Changes in intracellular activation-related gene expression and induction of Akt contribute to acquired resistance towards nelarabine in CCRF-CEM cell line

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Drug resistance is a major problem in treatment with nelarabine, and its resolution requires elucidation of the underlying mechanisms. We established two nelarabine-resistant subclones of the human T-cell lymphoblastic leukemia cell line CCRF-CEM. The resistant subclones showed changes in the expression of several genes related to nelarabine intracellular activation and inhibition of apoptosis. Activation of the Akt protein upon nelarabine treatment was observed in both subclones. The combination treatment with nelarabine and PI3K/Akt inhibitors was shown to inhibit cell growth. Cross-resistance was observed with ara-C and not with vincristine, daunorubicin, or etoposide treatment. Thus, changes in the expression of cellular activation-related genes, inhibition of apoptosis, and induction of Akt may be involved in the development of nelarabine-resistance in the CCRF-CEM cell model. The use of different classes of chemotherapeutic agents and combination therapy with PI3K/Akt pathway inhibitors may be used to overcome resistance to nelarabine.

Keywords: T-cell acute lymphoblastic leukemia; drug resistance; nelarabine; combination therapy; PI3K/Akt pathway

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) accounts for 10 to 15% of pediatric ALL cases, and despite the availability of aggressive treatment regimens, relapse is observed in ~15% of cases due to therapy resistance [1,2]. Nelarabine, a pro-drug of 9-β-D-arabinofuranosylguanine (ara-G), is a purine nucleoside analogue with lineage-specific cytotoxicity towards T-ALL and used as chemotherapy for relapsed, and recently in

newly diagnosed T-ALL[3,–6].

Nelarabine requires transport by specific membrane transporters, metabolism, and interaction with intracellular targets to confer cytotoxic effect [7,8]. *In vivo*, it is demethylated to ara-G in plasma, transported into cells by ENT1, and phospholrylated to ara-GTP by DCK and DGUOK enzymes. Ara-GTP accumulation results in cell death due to its incorporation into DNA, leading to termination of DNA elongation and inhibition of ribonucleotide reductase; blocking further DNA synthesis [7]. SAMHD1 is a deoxynucleotide triphosphate (dNTP) hydrolase that cleaves physiological dNTPs and tri-phosphorylated nucleoside analogues, including ara-GTP [9].

A major block in treatments using nucleoside analogues including nelarabine is its acquired resistance. The mechanism involves multiple pathways mainly related to drug metabolism and intracellular active compound concentration that affect drug activity and do not necessarily result due to genetic changes [10,11]. Studies have shown that chemosensitivity is influenced by the expression of genes involved in metabolism that affect the level of drug incorporation in leukemia cells [12–15].

Final target of chemotherapy is the induction of apoptosis leading to cell death, and changes affecting apoptotic factors disturb this process [16,17]. Aberrant activation of signal transduction pathways is also thought to influence drug resistance in T-ALL, as activation of the PI3K/Akt and MEK/ERK1/2 pathway, which are related to cell survival and drug resistance, is common [18,19].

In this study, we established nelarabine-resistant sub-clones of the human T-cell lymphoblastic leukemia cell line CCRF-CEM to investigate the factors related to the acquisition of nelarabine resistance *in vitro*, and to identify strategies to overcome resistance to nelarabine, focusing on intracellular activation-related genes, changes in apoptosis, and signaling pathways.

Materials and methods

Reagents

Nelarabine (LKT Laboratories Inc., St. Paul, MN, USA), ara-G hydrate (Sigma-Aldrich, St. Louis, MO, USA), etoposide and daunorubicin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), cytarabine (ara-C) and vincristine (Cayman Chemical, Ann Arbor, MI, USA), ZSTK474, PKI-587, MK-2206, trametinib and selumetinib (Selleck Chemicals, Houston, TX, USA) were purchased from the indicated vendors. All drugs were dissolved in dimethyl sulphoxide (DMSO) except vincristine and ara-C which were dissolved in dH2O.

Cell culture and development of nelarabine-resistant subclones

CCRF-CEM cells were obtained from JCRB Cell Bank Japan and were cultured at 37ºC with 5% CO_2 in RPMI-1640 medium (Fujifilm Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum. Nelarabine-resistant cells were established by serial incubation with an increasing dose of nelarabine followed by limiting dilutions for cloning. Parental CCRF-CEM cells were maintained with increasing concentrations of nelarabine, with initial concentration of 2 μM nelarabine in 0.1% DMSO. The cultures were allowed to grow, and cell proliferation was observed. When cells reached the logarithmic growth phase (1-2 weeks culture), concentration of nelarabine was gradually increased over 3 months. Using nelarabine at the concentration of 10 μM for Clone 1, cells were cloned using the limiting dilution method by growing ~0.5 cells/well in a 96-well plate. Clone 2 was developed by further incubation of cells with up to 33.33 μM of nelarabine, followed by cloning with limiting dilution method. The independent nelarabine-resistant CCRF-CEM variants were named Clones 1 and 2.

Cell proliferation assay and IC_{50} calculation

The growth-inhibitory effect of nelarabine was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay, Dojindo, Tokyo, Japan). We incubated 1 mL of cells (5×10^4/mL) for 72 h in 24-well plate with indicated dose of drugs. Next, 100-µL aliquot of cells was transferred to 96-well microplate and MTT reagent was added. After 4 h of incubation, formazan precipitates were dissolved in DMSO. Spectrophotometry analysis was performed at absorbance of 570 nm/630 nm using fluorescence microplate reader (Infinite M200, TECAN, Switzerland). The growth-inhibition curve was plotted and the IC_{50} value was calculated to measure the drug resistance using the Quest Graph™ IC50 Calculator (AAT Bioquest,Inc., Sunnyvale, CA, USA). The numbers obtained are rounded to 0 decimal.

Caspase activity assay

Cell apoptosis was determined using Caspase Glo®3/7 assay (Promega, Madison, WI, USA). Briefly, cells were treated with nelarabine 10 µM, dose equal to IC_{50} and 2-3 times IC_{50} of each cells for 72 h, and caspase 3/7 activity was evaluated using the luminescence assay as per the manufacturer's instruction using the TriStar LB 941 multimode microplate reader (Berthold Technologies GmbH & Co KG, Germany).

Evaluation of mRNA expression by real-time quantitative PCR (qPCR)

Total cellular RNA was extracted using RNeasy® Minikit (Qiagen, Germany) and first-strand cDNA synthesis were done using PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan) as per the manufacturer's protocol. The mRNA expression levels of nelarabine intracellular activation-related genes and Bcl2 family genes were measured

as described previously [13] using qPCR performed with the Thermal Cycler Dice Real-Time System II TP800 (Takara). Primers are shown in Supplement Data 1. The mRNA expression of each sample was normalized to that of *GAPDH* (Takara Bio Human Housekeeping gene primer set. ID: HA067812), and relative expression was calculated via the $\Delta\Delta C_t$ method as described previously [20].

Western blot

Protein expression was detected via western blot analysis, as described previously [21]. Briefly, 2×10^6 cells were lysed using Minute^{TM} Total Protein Extraction Kit (Invent Biotechnologies Inc., Plymouth, MN, USA) and protein concentrations were determined with Takara BCA protein assay (Takara). Detection was performed using primary antibodies for DCK (ab151966, Abcam, Cambridge, MA, USA), ENT1 (sc-377283) and DGUOK (sc-376267) from Santa Cruz Biotechnology, Dallas, TX, USA; AKT (9272S), pAKT (9271S), ERK (9102), pERK (4370), PARP (9254S) from Cell Signaling Technology (CST), Danvers, MA, USA; β-actin and Caspase-3 (ab136812, Abcam); and GAPDH (2118, CST), followed by an anti-rabbit or anti-mouse horseradish peroxidase-linked secondary antibody (7074S/7076, CST). Immunocomplexes were visualized by chemiluminescence using SignalFire^{TM} enhanced chemiluminescence (ECL) reagent (CST) and the fluorescence was documented using the FluorChem F2 imaging system (Alpha Innotech, San Jose, CA, USA). Band quantification of westernblot image were done using ImageJ 1.53a.

Statistical analyses

Experiments were performed thrice in duplicate for reproducible results. Data are expressed as mean ± SD and were analyzed using the R statistical software (version 3.4.2). Statistical differences were evaluated using one-way ANOVA followed by a

Bonferroni correction, or a two-way ANOVA followed by Tukey's honestly significant difference (HSD) test. A $p \leq 0.05$ was considered as statistically significant.

Results

Establishment of nelarabine-resistant CCRF-CEM cells

We developed nelarabine resistant subclones Clones 1 and 2 from CCRF-CEM cells. MTT assay demonstrated that drug-resistant subclones had an IC_{50} that was 50 and 71.6 times higher than that for CCRF-CEM cells. Differences in cell viability between the drug-sensitive and resistant cell lines were observed when treated in nelarabine 10 μM. Consequently, we used this concentration as the treatment comparison dose for all the cell lines to observe characteristic difference after nelarabine treatment (Fig 1a). Further, cells showed comparable cell viability after ara-G treatment *in vitro* (Fig 1b). *[Figure 1 near here]*

Changes in gene expression related to nelarabine intracellular activation

We examined several genes related to nelarabine intracellular activation to identify gene expression differences between cells in basal condition. The expression levels of *SLC29A1* (ENT1) ($p \leq 0.001$) and *DCK* ($p \leq 0.001$) were downregulated in both the resistant subclones, *DGUOK* was downregulated in Clone 2 ($p = 0.0015$), whereas *SAMHD1* was upregulated in both resistant subclones ($p = 0.012$). The expression of *RRM1* and *NT5C2* was not significantly different relative to the CCRF-CEM ($p > 0.05$) (Fig 2a). Western blot showed downregulation of DGUOK, DCK, and ENT1 protein expression in Clone 1 and 2 (Fig 2b). *[Figure 2 near here]*

Resistant clones showed apoptosis inhibition after nelarabine treatment

We detected significant increase in luminescence signals from drug-sensitive cells after 3 μM (equal to IC_{50}) and 10 μM (3 times IC_{50}) nelarabine treatment for 72 h indicating caspase 3/7 activityas apoptosis marker ($p \leq 0.001$). As for the resistant subclones, there were no increase observed after 10 μM nelarabine treatment ($p > 0.05$). Caspase activity were observed in Clone 1 after treatment with nelarabine 400 μM and in Clone 2 after 215 and 400 μM ($p \leq 0.05$). (Fig 3a). Western blot showed PARP and caspase 3 cleavage in CCRF-CEM cells after treatment with 10 μM nelarabine for 48 h and 72 h (Fig 3b).

Additionally, we examined genes belonging to the Bcl2 family via qPCR using mRNA from untreated and nelarabine-treated cells. The expression of *BCL2, BCL2L1* (Bcl-xL), *BAD,* and *BAX* in CCRF-CEM, Clone 1, and Clone 2 in basal condition did not differ significantly ($p > 0.05$). However, after treatment with 3 μM (IC_{50}) and 10 μM (3 times IC_{50}) nelarabine for 72 h, we observed statistically significant differences indicating the upregulation of *BCL2, BAD*, and *BAX* in CCRF-CEM cells ($p \leq 0.001$). There were no significant changes in the BCL2 family genes expression in Clone 1. Clone 2 showed upregulated expression of the anti-apoptotic gene *BCL2L1* after treatment with 10 μM nelarabine ($p = 0.003$), the pro-apoptotic gene *BAD* after treatment with 400 μM (2 times IC_{50}) ($p \leq 0.001$); and *BAX* after treatment with 215 μM (IC50) and 400 μM ($p \leq 0.001$) (Fig 3c). *[Figure 3 near here]*

Resistant subclones showed upregulation of Akt

We investigated the status of Akt as the Akt signaling pathway influences cell survival and drug resistance. CCRF-CEM, Clone 1, and Clone 2 cells were treated with 10 μM nelarabine for indicated times, and were subsequently analyzed by western blotting to

examine the activation of Akt and band quantification were done to see pAkt/Akt ratio.

Clone 2 showed higher expression for p-Akt in basal conditions ($p = 0.015$) (Fig 4a). Decrease in p-Akt levels in CCRF-CEM cells was observed in a time-dependent manner after nelarabine treatment. Conversely, the hyperactivation of p-Akt was observed in Clone 1 and Clone 2 cells after nelarabine treatment, indicating that the PI3K/Akt pathways were upregulated (Fig 4b).

Combination treatment with PI3K/Akt inhibitor induce cell death

Cells were treated with several PI3K/Akt pathway inhibitors to observe the effect of p-Akt modulation on nelarabine resensitization. Cells were incubated with 10 μM nelarabine, 0.5 μM ZSTK474 (PI3K inhibitor), 0.01 μM PKI-587 (dual PI3K/mTOR inhibitor), 0.1 μM MK-2206 (allosteric Akt inhibitor), or a combination of nelarabine and PI3K/Akt inhibitors for 48 h. MTT assays were performed to analyze cytotoxicity. Cell viability was significantly reduced in the combination treatment of nelarabine and PI3K/Akt inhibitor compared to nelarabine only in all cells ($p \leq 0.001$). Differences in cell viability were seen between the treatment of cells with ZSTK-474 or MK-2206 alone and combination therapy of the drug with nelarabine, but not with the use of PKI-587 in single vs. combination therapy except in CCRF-CEM ($p = 0.03$) (Fig 4c).

Treatment with the PI3K/Akt inhibitor alone reduced the p-Akt signal without the induction of apoptosis. Combination treatment with nelarabine and PI3K/Akt inhibitors resulted in a reduced p-Akt signal in all cells and induction of cell death indicated by PARP cleavage (Fig 4d). *[Figure 4 near here]*

Combination treatment with MEK/ERK inhibitor did not induce cell death

To investigate if MEK/ERK pathway is involved in drug resistance, we performed western blotting of ERK and p-ERK. Although we observed high expression of p-ERK

in Clone 2, the difference was not significant ($p = 0.07$) (Fig 5a). To observe the effect of p-ERK modulation on nelarabine resensitization, cells were incubated with 10 μM nelarabine, 0.1 or 1 μM trametinib, 0.1 or 1 μM selumetinib, or a combination of these drugs for 48 h and MTT assays were performed. p-ERK expression was induced after nelarabine treatment and its levels were highest in Clone 2. Cell viability was not reduced in the combination treatment in CCRF-CEM cells and Clone 1 even though p-ERK expression was inhibited. In Clone 2, decrease in cell viability were seen after combination treatment of nelarabine with 1 μM selumetinib ($p = 0.002$) and trametinib ($p = 0.005$ for 0.1 μM and $p \leq 0.001$ for 1 μM) in comparison with nelarabine only treatment; however, this observation was not accompanied by PARP cleavage (Fig 5b and 5c). *[Figure 5 near here]*

Resistant subclones showed resistance to ara-C

To investigate whether the subclones were cross-resistant to other chemotherapeutic agents, we incubated cells with daunorubicin, vincristine, ara-C, and etoposide at various doses for 48 h and MTT assays were done. All three cell lines showed similar sensitivity to daunorubicin, vincristine, and etoposide. Resistant subclones showed cross-resistance to ara-C, a nucleoside analogue (Fig 6a). Treatment with ara-C induced p-Akt expression in the resistant subclones and did not cause the cleavage of PARP and caspase 3 (Fig 6b). *[Figure 6 near here]*

Discussion

In this study, we isolated two subclones resistant to nelarabine with different degrees of resistance to identify the common characteristics and pathways causing drug resistance. Cells showed comparable resistance to the active form of nelarabine, ara-G, as reported previously that both drugs have a comparable effect on T-ALL cells *in vitro* [19,22].

The resistant cells may reflect the acquired resistance towards nelarabine, and may be important in cancer therapeutic studies as preclinical models [23]. Although other studies have used similar models, the complete set of mechanisms underlying drug-resistance have not been elucidated; therefore, the cell lines developed can be beneficial in such studies [12,24,25]. The analysis of nelarabine resistance showed involvement of various different mechanisms, suggesting that variations may arise due to the randomness of natural selection, indicating the complexity of drug resistance development processes [26]. Here, we treated cell lines with the same comparison dose (10 μM) and doses corresponding to IC_{50} and 2-3 times IC_{50} of each cell to observe characteristic differences between the sensitive CCRF-CEM cells and the resistant subclones, especially with respect to cytotoxicity of the drugs and cell death process.

Changes in the expression of *SLC29A1* (ENT1), DCK and *SAMHD1* were noted in both the subclones in comparable degree, except DGUOK. which downregulated in Clone 2 only. Studies showed that the combined expression of intracellular activation-related factors could predict the response of T-ALL to nelarabine [13,27]. Downregulation of ENT1, DCK, and DGUOK leads to reduced incorporation of ara-G into cellular DNA, leading to resistance of the drug. [12] Changes in DGUOK expression, which phosphorylate ara-G to ara-GTP in mitochondria, might contribute to higher resistance of nelarabine in Clone 2. [28] High levels of SAMHD1 are associated with increased substrate hydrolysis, lower cellular dNTP levels, and reduced cytotoxicity with nelarabine [15,29]. Previous studies showed that SAMHD1 were transcriptionally regulated, with its mRNA levels correlated with the ara-G IC_{50}, and DNA methylation of SAMHD1 promoter leads to transcriptional repression in T-ALL cell lines [15,30]. Further study should be aimed to confirm the status of SAMHD1

promoter methylation and DNA methylation status generally in resistant cell lines to elucidate one of the nelarabine resistance mechanism.

Treatment with nelarabine caused reduction in cell viability due to apoptosis induction; whichwere prevented in the subclones as shown by the inhibition of caspase 3/7 activation and PARP cleavage. In sensitive cells, significant caspase activities were seen after treatment with 3 μM and 10 μM nelarabine, while caspase were induced in resistant subclones only after treatment with high dose nelarabine. These showed increased resistance in apoptosis induction in resistant subclones. Previous reports have shown that the Bcl2 family is related to the regulation of mitochondrial apoptosis in chemotherapy resistance, including nelarabine [12,19,31,32]. We found no significant difference in the expression of *BCL2, BCL2L1* (Bcl-xL), *BAD,* and *BAX* in basal condition; however, cells responded differently after nelarabine treatment. Notably, the expression of *BCL2, BAX* and *BAD* was increased after nelarabine treatment in sensitive cells. Clone 1 showed no changes in Bcl2 familyUpregulation of *BCL2L1* (Bcl-xL) was observed in Clone 2 after 10 μM nelarabine treatment, and upregulation of *BAX* and *BAD* were seen after treatment with high dose nelarabine. These results implicate the different behavior of Bcl2 family factors in sensitive and resistant cells upon nelarabine treatment, and its role, at least partially, in the inhibition of mitochondrial apoptosis leading to nelarabine resistance. Moreover, the expression of several factors belonging to the Bcl2 family is known to be modulated by Akt [33,34].

Aberrant activation of the PI3K/Akt pathway is commonly found in T-ALL (70-80%) [35]. Recent evidence indicates the dysregulation of the pathway is related to resistance to therapy [33]. Lonetti et al. showed that the PI3K/Akt pathway is activated in panel of nelarabine resistant leukemia cell lines and primary samples from patients with relapsed T-ALL [19]. We demonstrated that PI3K/Akt pathway activation occurs

in the *in vitro* model that showed resistance after drug exposure. Upregulation of p-Akt in untreated cells and upon nelarabine treatment were observed in resistant clones, notably in Clone 2. This result is in line with evidence that chemotherapy can modulate Akt activity which contributes to acquired resistance [33]. We found generally similar degree of changes in genes related to nelarabine intracellular activation in both subclones, with significant overexpression of p-Akt in Clone 2, which display higher resistance to nelarabine. The finding might indicate different mechanisms involved in various degree of nelarabine resistance.

As the subclones showed higher levels of Akt signaling, we treated resistant cells with a combination of nelarabine and several PI3K/Akt inhibitors. Combination therapy increased drug toxicity, reduced p-Akt levels, and induced cell death in resistant clones compared to the nelarabine only treatment. Further, we observed a difference in drug effects because PKI-587 did not show higher cell cytotoxicity when used as combination therapy compared to its use as single agent as did ZSTK-474 and MK-2206. Different targets for PI3K/Akt pathway inhibitors may affect leukemia cells differentially based on the genomic context, and use of drugs as single or combination therapy [36]. These results indicate a potential role for PI3K/Akt inhibition as part of combination therapy to overcome nelarabine resistance. Further studies should aim to elucidate the most effective PI3K/Akt inhibitor to counteract nelarabine resistance.

We also investigated the expression level of p-ERK to evaluate the involvement of MEK/ERK pathway in nelarabine resistance. ERK1/2 is upregulated in approximately 34.5% cases of T-ALL and majorly contributes to chemoresistance [37,38]. We found that p-ERK was upregulated after treatment with nelarabine. A similar observation was shown by Nishioka et al. that exposure of AML cells to ara-C activated ERK signalling, and both simultaneous treatment and pre-incubation of ara-C

with MEK inhibitor enhanced cell growth inhibition. [39] However, we observed no effect on cell viability after treating the cells with the MEK/ERK inhibitors individually or in simultaneous combination with nelarabine. Combination treatment showed decrease of cell viability in Clone 2 without PARP cleavage which indicates cell death, to corroborate this observation. Therefore, we consider the decrease in cell viability could be due to synergistic effect of the drugs. CCRF-CEM cells have aberrantly activated PI3K/AKT pathway due to several mutations in the genes that encode its upstream kinases, including PTEN [40]. These reports possibly explain the difference in induction of Akt and ERK activity and the effect of Akt pathway inhibition in resistant subclones. Further studies are needed to elucidate the effects of drug combination sequence to supress nelarabine resistance.

Treatment with other chemotherapeutic agents showed similar sensitivity across cells, which suggests that the resistance is specific to nelarabine exposure, and is not related to multi-drug resistance factors. Cross-resistance with ara-C is expected because as nucleoside analogues, nelarabine and ara-C have common pathways for intracellular activation, that has changed in resistant subclones. Downregulation of ENT1 and DCK, as seen in Clone 1 and 2, play key role in developing resistance to cytarabine as well as nelarabine [10,11]. Treatment with ara-C in the subclones showed the induction of p-Akt and the inhibition of PARP and caspase 3 cleavage, indicating a similar resistance pattern. High induction of p-Akt activity in Clone 2 may have affected the degree of drug resistance, as Clone 1 and 2 were differentially sensitive to the drug. Thus, the use of other classes of chemotherapeutic agents beside nucleoside analogues may be used as a strategy to overcome drug resistance after treatment with nelarabine.

Further studies are required to elucidate the interactions between the factors involved in the development of nelarabine resistance, the predominant mechanism

underlying the nelarabine resistance in time and dose-wise manner, and the molecular background that initiates it. Moreover, as these findings were obtained *in vitro* in single cell lines, further evaluation is needed to confirm the results with studies performed *in vivo* and in clinical settings.

To summarize, we established nelarabine resistant subclones from CCRF-CEM cell line. Cell models showed that nelarabine resistance involved changes in the expression of genes related to nelarabine activation, inhibition of apoptosis accompanied by changes in the response of Bcl2 family genes, and Akt induction. Use of other chemotherapeutic agents or combination therapy with PI3K/Akt pathway inhibitors may be used as a method to overcome drug resistance during treatment.

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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.

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Figure 1. Establishment of nelarabine-resistant CCRF-CEM cells.

a) MTT assay showing the viability of CCRF-CEM, Clone 1, and Clone 2 cells after treatment with various concentrations of nelarabine for 72 h and table showing IC50 and relative resistance of resistant subclones. b) Comparison of cell viability after treatment with nelarabine or ara-G. * $p \leq 0.05$

Figure 2. Changes in gene expression related to nelarabine intracellular activation in basal condition.

a) Relative expression of mRNA of genes related to nelarabine intracellular activation in untreated cells as determined through qPCR and calculated using the $\Delta\Delta C_t$ method. **b)** Western blot image showing the downregulation of DCK, DGuoK, and ENT1 proteins in the subclones. * $p \leq 0.05$, ** $p \leq 0.001$.

Figure 3. Resistant clones showed apoptosis inhibition after nelarabine treatment.

a) Caspase 3/7 assay after treatment with nelarabine (dose equal to IC_{50}, 2-3 times IC_{50} for each cell type, and 10 μM) for 72 h. Caspase activity levels are shown as fold-change in luminescent activity that was normalized to the signal from cells with no nelarabine treatment, which were used as control. b) Western blot for PARP, cleaved PARP, pro-caspase 3, and caspase 3 after treatment with 10 μM nelarabine. β-actin was used as a loading control. c) qPCR for *BCL2, BCL2L1* (Bcl-xL), *BAD* and *BAX* was performed in untreated cells and after nelarabine treatment for 72 h. CCRF-CEM cells served as a control and the relative mRNA expression that was normalized to an endogenous housing keeping gene (*GAPDH*) was calculated using the $\Delta\Delta C_t$ method. n.s = not significant, * $p \leq 0.05$, ** $p \leq 0.001$.

Figure 4. Resistant subclones showed p-Akt induction after treatment with nelarabine and combination therapy of nelarabine and PI3K/Akt inhibitors induce cell death.

Representative western blot image and p-Akt/Akt quantitative ratio analysis in a) untreated cells, and b) cells treated with 10 μM nelarabine for the indicated times. *GAPDH* served as a loading control. c) Graph for cytotoxicity determined after 48 h treatment of cells with 10 μM nelarabine, 0.5 μM ZSTK-474, 0.01 μM PKI-587, 0.1 μM MK-2206, or a combination of nelarabine and PI3K/Akt inhibitor using the MTT assay. d) Western blot images for PARP cleavage, p-Akt/Akt, and β-actin after treatment of

cells with an individual drug or a combination of drugs. n.s = not significant, * $p \leq 0.05$ ** $p \leq 0.001$.

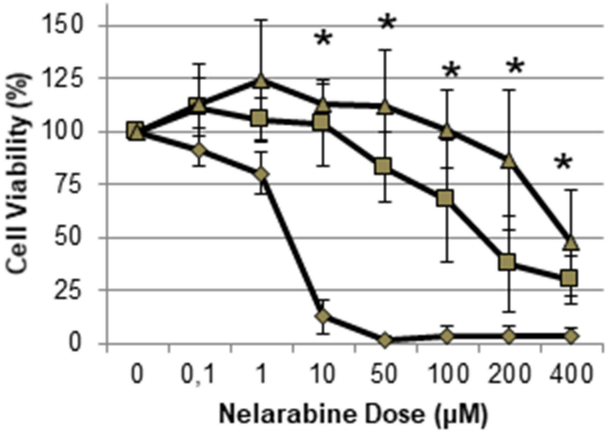
Figure 5. Combination treatment of nelarabine and MEK/ERK inhibitors did not induce cell death.

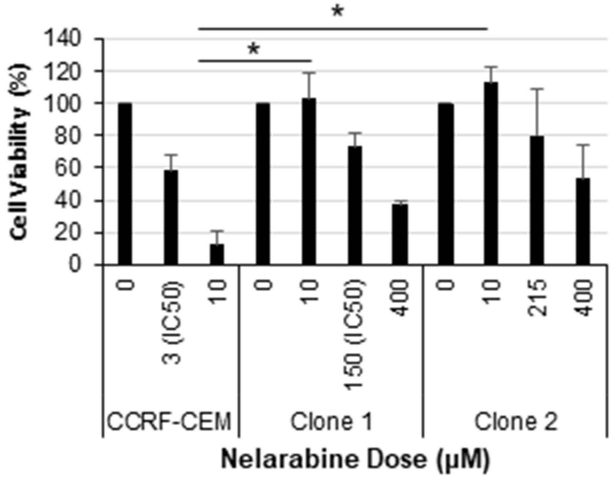
a) Representative western blot image and p-ERK/ERK quantitative ratio analysis in untreated cells. Graph for cytotoxicity determined after 48 h treatment of cells with 10 μM nelarabine, 0.1 μM and 1 μM selumetinib b) and 0.1 μM and 1 μM trametinib c), or a combination of nelarabine and MERK/ERK inhibitor using the MTT assay; Western blot image for PARP cleavage, p-ERK/ERK, and GAPDH after treatment of cells with the drug individually or in combination. * $p \leq 0.05$, ** $p \leq 0.001$.

Figure 6. Resistant subclones showed resistance to ara-C but not with daunorubicin, vincristine, and etoposide.

a) MTT assay showing the viability of CCRF-CEM, Clone 1, and Clone 2 cells after treatment with various concentrations of chemotherapeutic agents for 72 h. b) Western blot images for PARP, cleaved PARP, p-Akt, Akt, β-actin, caspase 3, and cleaved caspase 3 after treatment of cells with 0.1 μM ara-C, 0.01 μM daunorubicin, 0.1 nM vincristine, and 0.1 μM etoposide.

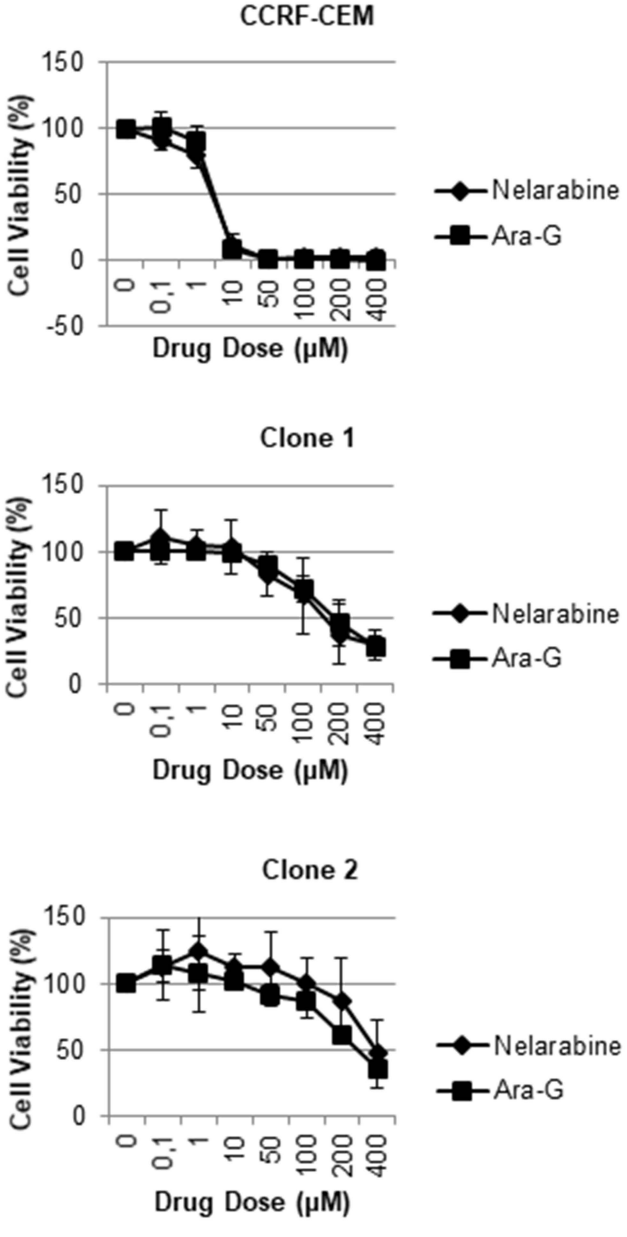
a)

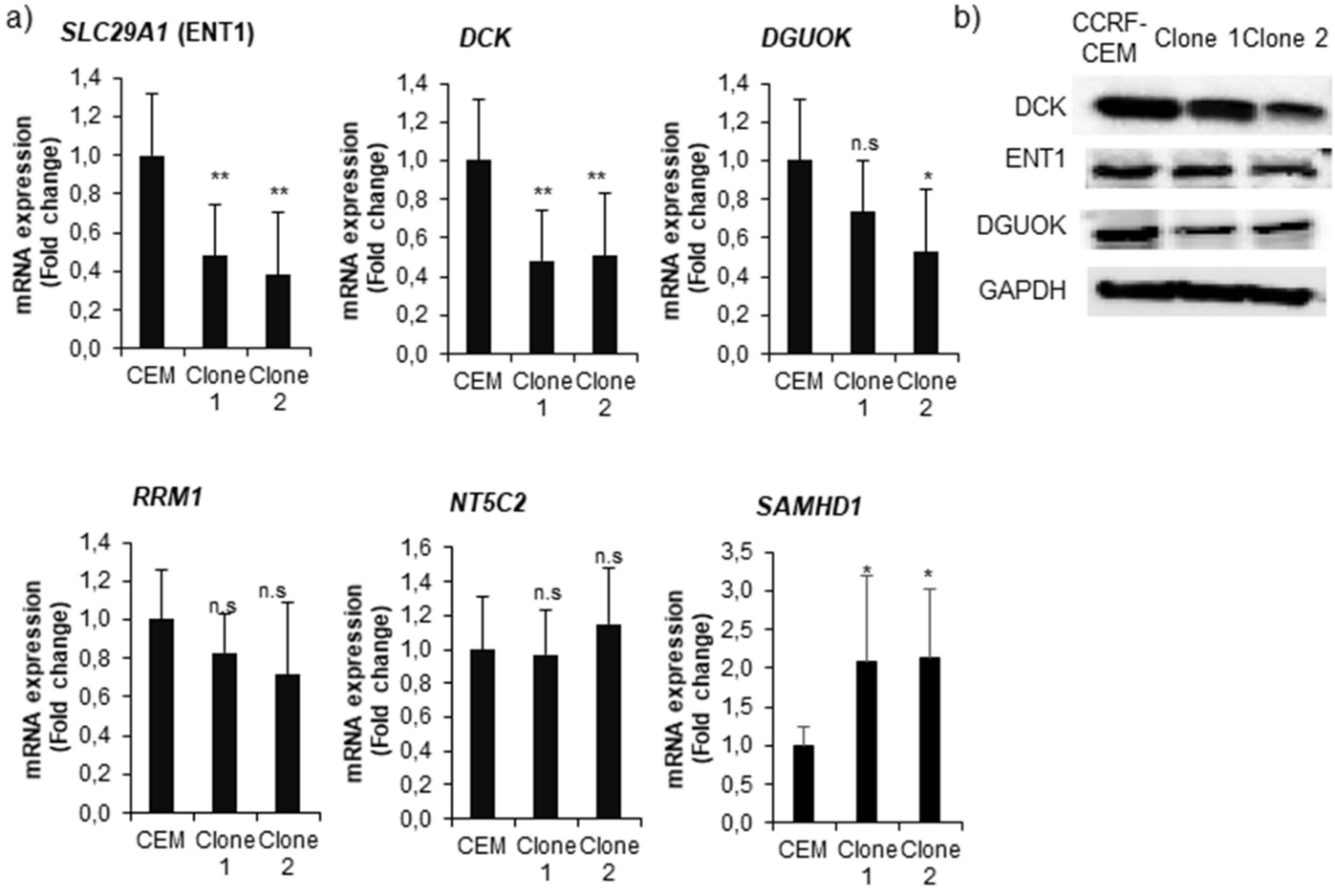


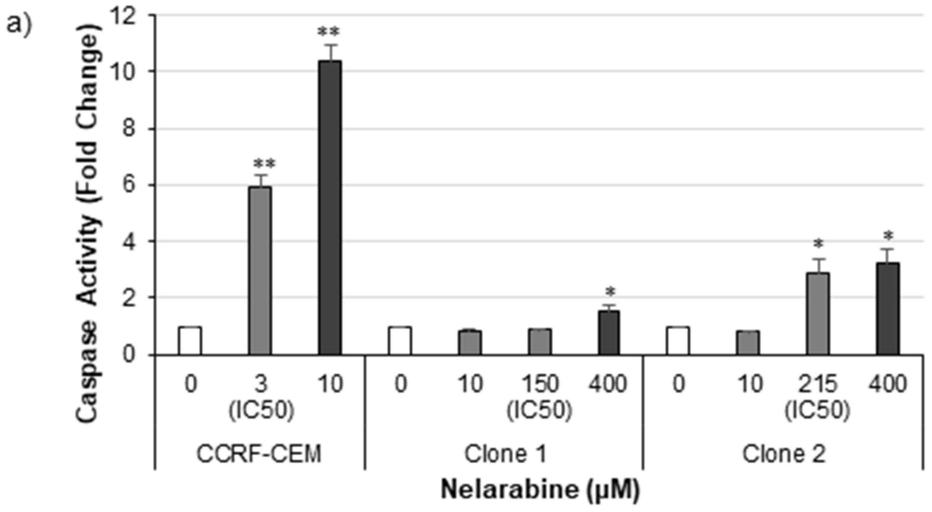


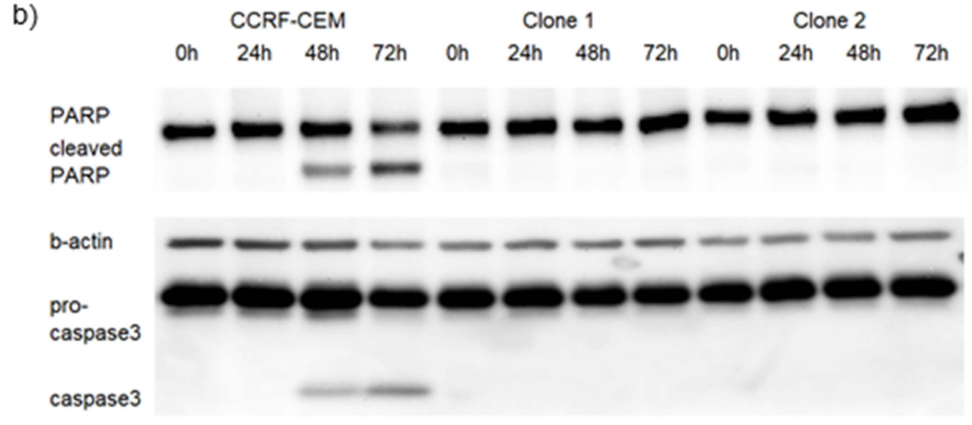
Cell Line	Nelarabine Resistance	
	IC50 (µM)	Relative Resistance
CCRF-CEM	3	-
Clone1	150	50
Clone 2	215	71.6

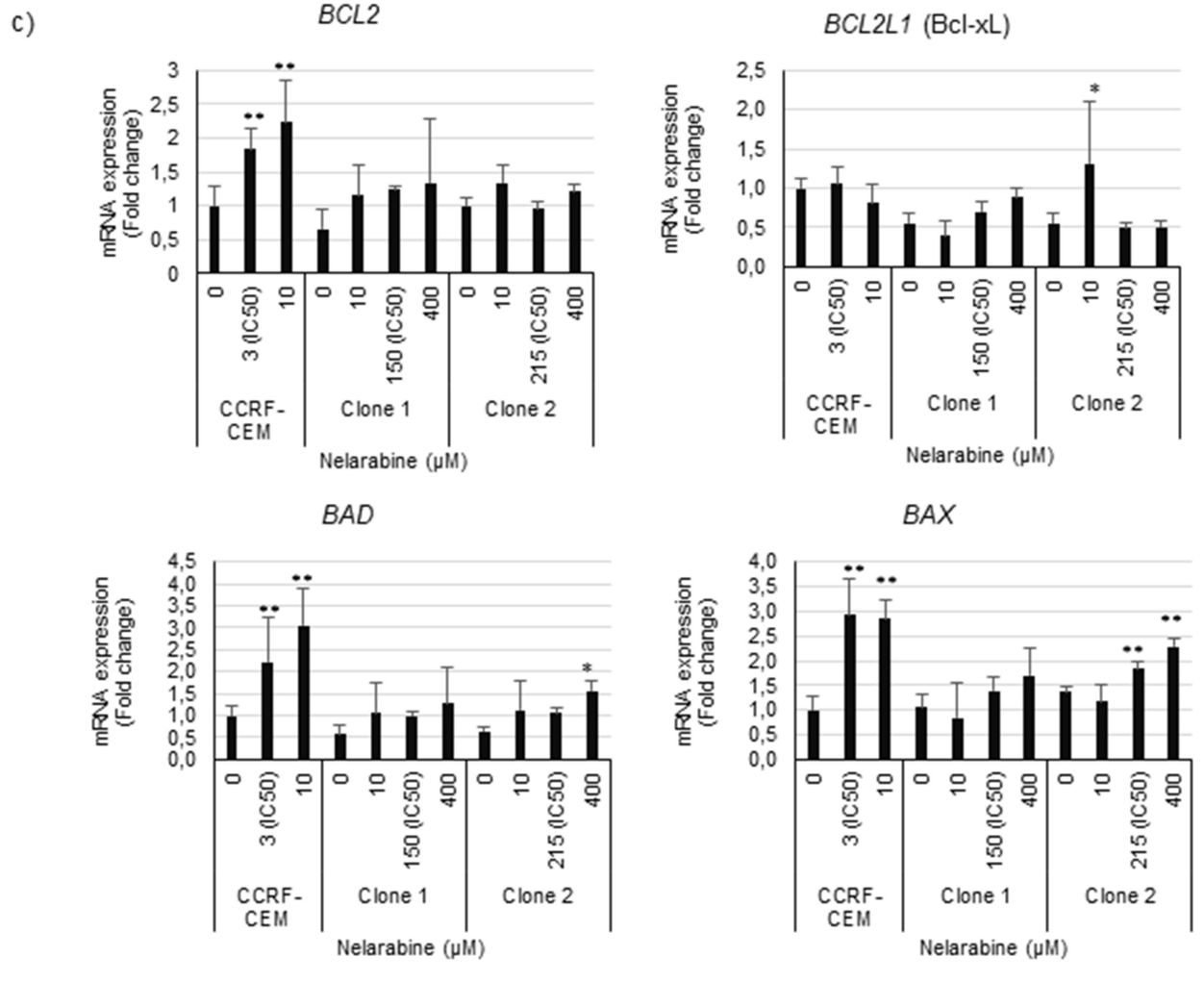
b)

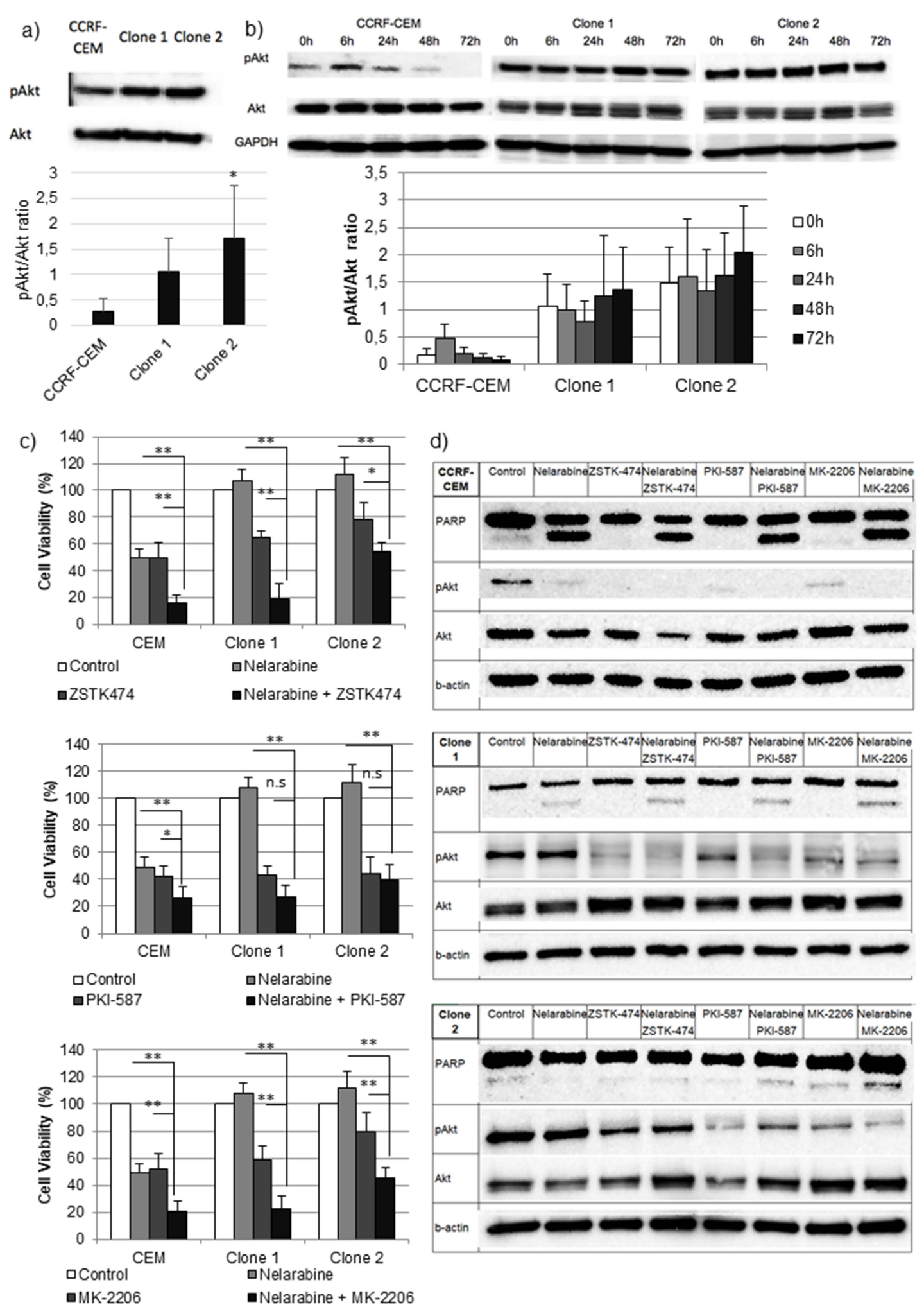


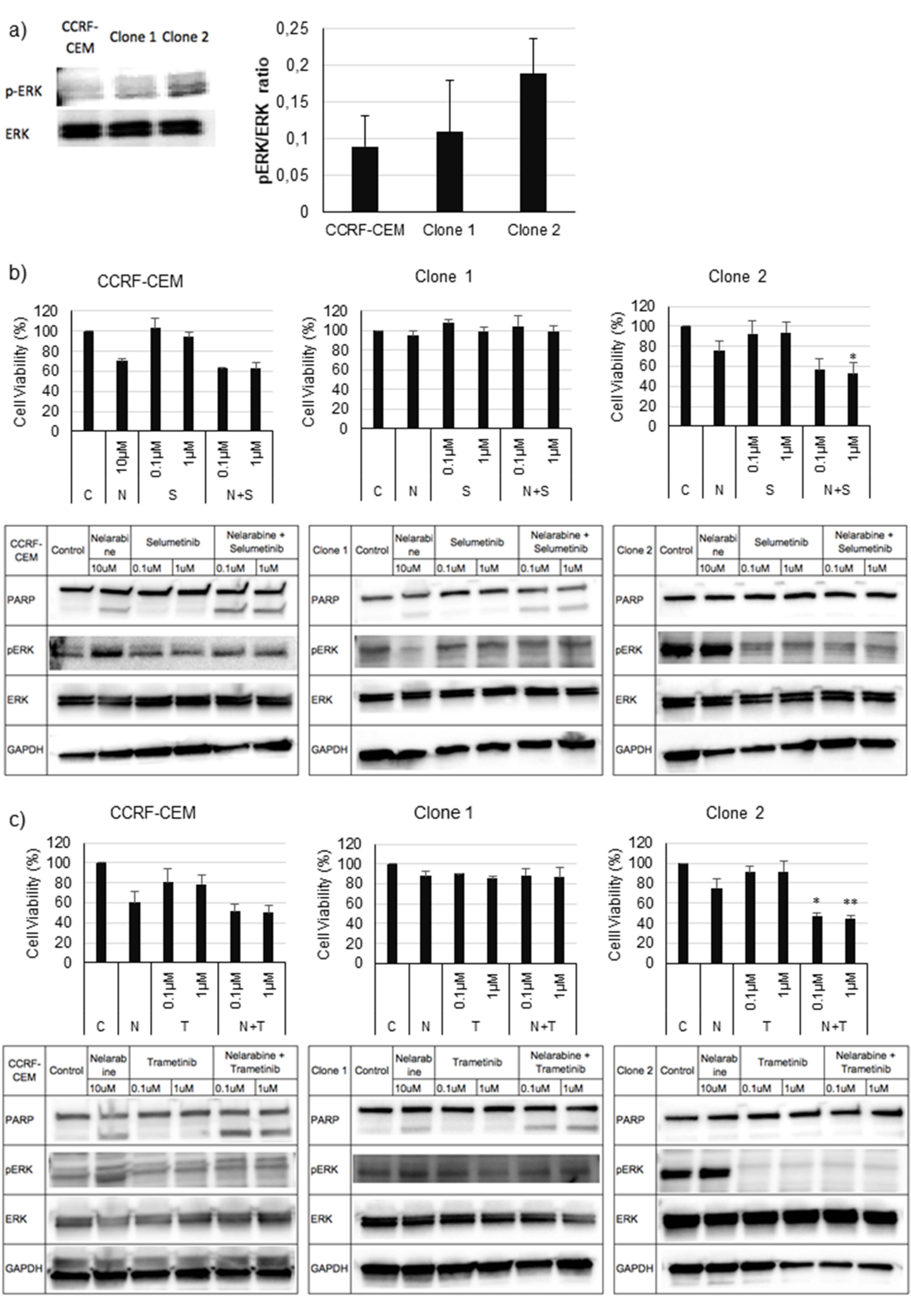

a)
SLC29A1 (ENT1)
DCK
DGUOK
RRM1
NT5C2
SAMHD1
mRNA expression (Fold change)
CEM
Clone 1
Clone 2
**
n.s
*
b)
CCRF-CEM
Clone 1
Clone 2
DCK
ENT1
DGUOK
GAPDH


a)
Caspase Activity (Fold Change)
0
2
4
6
8
10
12
**
**
*
*
*
0
3
(IC50)
10
0
10
150
(IC50)
400
0
10
215
(IC50)
400
CCRF-CEM
Clone 1
Clone 2
Nelarabine (µM)

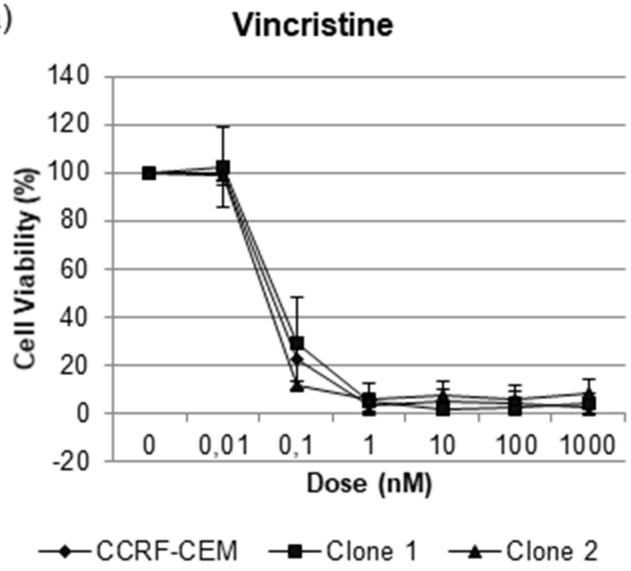

b)
CCRF-CEM
Clone 1
Clone 2
0h
24h
48h
72h
0h
24h
48h
72h
0h
24h
48h
72h
PARP
cleaved
PARP
b-actin
pro-
caspase3
caspase3

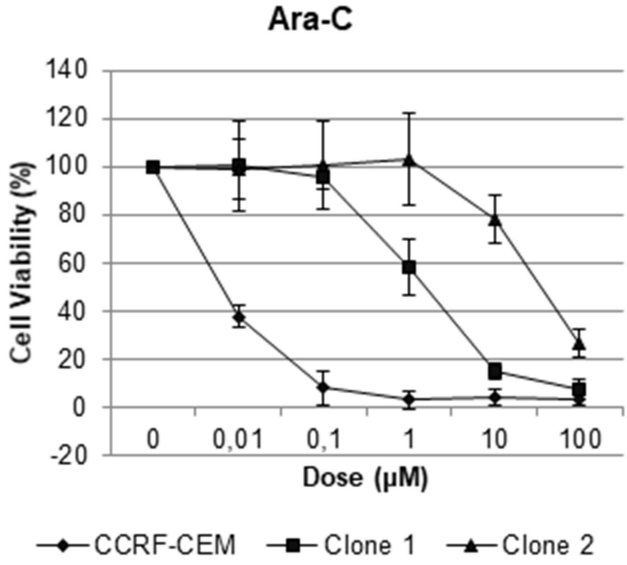

c)
BCL2
mRNA expression
(Fold change)
0
0,5
1
1,5
2
2,5
3
••
••
0
3 (IC50)
10
0
10
150 (IC50)
400
0
10
215 (IC50)
400
CCRF-
CEM
Clone 1
Clone 2
Nelarabine (µM)
BCL2L1 (Bcl-xL)
mRNA expression
(Fold change)
0,0
0,5
1,0
1,5
2,0
2,5
*
0
3 (IC50)
10
0
10
150 (IC50)
400
0
10
215 (IC50)
400
CCRF-
CEM
Clone 1
Clone 2
Nelarabine (µM)
BAD
mRNA expression
(Fold change)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
••
••
*
0
3 (IC50)
10
0
10
150 (IC50)
400
0
10
215 (IC50)
400
CCRF-
CEM
Clone 1
Clone 2
Nelarabine (µM)
BAX
mRNA expression
(Fold change)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
••
••
••
••
0
3 (IC50)
10
0
10
150 (IC50)
400
0
10
215 (IC50)
400
CCRF-
CEM
Clone 1
Clone 2
Nelarabine (µM)

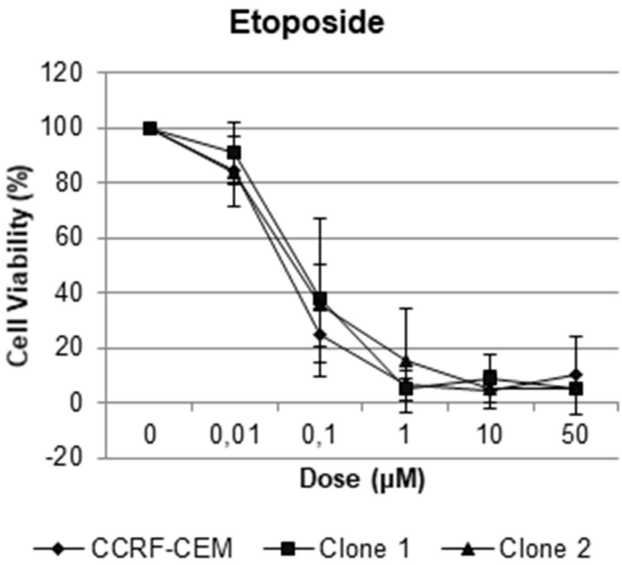

a)
CCRF-CEM
Clone 1
Clone 2
pAkt
Akt
pAkt/Akt ratio
CCRF-CEM
Clone 1
Clone 2
b)
CCRF-CEM
Clone 1
Clone 2
0h
6h
24h
48h
72h
pAkt
Akt
GAPDH
pAkt/Akt ratio
0h
6h
24h
48h
72h
c)
Cell Viability (%)
CEM
Clone 1
Clone 2
Control
Nelarabine
ZSTK474
Nelarabine + ZSTK474
PKI-587
Nelarabine + PKI-587
MK-2206
Nelarabine + MK-2206
n.s
d)
CCRF-CEM
Clone 1
Clone 2
Control
Nelarabine
ZSTK-474
Nelarabine ZSTK-474
PKI-587
Nelarabine PKI-587
MK-2206
Nelarabine MK-2206
PARP
pAkt
Akt
b-actin

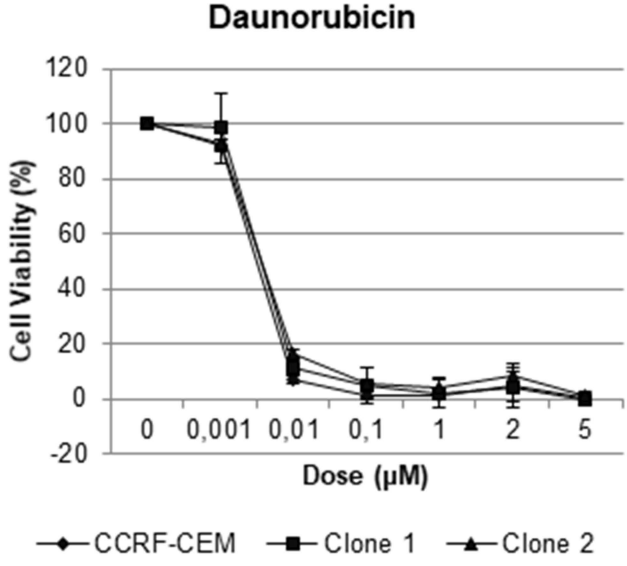

a)
CCRF-CEM
Clone 1
Clone 2
p-ERK
ERK
pERK/ERK ratio
0,25
0,2
0,15
0,1
0,05
0
CCRF-CEM
Clone 1
Clone 2
b)
CCRF-CEM
Clone 1
Clone 2
Cell Viability (%)
120
100
80
60
40
20
0
C
N
10µM
0.1µM
1µM
S
N+S
*
CCRF-CEM
Control
Nelarabine
Selumetinib
Nelarabine + Selumetinib
10uM
0.1uM
1uM
PARP
pERK
ERK
GAPDH
Clone 1
Clone 2
c)
CCRF-CEM
Clone 1
Clone 2
Cell Viability (%)
C
N
T
N+T
**
Trametinib
Nelarabine + Trametinib

a)

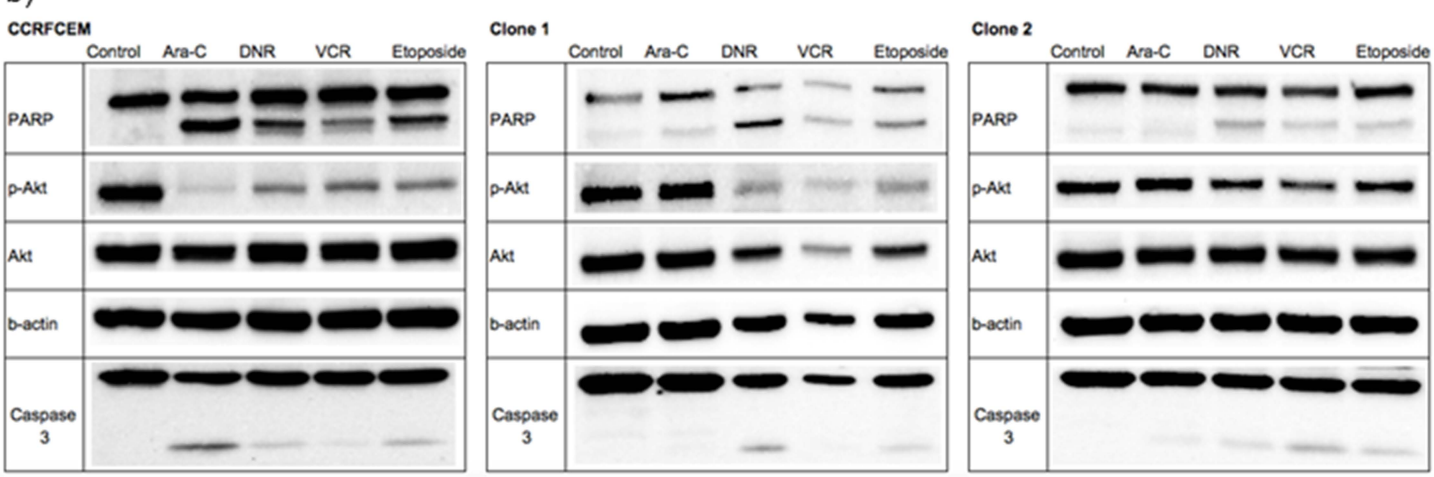

Vincristine
Cell Viability (%)
140
120
100
80
60
40
20
0
-20
0
0,01
0,1
1
10
100
1000
Dose (nM)
CCRF-CEM
Clone 1
Clone 2


Ara-C
Cell Viability (%)
140
120
100
80
60
40
20
0
-20
0
0,01
0,1
1
10
100
Dose (µM)
CCRF-CEM
Clone 1
Clone 2


Etoposide
Cell Viability (%)
120
100
80
60
40
20
0
-20
0
0,01
0,1
1
10
50
Dose (µM)
CCRF-CEM
Clone 1
Clone 2


Daunorubicin
Cell Viability (%)
120
100
80
60
40
20
0
-20
0
0,001
0,01
0,1
1
2
5
Dose (µM)
CCRF-CEM
Clone 1
Clone 2

b)


CCRFCEM
Control
Ara-C
DNR
VCR
Etoposide
PARP
p-Akt
Akt
b-actin
Caspase 3
Clone 1
Control
Ara-C
DNR
VCR
Etoposide
PARP
p-Akt
Akt
b-actin
Caspase 3
Clone 2
Control
Ara-C
DNR
VCR
Etoposide
PARP
p-Akt
Akt
b-actin
Caspase 3