin served as a loading control. (E) TL-Om1 cells were treated in triplicate with LL-Z1640-2 at the indicated concentrations with or without SH4-54 at the indicated concentrations in the presence or absence of IL-2 (100 U/mL) for 48 h. (F) ST1 cells were treated in triplicate with LL-Z1640-2 at the indicated concentrations with or without SH4-54 at the indicated concentrations in the presence of IL-2 (100 U/mL) for 48 h. Cell viability was assessed by a CCK-8 assay. The results were expressed as percentage changes from the controls without treatment (means ± SD). The cells were cultured in the presence of LL-Z1640-2 and/or SH4-54 at the indicated concentrations, and potential synergy was evaluated. Bliss synergy scores were calculated by SynergyFinder 3.0 (https://synergyfinder.fimm.fi), and interactive heatmaps are shown.

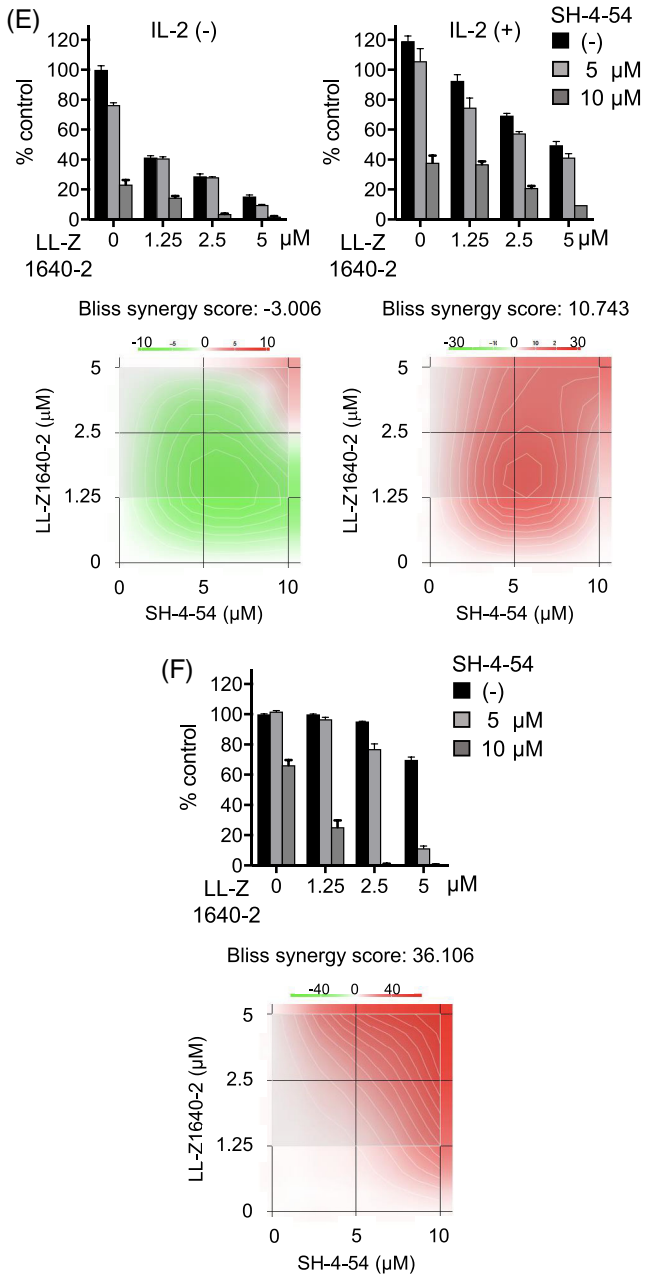


**FIGURE 6** Continued

was effective against ATL cells. However, CCR4 expression and phosphorylated p38 levels recovered in ATL cells in a time-dependent manner after the removal of LL-Z1640-2 (Figure S4). Although LL-Z1640-2 irreversibly binds the ATP pocket in TAK1, nascent TAK1 over-produced in ATL cells can mitigate the suppression of the activity of TAK1 and TAK1-dependent p38 MAPK after the removal of LL-Z1640-2. Repeated exposure to LL-Z1640-2 is needed to maintain its anti-ATL activity as we did in the in vivo models (Figure 1C,D). In addition, LL-Z1640-2 suppressed the incorporation of puromycin in ATL cells (Figure S5), which is indicative of the suppression of translation. The suppression of translation may explain the unique activity of LL-Z1640-2 in terms of potent cytotoxic activity against ATL cells with multiple mutations. However, the mechanisms underlying the anti-ATL effects of LL-Z1640-2 are yet to be elucidated in detail.

Due to its potent activity, we showed the therapeutic efficacy of LL-Z1640-2 for MM [15]. Adhesion between MM cells and bone marrow stromal cells (BMSCs) has been shown to induce the production of IL-6 and expression of receptor activator of NF-κB ligand (RANKL) in BMSCs. Treatment with LL-Z1640-2 abolishes MM cell–BMSC adhesion, thereby suppressing the production of IL-6 and expression of RANKL in BMSCs. LL-Z1640-2 has also been shown to inhibit RANKL-mediated osteoclastogenesis. These results indicate the therapeutic impact of LL-Z1640-2 targeting the tumour microenvironment. Therefore, LL-Z1640-2 may also affect cellular signalling pathways in

different cell types in the tumour macroenvironment together with ATL cells. In the present study, we found that LL-Z1640-2 decreased the expression of CCR4 on the surface of ATL cells, which has been suggested to mitigate the activation [26] and migratory function [27] of ATL cells in response to microenvironmental stimuli. Regulatory T cells (Tregs), normal counterparts of ATL cells, play an important role in the evasion of cancer from immune surveillance [28]. LL-Z1640-2 may suppress the expression of CCR4 on the surface of Tregs, thereby reducing their immunosuppressive function. TAK1 is an essential mediator of TGF-$\beta$ signalling, which is required for the induction of Tregs [29]. Therefore, the inhibition of TAK1 may suppress the induction of Tregs. The broader functions of TAK1 are yet to be clarified in other immune and non-immune cell types in the tumour microenvironment.

The present results demonstrated that the combination of LL-Z1640-2 and STAT3/5 inhibitors exhibited cooperative therapeutic activities against ATL cells, which warrants further study on a precise cross-talk between the TAK1 and STAT3/5-mediated signalling pathways in IL-2-responsive ATL cells. Based on the safety profiles of LL-Z1640-2 in the in vivo models, the combination of LL-Z1640-2 with other anti-ATL drugs, including STAT3/5 inhibitors, may be envisioned. The potent clinical efficacy and safety profiles of LL-Z1640-2 in the in vivo models of ATL and MM prompted us to synthesise new compounds based on the structure–activity relationship of LL-Z1640-2. To further enhance their targeting efficiency, we are examining a liposome-mediated drug delivery system with LL-Z1640-2 and its derived compounds.

## AUTHOR CONTRIBUTIONS

Masahiro Oura and Takeshi Harada designed the study. Masahiro Oura, Takeshi Harada, Asuka Oda, Jumpei Teramachi, Ryohei Sumitani and Yusuke Inoue performed experiments and analysed data. Atushi Nakayama synthesised LL-Z1640-2. Masahiro Oura, Takeshi Harada, Ryohei Sumitani, Kimiko Sogabe, Tomoko Maruhashi, Mamiko Takahashi, Shiro Fujii, Shingen Nakamura, Hirokazu Miki, Masafumi Nakamura, Yusaku Maeda, Tomoyo Hara and Hiroki Yamagami provided clinical samples. Hiroo Hasegawa and Hiroshi Fujiwara provided ATL cell lines and reviewed data. Kiyoe Kurahashi and Itsuro Endo reviewed data. Masahiro Abe supervised the study design and experiments. Masahiro Oura, Takeshi Harada and Masahiro Abe wrote the manuscript. Masahiro Oura, Takeshi Harada and Masahiro Abe reviewed and edited the manuscript.

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## CONFLICT OF INTEREST STATEMENT

Takeshi Harada received research funding from Nippon Shinyaku. Masahiro Abe received research funding from Chugai Pharmaceutical, Sanofi K.K., Pfizer Seiyaku K.K., Kyowa Hakko Kirin, MSD K.K., GSK, Nippon Shinyaku, Astellas Pharma, Takeda Pharmaceutical, Teijin Pharma and Ono Pharmaceutical, and honoraria from Janssen. The remaining authors declare no conflicts of interest.

## CLINICAL TRIAL REGISTRATION

The authors have confirmed clinical trial registration is not needed for this submission.

## DATA AVAILABILITY STATEMENT

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

## ETHICS APPROVAL STATEMENT

All animal studies were performed under a protocol approved by the Animal Ethics Committee of Tokushima University (T2019-80).

## PATIENT CONSENT STATEMENT

All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki using a protocol approved by the Institutional Review Board (3582 in Tokushima University Hospital).

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## SUPPORTING INFORMATION

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