Characterization of Newly Established Pralatrexate-resistant Cell Lines and the Mechanisms of Resistance

Naoko Hosono (hosono@u-fukui.ac.jp)
University of Fukui https://orcid.org/0000-0002-6336-3250
Kana Oiwa
Univeroty of Fukui
Rie Nishi
University of Fukui
Luigi Scott
Columbia University
Owen A. O'Connor
Columbia University
Takahiro Yamauchi
University of Fukui

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Abstract

Background

Pralatrexate (PDX) is a novel antifolate approved for the treatment of patients with relapsed/refractory peripheral T-cell lymphoma. However, some patients exhibit intrinsic resistance or develop acquired resistance to PDX. Here, we evaluated the mechanisms underlying acquired drug resistance and identified strategies to prevent resistance.

Methods

We established two PDX-resistant T-lymphoblastic leukemia cell lines (CEM and MOLT4) through repeated escalating exposure to PDX. Gene expression analysis and methylation profiling were performed to identify the mechanisms of resistance. We then explored rational drug:drug combinations to prevent resistance.

Results

PDX-resistant cells exhibited a 30-fold increase in half-maximal inhibitory concentration values compared with those of their parental cells. Induction of apoptosis by PDX was significantly decreased in both PDX-resistant cell lines. Intracellular uptake of [^{14}C]-PDX decreased in PDX-resistant CEM cells, and dihydrofolate reductase (DHFR) expression was increased in PDX-resistant MOLT4 cells. Gene expression array analysis revealed that DNA-methyltransferase 3β expression was significantly elevated in both cell lines. Moreover, decitabine plus PDX showed synergistic effects in drug-resistant cell lines compared with parental lines. In addition, both PDX-resistant cell lines showed sensitivity to nucleoside analogs, i.e., cytarabine and forodesine.

Conclusions

This is the first study to explore the specific mechanisms of PDX resistance in T-cell lymphoma. The resistance mechanisms were associated with reduced cellular uptake of PDX and overexpression of DHFR. Epigenetic alterations were also considered to play a role in the resistance mechanism. The cells exhibited increased sensitivity to nucleoside analogs. These results could facilitate rational combinations to improve the clinical efficacy of PDX.

Background

Peripheral T-cell lymphomas (PTCLs) are a heterogeneous group of mature T-cell and natural killer-cell neoplasms accounting for approximately 5–15% of non-Hodgkin lymphomas [1]. Owing to intrinsic chemotherapy resistance, the prognosis of patients with PTCL is extremely poor. The overall survival of

patients after first relapse is estimated to be only 5.5 months [2, 3]. Conventional chemotherapy regimens have shown limited activity in the second-line setting and beyond, thereby creating a need for new drugs that are selectively active against T-cell malignancies.

Recently, pralatrexate (10-propargyl 10-deazaminopterin; PDX) has been approved for the treatment of PTCL in the USA, Japan, and other countries. PDX is a folate analog designed to have a high affinity for reduced folate carrier (RFC1), dihydrofolate reductase (DHFR), and folylpolyglutamate synthetase (FPGS), resulting in increased cytotoxic activity compared with methotrexate (MTX) [4, 5]. PDX is transported into cancer cells via RFC1 and undergoes polyglutamylation by FPGS, leading to subsequent inhibition of DHFR and termination of DNA synthesis. Although this mechanism is common for antifolates, PDX may also have other effects in PTCLs in addition to DHFR inhibition.

MTX is a well-established antifolate that blocks the action of DHFR, and has been used to treat various types of non-Hodgkin's lymphomas, including primary central nervous system lymphoma and Burkitt's lymphoma. However, its clinical efficacy in PTCL is limited. PDX has been studied across a variety of non-Hodgkin's lymphomas [6–8] and is most efficacious in patients with PTCL, leading to its accelerated approval by the US Food and Drug Administration for patients with relapsed/refractory PTCL. A recent case match control analysis confirmed that there was a survival advantage for patients enrolled in the Pralatrexate in Patients with Relapsed or Refractory Peripheral T-Cell Lymphoma (PROPEL) study that received PDX compared with a well-matched population of patients receiving the standard of care. These data suggested an overall survival advantage of 14.5 months for patients in the PROPEL trial, compared with only 4 months for the control population [7]. Because of the apparent T-cell selective activity of PDX, this drug has been studied in combination effects with a host of agents, including romidepsin and 5-azacytidine.

Despite this promising activity, some patients can acquire resistance to PDX over time. Identifying the underlying mechanisms of drug resistance could lead to rational strategies to prevent or overcome these mechanisms of resistance, thereby improving the clinical efficacy. Accordingly, in this study, we newly established two PDX-resistant T-lymphoblastic leukemia cell lines designated CCRF-CEM (CEM) and MOLT-4 (MOLT4) in order to explore these mechanisms of drug resistance. These cell lines were then used to develop and evaluate complementary drug combinations as a means to overcome acquired resistance to PDX.

Methods

Cell lines

The human acute T-lymphoblastic leukemia cell lines CEM and MOLT4 were purchased from American Type Culture Collection (Manassas, VA, USA).

Reagents

PDX, forodesine (FDS) [9], MTX, cytarabine (AraC), bortezomib (BOR), decitabine (DAC), deoxyguanosine (dGuo) and panobinostat (LBH589) were used in this research.

Establishment of resistant cell lines

CEM and MOLT4 cells were initially incubated with 0.01 nM PDX, and the concentration of PDX was then gradually increased by 0.01–0.02 nM over a period of 10 months. The initial concentration was 1/100 the concentration required to inhibit 50% growth of the cells (IC_{50}). After acquisition of PDX-resistant cells, single-cell cloning was performed by a limiting dilution method according to previous reports [10–13].

Growth inhibition assay

The IC_{50} value of each drug was calculated from an analysis of growth inhibition. To evaluate the proliferative activity of each cell line, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 h-tetrazolium-5-carboxanilide (XTT) assays were performed according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) [10, 14–18]. Cross-resistance to other antileukemic agents was also assessed. The combination index (CI) of PDX and other agents was calculated using COMPUSYN software (http://www.combosyn.com).

Quantification of apoptotic cell death

The percentage of apoptotic cells was assessed by flow cytometry using an Annexin V-FLUOS Staining kit (Roche Japan). The cells were treated with PDX or FDS for 48 h, washed, and stained with propidium iodide, annexin V-FITC, or both, according to the manufacturer's instructions. The cells were then analyzed using a FACSCant flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Annexin V-positive cells were considered to be apoptotic.

Gene expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and microarray

RT-qPCR was performed by a two-step reaction. RNA from each cell line was prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was then synthesized using a PrimeScript RT reagent kit (Takara, Shiga, Japan), and qPCR was conducted using a TaqMan Fast Advanced Master Mix kit (Applied Biosystems, Waltham, MA, USA). Primers for *RFC1* (Hs01099126), DNA methyltransferase 3β (*DNMT3B*; Hs00171876), and glyceraldehyde 3-phosphate dehydrogenase (Hs2786624) were purchased from Applied Biosystems. The microarray analysis was performed by Clariom S Arrays (Affymetrix Japan) using 10 ng total RNA extracted from parental and PDX-resistant cells.

Western blot analysis

Western blotting analysis was performed [10, 14]. Anti-FPGS, anti-DHFR, anti-DNMT3B, and anti-β-actin antibodies were used as primary antibodies, and anti-rabbit polyclonal antibodies were used as secondary antibodies.

Cellular uptake of [^{14}C]-PDX

Cellular uptake of PDX was calculated by a radioisotope assay. The cells (5×10^6) were incubated with 1 nM [^{14}C]-PDX for 0, 5, 10, 20, or 30 min, and cell pellets were dissolved using SOLUENE-350 and Clear-sol I (Nacalai Tesque, Kyoto, Japan). Radioactivity was measured using a liquid scintillation counter.

Lentiviral-mediated small hairpin RNA (shRNA) knockdown

Viral supernatants containing DNMT3B-shRNA (TRCN0000035686) and the control non-target shRNA were purchased from Sigma-Aldrich. CEM/P cells were infected in the presence of 25 mg/mL retronectin (Takara Bio) for 6 h and selected with puromycin (10 mg/mL).

Results

Establishment of two PDX-resistant cell lines

To generate PDX-resistant cell lines, the human acute T-lymphoblastic leukemia cell lines CEM and MOLT4 were exposed to gradually escalating PDX concentrations for 10 months. The IC_{50} values for the PDX-resistant cell lines (CEM/P and MOLT4/P) were 20 nM and 80 nM, respectively. In comparison with the IC_{50} values of the parental cells (CEM: 0.6 nM, MOLT4: 2.4 nM), the relative level of PDX resistance was increased by approximately 33-fold in both cell lines (Table 1). The doubling times of PDX-resistant cells were similar to those of their parental counterparts (Supplementary Data 1), and the degree of resistance in these cells did not change for 6 months despite culturing the cells in medium without PDX.

Table 1. Growth inhibitory effects of PDX.

	IC50 (nM)		
	Parental	PDX-R	[ratio]
CEM	0.6	20	33
MOLT4	2.4	80	33

IC_{50}, 50% growth inhibitory concentration; PDX, pralatrexate.

To assess PDX-induced cytotoxicity, we evaluated the induction of apoptosis using flow cytometry. After 48 h of treatment with PDX at the IC_{75} (5 nM for CEM cells, 10 nM for MOLT4 cells), induction of apoptosis was observed in 78.7% of CEM cells and 77.7% of MOLT4 cells, whereas only 4.4% of CEM/P cells and 5.9% of MOLT4/P cells were apoptotic at the same concentration as used for parental cell lines (Fig. 1).

Intracellular uptake of PDX

We next focused on the intracellular uptake of PDX because acquired mechanisms of resistance to MTX have been attributed to loss of RFC1, resulting in decreased MTX internalization [19, 20]. RT-qPCR analysis showed that the expression level of *RFC1* mRNA in CEM/P cells was significantly decreased compared with that in parental CEM cells (Fig. 2a). Accordingly, intracellular uptake of [^{14}C]-PDX was significantly decreased in CEM/P cells. Area under the curve values were 21,646.7 dpm·min in CEM cells and 14,337.7 dpm·min in CEM/P cells ($p = 0.0097$; Fig. 2c). There were no significant differences in *RFC1* expression and intracellular uptake of [^{14}C]-PDX between MOLT4 and MOLT4/P cells (Fig. 2b and 2d). Sequencing analysis of exons 2 and 3 in *RFC1* showed that no acquired somatic mutations in PDX-resistant cells (Supplementary data 2).

Expression levels of FPGS and DHFR

The resistance mechanisms of intracellular folate are thought to be related to deficiencies in FPGS activity [17, 18, 21] and/or increased DHFR expression [22–24]. FPGS catalyzes the formation of polyglutamate chains, yielding active PDX polyglutamates. Therefore, we examined the expression levels of FPGS protein in PDX-resistant cells and parental cells, demonstrating no significant differences (Fig. 3a, 3b). In contrast, the protein expression level of DHFR, a key enzyme involved in intracellular folate metabolism and induction of intrinsic resistance to MTX, was increased in MOLT4/P cells compared with that in the parental cell line (Fig. 3c, 3d).

Patterns of cross-resistance to other anticancer agents

To assess acquired drug resistance in PDX-resistant cells, the growth inhibitory effects of other anticancer drugs were compared between parental cells and PDX-resistant cells, and the IC_{50} values and relative degree of resistance were determined (Fig. 4, Supplementary Data 3). As expected, CEM/P and MOLT4/P cells were 2.1- and 3.0-times more resistant to MTX, respectively. The sensitivity of the cells to nucleoside analogs such as FDS and DAC was more prominent in PDX-resistant cells; the relative degree of resistance to each drug was 0.4 and 0.4, respectively, in CEM/P cells, and 0.9, and 0.2, respectively, in MOLT4/P cells. There was no cross-resistance to BOR or LBH589 observed in PDX-resistant cell lines.

Next, we examined whether FDS induced cell death through apoptosis in PDX-resistant cells. After 72 h of treatment with FDS with dGuo (10 μM), the proportion of Annexin V-positive cells slightly decreased in PDX-resistant cells compared with the parental cells, suggesting that apoptosis may contribute to the acquisition of PDX resistance (Fig. 4b).

Gene expression analysis

To identify the potential mechanisms underlying PDX resistance, we performed mRNA expression profiling of PDX-resistant and parental cells using microarray analysis. In comparison with mRNA profiles, we identified 4,227 and 4,034 significantly deregulated transcripts in CEM/R and MOLT4/R cells, respectively (Fig. 5a). The five most highly up- or downregulated transcripts in PDX-resistant cell lines are shown in Fig. 5b and 5c. There were no significant differences in the expression levels of folate metabolism pathway components such as *FPGS, DHFR*, and thymidylate synthase (*TS*) in both PDX-

resistant cell lines (Fig. 5d). When we focused on transcripts that were commonly deregulated among the two PDX-resistant cell lines, we found that the *DNMT3B* expression level was significantly elevated in both CEM/P and MOLT4/P cells. When PDX sensitivity was examined using DNMT3B-knockdown cells, the IC_{50} values were 30 and 18 nM in CEM/Pmock and CEM/PshDNMT3B cells, respectively, suggesting limited recovery of PDX sensitivity (Fig. 6).

Enhancement of the cytotoxicity of PDX by DAC in PDX-resistant cells

Finally, we evaluated the advantage of combining PDX with DAC, an inhibitor of DNMT, which could pharmacologically counterbalance the high expression of DNMT3B. Treatment with DAC significantly inhibited cell growth in combination with PDX, even at a DAC concentration of 1 μM. Strong synergistic effects (CI = 0.15–0.31) were observed at the IC_{50} in both PDX-resistant cell lines (Fig. 7). This synergism was specific to PDX-resistant cells, as the combination of PDX and DAC in parental cells only showed an additive effect (CI = 0.97–1.02). These results suggested that epigenetic agents may overcome the cellular resistance to PDX.

Discussion

Using classical methods to produce drug-resistant cell lines, we generated two cell lines that showed approximately 30-fold greater resistance to PDX than their parental counterparts. The characteristics of the PDX-resistant cells were as follows: (i) decreased internalization of PDX in CEM/P cells owing to reduced RFC expression; (ii) increased expression of DHFR protein in MOLT4/P cells; (iii) collateral sensitivity to nucleoside analogs such as FDS and AraC; and (iv) synergism with the hypomethylating agent DAC.

These data are the first to address the potential mechanisms of acquired PDX resistance, although the molecular mechanisms of antifolate resistance to MTX in tumor cells have been extensively studied [25, 26]. The most frequent mechanism responsible for mediating antifolate resistance is related to impairment of antifolate uptake, typically due to reduced expression of RFC1 and/or inactivating mutations in RFC1. Increased antifolate efflux owing to overexpression of multidrug resistance efflux transporters has also been reported [25, 26]. Mechanisms related to defective polyglutamylation have been shown to be attributed to decreased FPGS expression, inactivating mutations in FPGS, and increased expression of gamma glutamyl hydrolase (GGH) [25, 26]. In addition, amplification of *DHFR* and/or *TS* caused by overexpression has been reported, and mutations in these genes can decrease their affinity for antifolates, resulting in antifolate resistance [25, 26]. Our results indicated that impaired internalization of PDX owing to decreased expression of RFC1 may be one of the mechanisms of PDX resistance in CEM/P cells. In contrast, in MOLT4/P cells, increased DHFR expression was observed, suggesting that increased DHFR protein levels may be one of the mechanisms of PDX resistance in MOLT4/P cells.

In our study, PDX-resistant cells were highly sensitive to nucleoside analogs. MTX inhibits cell growth by interfering with enzymatic reactions that require thymidine and purine synthesis. According to previous reports, nucleobase salvage is necessary for the activity of MTX [27, 28]. Thus, collateral sensitivity to nucleoside analogs may have occurred. The single use of FDS or AraC may have cytotoxic effects on PDX-resistant cells *in vivo*. FDS-induced apoptosis was reduced in both PDX-resistant cell lines. Thus, the apoptotic pathway may be partly associated with the acquisition of PDX resistance, although the mechanisms responsible for this effect have not yet been clarified.

As shown in Fig. 5, the expression level of DNMT3B was increased in PDX-resistant cells, and the sensitivity to DAC was also significantly higher. DNMT3B has a prominent role in the methylation of CpG islands, and overexpression of DNMT3B is related to drug resistance [29, 30]. Knockdown of DNMT3B in CEM/P cells resulted in only partial restoration of PDX resistance. Although we did not perform methylation array analysis in our PDX-resistant cell lines, the products of methylated genes may confer high levels of PDX resistance. Thus, combination with DAC resulted in high susceptibility to PDX in resistant cells.

Recently, several studies have explored potentially synergistic combinations with PDX [15, 16, 31–35]. For example, T-cell lymphomas are driven by some epigenetic defects [34], and sensitivity to epigenetic therapies such as histone deacetylase (HDAC) inhibitors (e.g., romidepsin) or DNMT inhibitors [33] has been noted. Combination of PDX with romidepsin showed promising activity in patients with relapsed/refractory PTCL in a recently published phase I study. Among patients with T-cell lymphoma, the overall response rate was 71% (10/14), with 29% (4/14) achieving a complete response in the phase I trial [15, 31]. Participants are now being actively recruited for a phase II trial (clinical trial no. NCT01947140). Additionally, combinations of HDAC inhibitors and DNMT inhibitors such as romidepsin and azacytidine or DAC are also effective in clinical setting [33, 34]. Marchi et al. reported that the combination of romidepsin and DAC increased the number of modulated genes involved in apoptosis and cell cycle arrest [33]. A clinical trial evaluating the combination of DAC, PDX, and pembrolizumab in PTCL is now underway (NCT03240211). We are expecting that the combination of PDX with DAC may be effective in relapsed/refractory PTCLs in a clinical setting.

Conclusion

This is the first report to investigate the mechanisms of PDX resistance. The resistant cells demonstrated sensitivity to nucleoside analogs and hypomethylating agents. Reduced cellular uptake of PDX and epigenetic alterations may contribute to the development PDX resistance. FDS, a new T-lymphoma-targeting agent, may be effective in this setting. These findings will support clinical trials for patients with refractory/relapsed PTCL using PDX as a single agent or in combination.

Abbreviations

PDX

pralatrexate
DHFR
dihydrofolate reductase
PTCLs
peripheral T-cell lymphomas
RFC1
reduced folate carrier
FPGS
folylpolyglutamate synthetase
MTX
methotrexate
PROPEL
Patients with Relapsed or Refractory Peripheral T-Cell Lymphoma
CEM
CCRF-CEM
MOLT4
MOLT-4
FDS
forodesine
AraC
cytarabine
BOR
bortezomib
DAC
decitabine
dGuo
deoxyguanosine
LBH589
panobinostat
IC_{50}
inhibit 50% growth of the cells
XTT
2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 h-tetrazolium-5-carboxanilide
CI
combination index
RT-qPCR
reverse transcription-quantitative polymerase chain reaction
DNMT3B
DNA methyltransferase 3β
GGH

gamma glutamyl hydrolase
HDAC
histone deacetylase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed, together with the PDX-resistance cell line, during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Conceptualization and statistics by TY and OAO; methodology and design of experiment by NH and LS; experiments and editing by KO and RN; Project Administration by TY. All authors read and approved the final manuscript.

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Figures

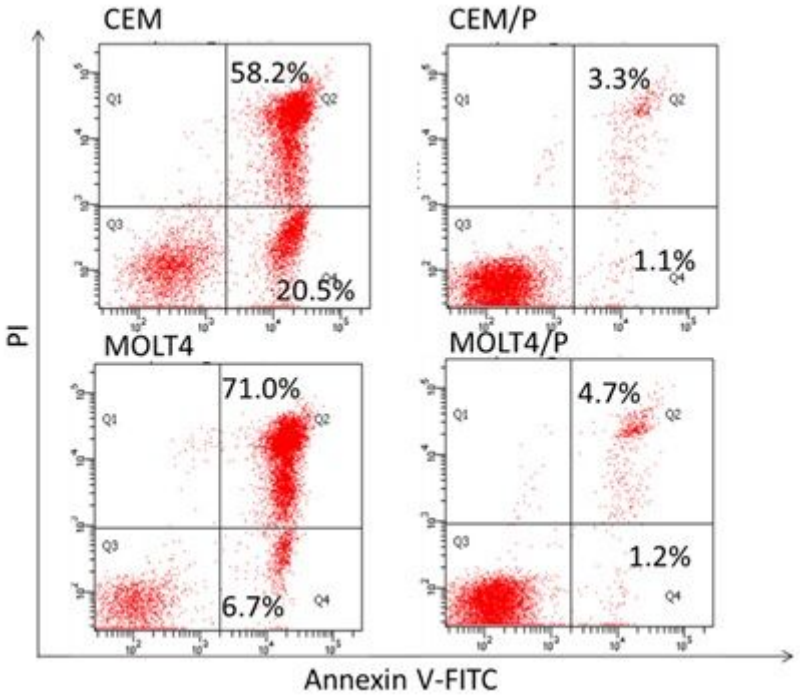

Figure 1

Induction of apoptosis by PDX. Apoptotic cells induced by PDX treatment were detected using Annexin V-FITC staining. After 48 h of treatment at the IC75 for each parental cell line (CEM and CEM/P: 5 nM, MOLT4 and MOLT4/P: 10 nM), cells were stained with both Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Dimethyl sulfoxide-treated cells were used as a control. IC75, 75% growth inhibitory concentration.

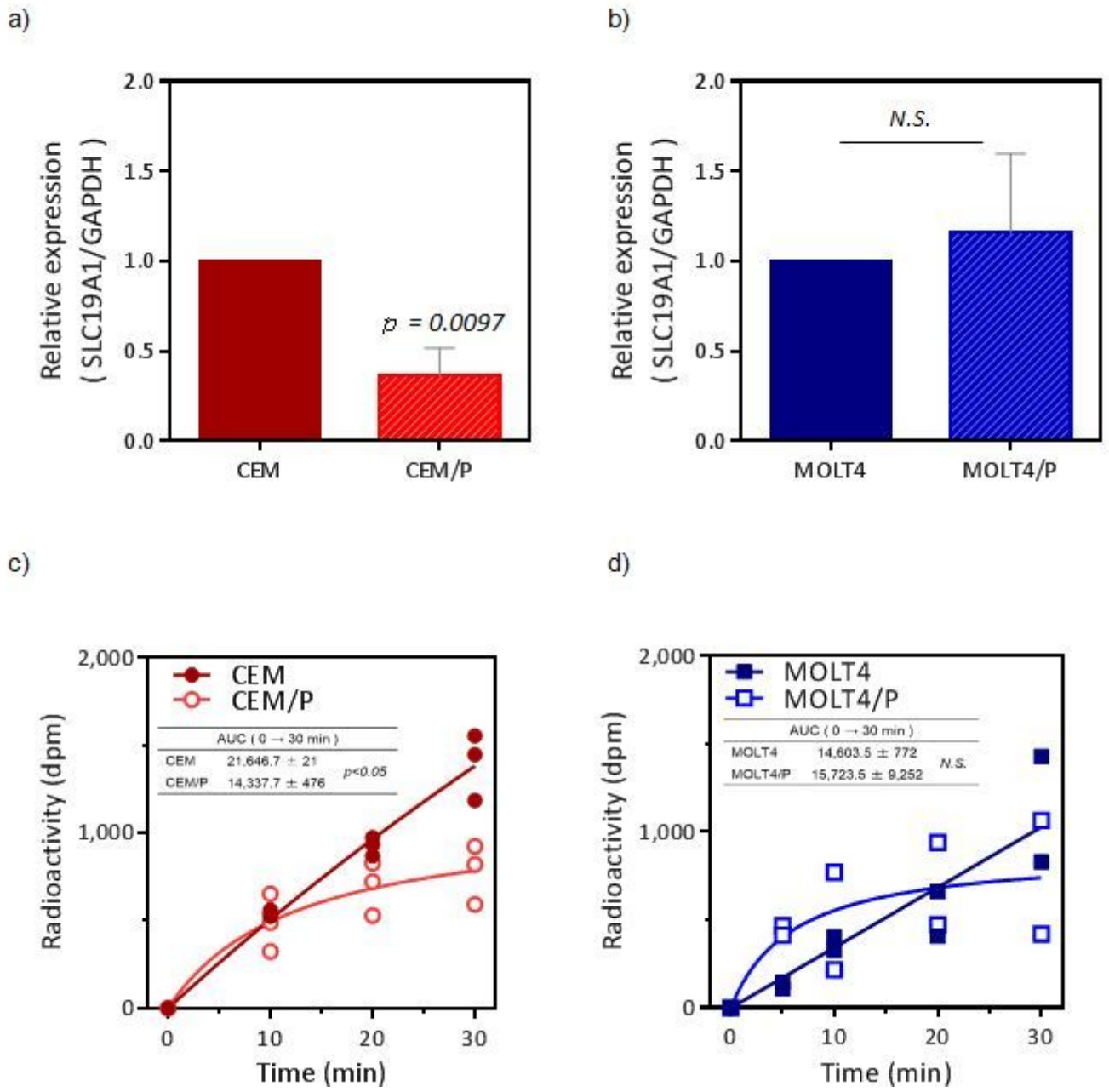

Figure 2

Expression levels of RFC1 and drug uptake. a, b) RFC1 transcript level. RQ-PCR analyses of RFC1 (SLC19A1) in parental and PDX-resistant cells are shown. Expression levels of mRNA of RFC1 were assessed by RQ-PCR. Statistical analysis was performed using two-tailed pares Student's t-tests. c, d) Cellular uptake of [14C]-PDX was assayed by radioimmunoassay. Cells were incubated with 1 nM [14C]-PDX for various times and evaluated for radioactivity using a liquid scintillation counter. Statistical analysis was performed using two-tailed pares Student's t-tests

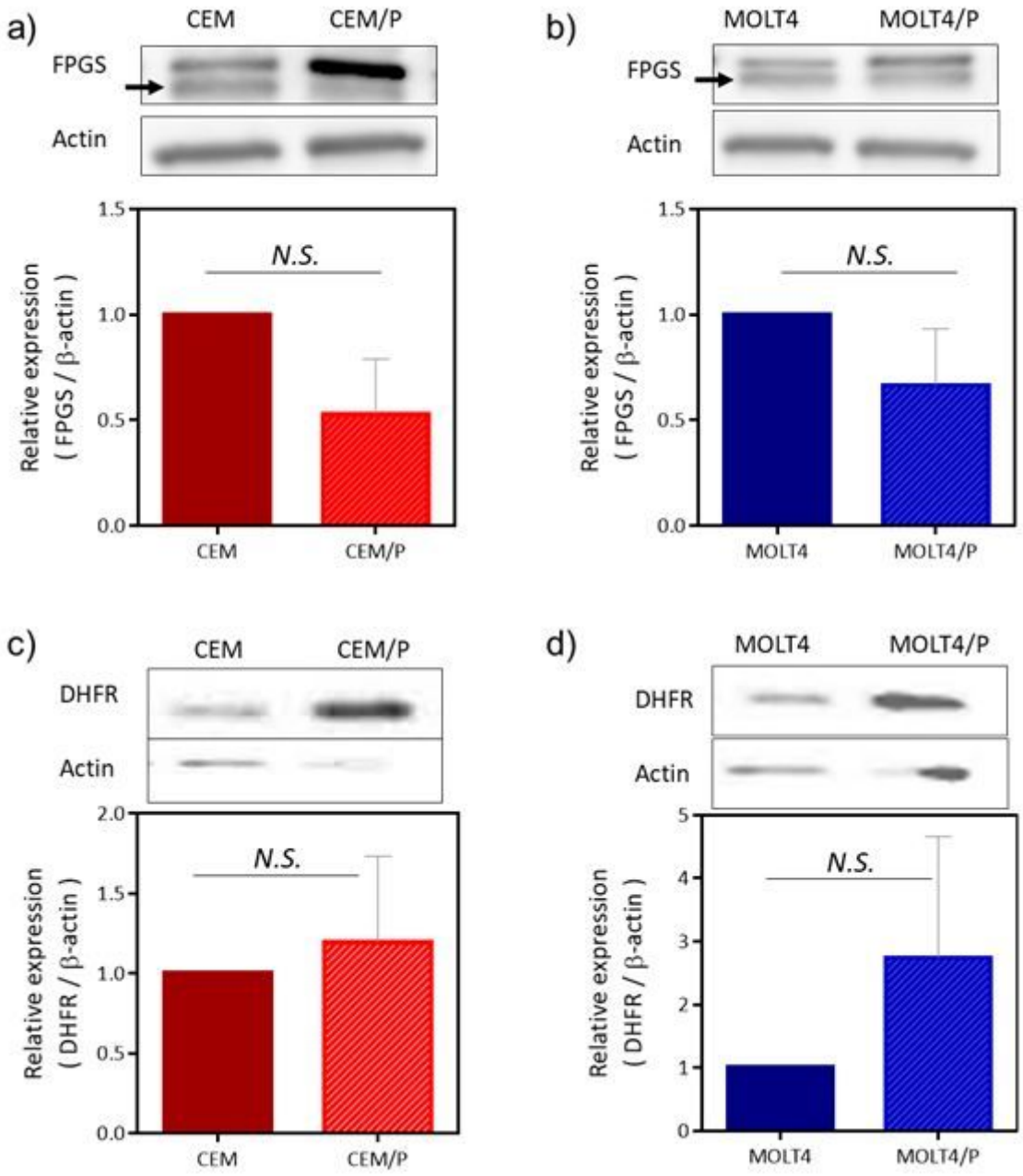

Figure 3

Expression levels of FPGS and DHFR. Western blotting of FPGS (a, b) and DHFR (c, d) in parental and PDX-resistant cells. Full-length blots/gels are presented in Supplementary Figure 1-2. N.S., not significant

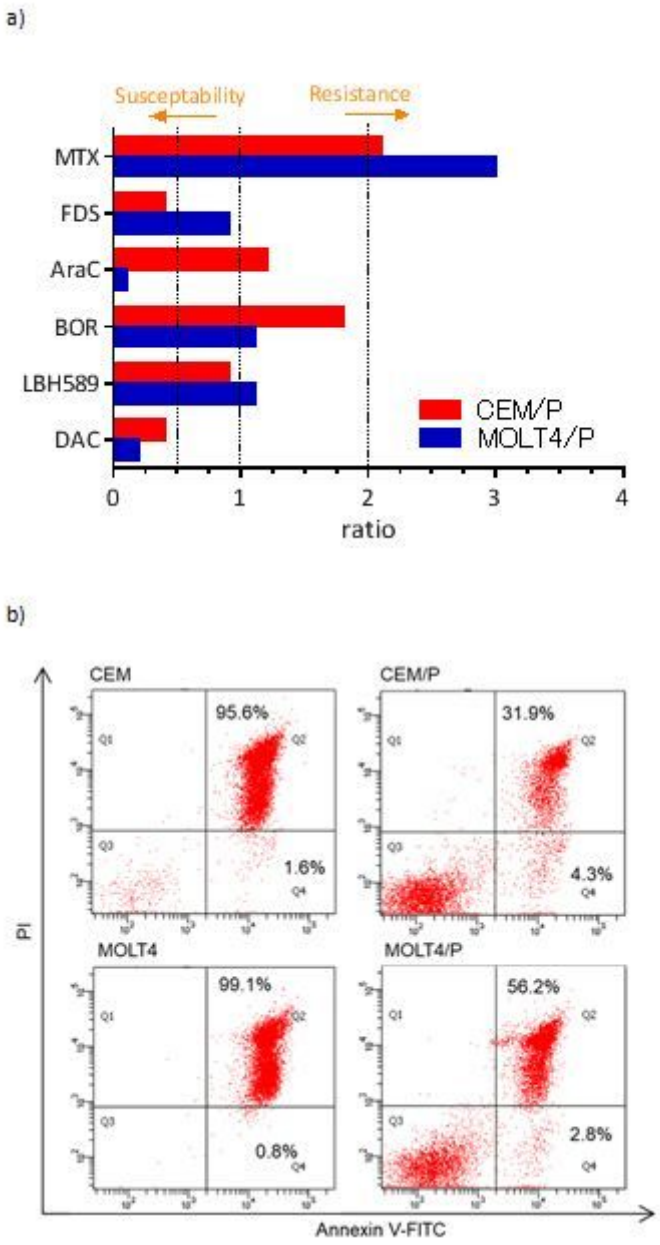

Figure 4

Cross-resistance profile in PDX-R cells. a) Cross-resistance for other anti-lymphoma agents. Parental or PDX-resistant cells were incubated with various concentrations of MTX, FDS*, AraC, BOR, or LBH589. IC50 values were calculated by XTT assays. Ratios were calculated by dividing the IC50 of PDX-R cells by that of each parental cell line. b) Induction of apoptosis by FDS. After 72 hours of FDS* treatment at the indicated concentration (CEM and CEM/P cells: 5 nM, MOLT4 and MOLT4/P cells: 8 nM), cells were stained with both Annexin V-FITC and PI and analyzed by flow cytometry. *In assays using FDS, cells were incubated with deoxyguanosine (10 μM) because the cytotoxicity of FDS requires the presence of deoxyguanosine in vitro. AraC, cytarabine; BOR, bortezomib; FDS, forodesine; LBH589, panobinostat; MTX, methotrexate

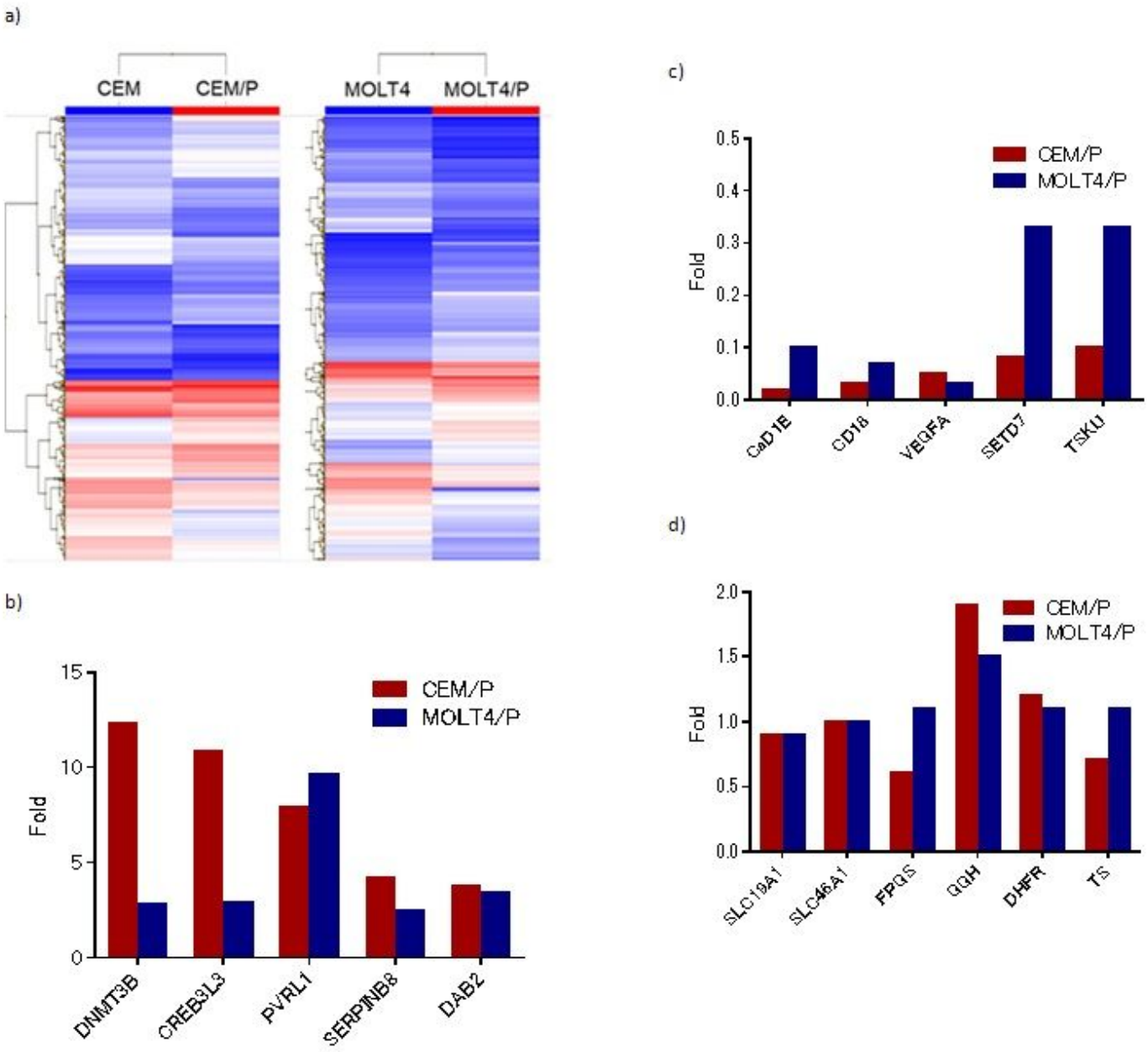

Figure 5

Microarray analysis. Total RNA (10 ng) from parental and PDX-resistant cells was obtained, and mRNA expression was evaluated using Clariom S Arrays. a) Heat map of genes with expression changes of more than 2-fold or less than 0.5-fold. b, c) Top five identical genes with expression changes in both PDX-resistant cell lines. b) Upregulated genes, and c) downregulated genes. d) Some major genes related to folate metabolism in PDX-resistant cells.

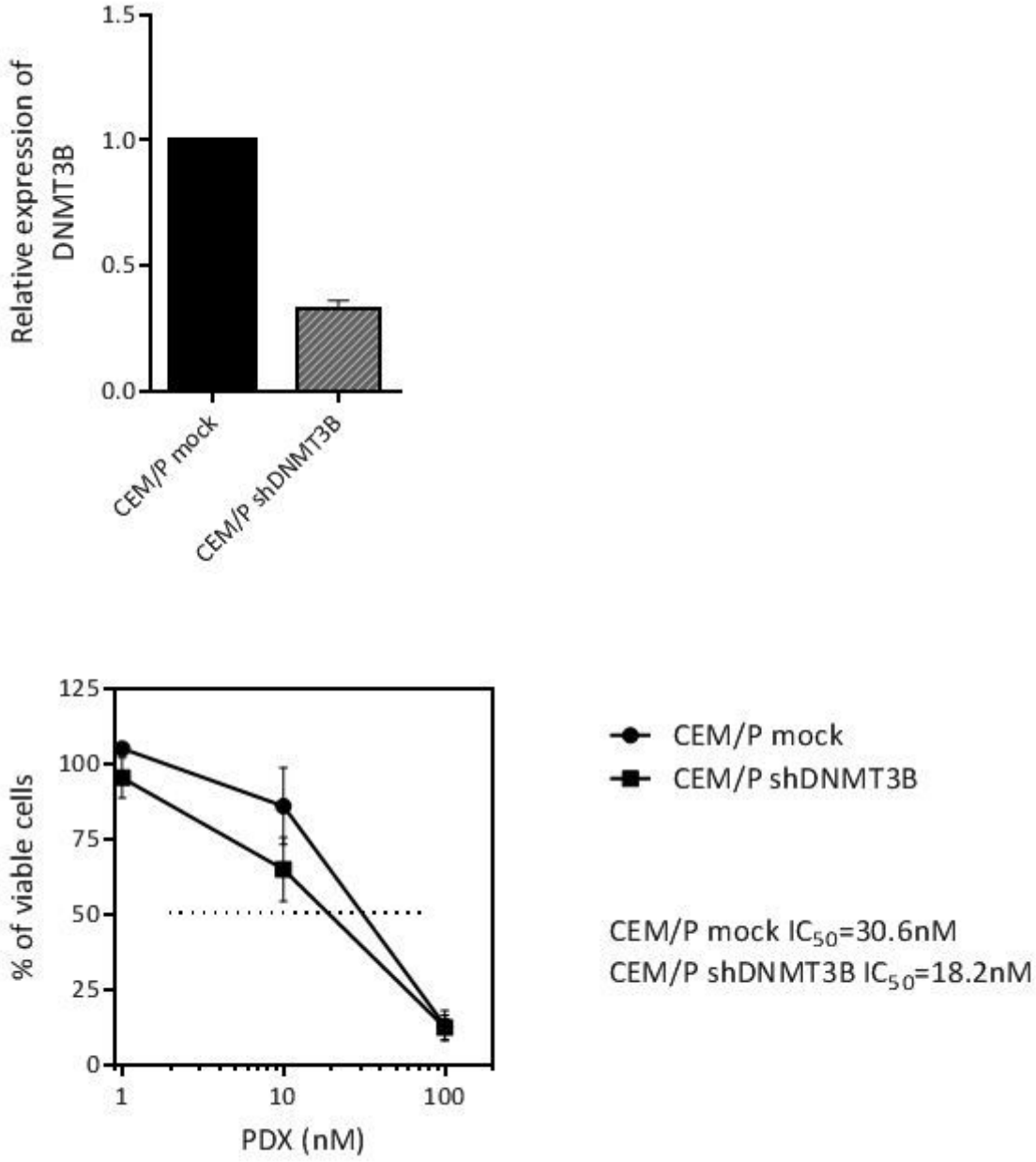

Figure 6

Comparison of PDX-resistant cells and DNMT3B knockdown cells Using viral supernatants containing DNMT3B-shRNA (TRCN0000035686) and the control non-target shRNA, PDX-resistant CEM cells were infected in the presence of 25 mg/mL of Retronectin for 6 hr and selected with puromycin (10 mg/mL). DNMT3B, DNA methyltransferase 3B.

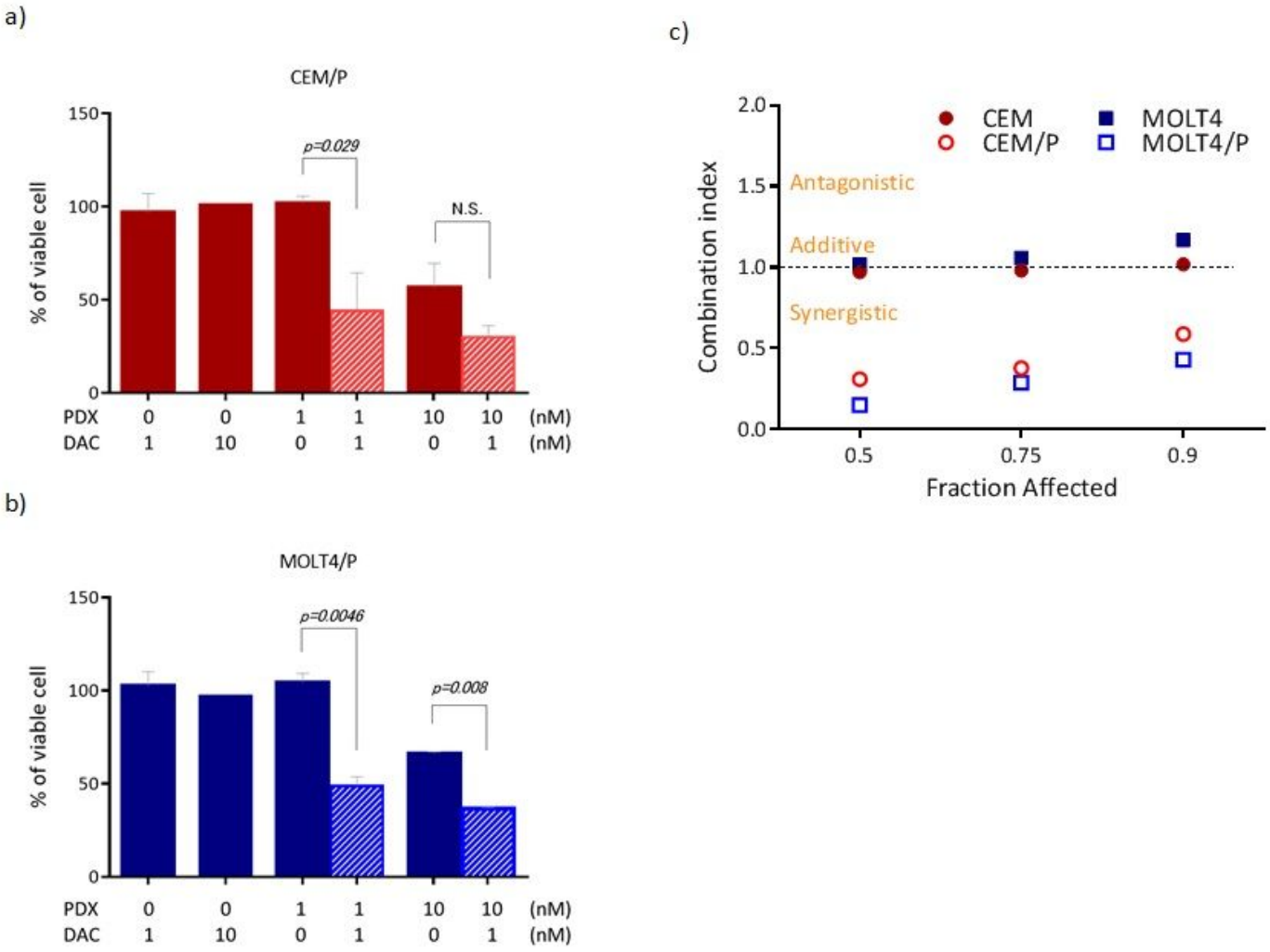

Figure 7

Combination of PDX with DAC. Parental or PDX-resistant cells were incubated with various concentrations of PDX and DAC for 72 hours. Viable cells were assayed by XTT assay. The combination index (CI) of PDX and DAC was calculated using COMPUSYN software (http://www.combosyn.com) (PDX:DAC = 1:100). Statistical analysis was performed using two-tailed paired Student's t-tests

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DHFRsupple0829.pptx
- FPGSsupple0829.pptx
- SupplementaryData.docx