Published in final edited form as:

Leuk Res. 2016 August ; 47: 100–108. doi:10.1016/j.leukres.2016.05.019.

Romidepsin enhances the cytotoxicity of fludarabine, clofarabine and busulfan combination in malignant T-cells

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Abstract

Novel approaches to pre-transplant conditioning are needed to improve treatment of advanced T-cell malignancies. We investigated the synergism of fludarabine (Flu), clofarabine (Clo), busulfan (Bu), and romidepsin (Rom) in T-cell lines and patient-derived cell samples. [Flu+Clo+Bu+Rom] had combination indexes of 0.4–0.5 at ~50% cytotoxicity in PEER and SUPT1 cells, suggesting synergism. Drug exposure resulted in histone modifications, DNA-damage response (DDR), increased reactive oxygen species (ROS), decreased glutathione (GSH) and mitochondrial membrane (MM) potential, and apoptosis. Similar activation of DDR and apoptosis was observed in patient samples. The PI3K-AKT-mTOR, NF κ B, Raf-MEK-ERK, JAK-STAT and Wnt/ β -catenin pro-survival pathways were inhibited by the 4-drug combination. The SAPK/JNK stress pathway was activated. A novel finding was the down-regulation of the drug transporter MRP1. We propose the following mechanisms of synergism: Flu, Clo and Rom induce histone modifications and chromatin remodeling, exposing DNA to Bu alkylation; the increased production of ROS, due to drug-mediated stress response and decreased GSH, damages the MM causing leakage of pro-apoptotic factors; down-regulation of MRP1 increases intracellular Bu concentration and exacerbates the DDR; and inhibition of multiple survival pathways. Our results provide the basis for a clinical trial to evaluate [Flu+Clo+Bu+Rom] as part of conditioning regimen for refractory T-cell malignancy patients undergoing stem cell transplantation.

Graphical Abstract

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Conflict of interests

The authors declare no financial conflict(s) of interest.

1. Introduction

T-cell malignancies are lymphoid hematologic disorders, which include T-cell acute lymphoblastic leukemia (T-ALL), prolymphocytic leukemia (T-PLL), cutaneous T-cell lymphoma (CTCL), and peripheral T-cell lymphoma (PTCL). With modern chemotherapy regimens, up to 60% of patients with T-ALL and ~40% of patients with PTCL can be cured. However, many patients relapse, and the only potentially curative therapy is allogeneic stem cell transplantation (allo-SCT). Likewise, for patients with T-PLL and CTCL there is no curative therapy aside from allo-SCT. In these high risk populations, long-term survival rates after allo-SCT range from ~25–40% [1–5]. A novel approach that targets multiple disease subtypes is needed due to disease heterogeneity.

Romidepsin (Rom) is approved for treatment of relapsed/refractory CTCL and PTCL. It is a histone deacetylase (HDAC) inhibitor. Since dysregulation of HDACs is associated with T-cell malignancies [6], treatment with Rom plus other drugs that invoke different mechanisms of cell death may improve patient prognosis. Rom-mediated cell death may be due to restoration of normal gene expression [7, 8], production of reactive oxygen species [9, 10], mitochondrial membrane dysfunction and caspase activation [11], and inhibition of pro-survival pathways [12, 13]. Recently, we reported a Rom-associated DNA hypomethylating activity, which increased SFRP1 expression and inhibited the Wnt/β-catenin pathway [14].

Based on these effects, we hypothesized that Rom in combination with the nucleoside analogs fludarabine (Flu) and clofarabine (Clo) and the DNA alkylator busulfan (Bu) would be synergistically cytotoxic to malignant T-cells. Since the efficacy of Flu, Clo and Bu as part of pre-transplant regimens has been reported [15], we performed preclinical studies to elucidate whether [Flu+Clo+Bu+Rom] can be used as a pre-transplant therapy for T-cell leukemia/lymphoma. Our results show synergistic cytotoxicity of the four drugs in malignant T-cell lines and primary patient samples, which may be used to design a clinical trial.

2. Materials and Methods

2.1. Cell culture and patient samples

Two established T-cell lines were used: PEER (from Dr. Garcia-Manero, University of Texas MD Anderson Cancer Center (UTMDACC)) was originally established from the peripheral blood of a T-ALL patient [16], and SUPT1 (ATCC, Manassas, VA) was derived from a pleural effusion of another T-ALL patient [17]. Blood samples from three patients with T-cell malignancies were collected after obtaining written informed consent. This study was approved by the Institutional Review Board of the UTMDACC. All cell cultures were performed as previously described [14].

2.2. Drugs

Flu and Bu were obtained from Sigma-Aldrich (St. Louis, MO), Clo from Genzyme Corporation (Cambridge, MA), and romidepsin from Selleck Chemicals (Houston, TX). Flu and Bu were dissolved in dimethyl sulfoxide (DMSO) to a final DMSO concentration <0.08%. Z-VAD-FMK (Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO.

2.3. Drug cytotoxicity and Western blotting

Cytotoxicity assays (including the determination of IC_{10-20} values) and Western blotting (see Table 1, Supplementary Data for antibodies) were performed as described [18]. Drug synergisms were estimated based on the combination index (CI) values [19] determined using the CalcuSyn software (Biosoft, Ferguson, MO). $CI < 1$ indicates synergism, $CI \approx 1$ is additive, and $CI > 1$ suggests antagonism.

2.4. Determination of production of reactive oxygen species (ROS) and changes in mitochondrial membrane potential (Ψ_m)

Cells were exposed to drugs for 48 hrs and analyzed for production of ROS using the CM- H_2DCFDA probe and changes in Ψ_m using the JC-1 probe as described [18]. Results were calculated relative to the untreated control.

2.5. Assay for caspase 3 activity

Cells were exposed to drugs for 48 hrs and harvested by centrifugation and washing with ice-cold PBS. Cell extracts were prepared and analyzed for caspase 3 enzymatic activity based on cleavage of the DEVD sequence using a colorimetric assay kit (Millipore, Billerica, MA).

2.6. Assay for glutathione (GSH)

Cells were pelleted and lysed using a buffer included in the GSH assay kit (Cayman Chemical). Lysates from sonicated cells were centrifuged and the supernatants were analyzed for total protein concentration using a BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA). Supernatants containing equal amounts of protein were de-proteinated and analyzed for GSH relative to the control.

3. Results

3.1. [Flu+Clo+Bu+Rom] combination shows synergistic cytotoxicity in neoplastic T-cells

Cells were exposed to individual drugs to calculate their IC_{10-20} values, which were used in the drug combination experiments to determine their effects on cell proliferation and apoptosis. Exposure of PEER cells to 0.3 μ M Flu, 28 nM Clo, 134 μ M Bu or 5.2 nM Rom resulted in ~80% cell proliferation relative to control. [Flu+Bu], [Clo+Bu], and [Flu+Clo+Bu] had similar effects. Addition of Rom to the two-drug combinations decreased cell proliferation to ~45%–50% of control; an even greater effect was observed with [Flu+Clo+Bu+Rom] which resulted in ~70% growth inhibition (Fig. 1A). These results correlate with the Annexin V data. [Flu+Bu+Rom], [Clo+Bu+Rom] and [Flu+Clo+Bu+Rom] combinations caused ~57%–65% cell death as indicated by Annexin V positivity. To test for a synergistic interaction, cells were exposed to different concentrations of individual drugs or to the four-drug combination at a constant concentration ratio, and the Annexin V assay was performed. The calculated CI at increasing drug effects were graphically analyzed. Fig. 1B shows a CI of ~0.5 at a fraction affected (F_a) = 0.4, suggesting significant synergism.

Similar results were obtained using SUPT1 cells exposed to [Flu+Clo+Bu+Rom], which resulted in ~85% growth inhibition and ~46% Annexin V-positive cells whereas the individual drugs and other combinations showed much lesser effects (Fig. 1C). Combination indices of less than 0.5 were obtained at F_a = 0.4 (Fig. 1D), indicating significant synergism.

3.2. [Flu+Clo+Bu+Rom] activates the DNA-damage response (DDR) and induces histone modifications

Since [Flu+Clo+Bu] activates the DDR in AML cells [20], we examined if [Flu+Clo+Bu+Rom] would have similar effects in neoplastic T-cells. Since the activation of DDR is an early event, we exposed PEER cells to drugs for 24 hrs and analyzed them by Western blotting. Phosphorylation of the ATM kinase (Ser1981) increased in the presence of individual drugs, two-drug combinations and three-drug combinations (Fig. 2A). A more dramatic increase was observed with [Flu+Clo+Bu+Rom]. This observation is generally consistent with increased phosphorylation of the ATM substrates histone 2AX, CHK2 and SMC1 (Fig. 2A); an exception was seen with KAP1 where phosphorylation appeared to track more with the inclusion of Clo in the treatment regimen.

We next analyzed the effect of these combinations on various chromatin protein modifications and enzymes involved therein at 48 hrs. Acetylation of histone 3 at lysine 9 and 27 increased with [Flu+Bu+Rom] and [Clo+Bu+Rom], and more significantly with [Flu+Clo+Bu+Rom] (Fig. 2B). Methylation of histone 3 had a similar pattern, consistent with the reported functional interactions between histone methyltransferases and deacetylases [21]. Romidepsin also increased acetylation of α -tubulin when combined with [Flu+Bu], [Clo+Bu] or [Flu+Clo+Bu] (Fig. 2B). The level of histone deacetylases HDAC3 and HDAC4 and histone demethylases JMJD1B and JARID1B decreased, which may be responsible for the increased histone modifications; no significant change in the level of HDAC2 and LSD1 was observed, suggesting specificity (Fig. 2B).

3.3. [Flu+Clo+Bu+Rom] induces apoptosis

Whether the observed DDR might lead to apoptosis was assessed by analyzing the cleavage of PARP1 and caspases 3, 8 and 9, which are indicators of apoptosis. [Flu+Bu+Rom], [Clo+Bu+Rom] and [Flu+Clo+Bu+Rom] caused significant protein cleavage (Fig. 3A). Involvement of caspases is demonstrated by the reversal of Annexin V-positivity (Fig. 3B), cleavage of PARP1 and caspase 3, and phosphorylation of histone 2AX by the pan-caspase inhibitor Z-VAD-FMK (Fig. 3C). Levels of the pro-apoptotic protein BNIP3 increased in cells treated with Rom containing 3- or 4-drug combinations whereas the levels of the pro-survival/anti-apoptotic proteins BCL-xL, MCL-1, XIAP and c-MYC decreased; more profound effects occurred with [Flu+Clo+Bu+Rom], especially for XIAP (Fig. 3A).

3.4. [Flu+Clo+Bu+Rom] induces production of ROS

To further identify possible mechanisms of apoptosis activation, we examined the production of ROS in PEER cells exposed to [Flu+Clo+Bu] and [Flu+Clo+Bu+Rom]. Flow cytometry showed an ~2-fold increase in ROS levels in cells exposed to [Flu+Clo+Bu] versus control cells, which increased by a further ~4-fold with Rom (Fig. 4A). ROS production was inhibited by pre-exposure to N-acetylcysteine (NAC), a known anti-oxidant. This ROS production correlated with a decreased Ψ_m , which was abrogated in the presence of NAC (Fig. 4B). The decrease in Ψ_m may cause pro-apoptotic factors to leak from the mitochondria and activate caspases. Indeed, caspase 3 activity increased in cells exposed to [Flu+Clo+Bu+Rom], and was inhibited by NAC (Fig. 4C). This caspase 3 activation closely paralleled the triggering of apoptosis as shown by the Annexin V assay (Fig. 4D). Overall, cell exposure to [Flu+Clo+Bu+Rom] induces ROS production, decreases Ψ_m , causes leakage of pro-apoptotic factors and activates caspases, and commits cells to apoptosis as shown by the cleavage of PARP1, caspase 3 and caspase 9 (Fig. 4E). The relevance of ROS is supported by the reversal of these effects in cells pre-exposed to NAC.

GSH is another anti-oxidant that neutralizes ROS [22]. We hypothesized that the increased ROS level might be partly due to decreased cellular GSH. Indeed, our analysis showed an ~35% decrease in the level of GSH relative to the control in PEER cells exposed to [Flu+Clo+Bu+Rom] (Fig. 4F). Since Bu metabolism depends on GSH, the observed decrease in GSH may favor free Bu and contribute to the observed synergism.

3.5. The PI3K-AKT-mTOR and NF κ B signaling pathways are inhibited by [Flu+Clo+Bu+Rom]

Romidepsin binds to PI3K, thereby inhibiting its enzymatic activity *in vitro*; it also inhibits the AKT-mTOR pathway downstream of PI3K [12]. We wanted to determine if a low concentration of Rom combined with IC₁₀₋₂₀ concentrations of Flu, Clo and Bu would inhibit the PI3K-AKT-mTOR pathway, and if so, which PI3K isoforms might be affected. The level of all three isoforms of the catalytic subunit of Class 1A PI3K p110 α and p110 β and Class 1B PI3K p110 γ [23] decreased with exposure to [Flu+Clo+Bu+Rom], and the phosphorylation status of their regulatory subunit PI3K p85 decreased significantly (Fig. 5A). Class III PI3K was also down-regulated, suggesting a global effect of [Flu+Clo+Bu+Rom] on various classes of PI3K.

An early mediator of the PI3K effect is PDK1 kinase, which is negatively regulated by [Flu+Clo+Bu+Rom] (Fig. 5A). PDK1 signals may lead to the AKT-mTOR or NFκB pathways. AKT and mTOR were both down-regulated after [Flu+Clo+Bu+Rom] exposure (Fig. 5A). These effects may inhibit protein translation as suggested by the down-modulation of p70 S6 kinase, phosphorylated S6 ribosomal protein and phosphorylated translation elongation factors eEF2κ and eIF4B (Fig. 5A). The level of TRAF3, a surface receptor-associated adaptor protein which is important for continued PI3K/AKT signaling [24], was decreased in cells exposed to [Flu+Clo+Bu+Rom], again suggesting that the PI3K/AKT pathway was compromised; the negligible effect of the 4-drug combination on the level of TRAF2 suggests specificity (Fig. 5A).

As mentioned above, the inhibition of PDK1 may lead to down-modulation of the NFκB pathway. Exposure of cells to [Flu+Clo+Bu+Rom] decreased the level of Iκκ α , Rel B, c-Rel, NFκB p100/52, and pan- and phosphorylated NFκB2 p65; the NFκB2 downstream target genes *BCL-xL* and *COX2* were down-regulated, as anticipated (Fig. 5B). These results show that the [Flu+Clo+Bu+Rom] synergism may involve the targeting of PI3K which leads to the inhibition of the pro-survival AKT-mTOR and NFκB signaling pathways.

3.6. [Flu+Clo+Bu+Rom] induces caspase-dependent cleavage of MEK and inhibits the Raf-MEK-ERK pathway

Another pathway that interacts with PI3K is the Raf-MEK-ERK signaling axis, its up-regulation leads to unrestricted cell proliferation [25,26]. Exposure of cells to [Flu+Clo+Bu+Rom] decreased the levels of A-Raf, B-Raf and C-Raf and their phosphorylated forms (Fig. 5C). A key mediator of this survival pathway is MEK. Its phosphorylation status was decreased in cells exposed to [Flu+Clo+Bu+Rom], and its cleavage was also observed (Fig. 5C), consistent with caspase-mediated proteolysis of MEK [27]. Pre-exposure of cells to Z-VAD-FMK prior to [Flu+Clo+Bu+Rom] inhibited MEK cleavage (not shown), suggesting a caspase-dependent proteolysis of MEK in our cell model. The down-regulation of MEK correlated with a decreased phosphorylation of ERK1/2, a downstream target of MEK (Fig. 5C). These results demonstrate that the cytotoxicity of [Flu+Clo+Bu+Rom] is partly due to modulation of the Raf- MEK-ERK signaling pathway.

3.7. [Flu+Clo+Bu+Rom] inhibits the JAK-STAT pathway

Various upstream pathways regulate PI3K-AKT signaling, one of which is the JAK-STAT pathway. We therefore determined the effects of [Flu+Clo+Bu+Rom] on JAK-STAT components. The level of JAK1, JAK2, STAT3 and STAT5 proteins and the phosphorylation of STAT1 at threonine 701, STAT3 at serine 727 and STAT5 at threonine 694 all decreased following exposure to the 4-drug combination (Fig. 5D). Their target genes *c-MYC*, *MCL-1* (Fig. 3) and *PIM 2* (Fig. 5D) were down-regulated, suggesting that inhibition of the pro-survival JAK-STAT pathway may partly explain the synergism of [Flu+Clo+Bu+Rom].

3.8. [Flu+Clo+Bu+Rom] down-regulates the expression of the drug transporter MRP1

Busulfan conjugation with GSH is known to decrease its efficacy [28]. Further, the cellular efflux of GSH-conjugated DNA alkylating agents is catalyzed by the MRP1/ABCC1 protein [29]. We then examined the effects of Flu, Clo, Bu and Rom, individually or in combination,

on the expression of key drug transporters. Western blotting showed that higher concentrations of Flu (0.7 μ M and 0.9 μ M vs 0.3 μ M used in the combination experiments) and Rom (9 nM and 10 nM vs 5.2 nM) decreased the expression of MRP1; in contrast, Clo and Bu both increased the level of MRP1 protein (Fig. 6A). When lower concentrations of the four drugs were used in combination, a dramatic decrease in MRP1 expression was seen (Fig. 6B). The expression of another drug transporter, MDR1/ABCB1, was increased in cells exposed to a high concentration of Rom (Fig. 6A) or to low concentrations of [Flu+Clo+Bu+Rom] (Fig. 6B), consistent with the previously reported effects of Rom on MDR1 expression [30]. While 0.7–0.9 μ M Flu by itself decreased the level of MRP1, it had negligible effect on MDR1 expression. The expression of BCRP/ABCG2, another drug transporter, was also decreased in cells exposed to 0.7–0.9 μ M Flu or 9–10 nM Rom (Fig. 6A) or to low concentrations of [Flu+Clo+Bu+Rom] (Fig. 6B), but less markedly. These results suggest that the synergism of [Flu+Clo+Bu+Rom] may be partly attributed to a decreased expression of MRP1, which may result in increased cellular concentrations of Bu.

3.9. [Flu+Clo+Bu+Rom] has similar synergistic effects in patient-derived malignant T-cells

To assess the potential clinical extension of our results, cells from patients with refractory T-cell malignancies were exposed to Flu, Clo, Bu and Rom, individually or in combination. Western blotting showed increased phosphorylation of histone 2AX and p53 proteins in three patient samples exposed to [Flu+Clo+Bu+Rom], suggesting robust activation of the DDR pathway. Acetylation of histone 3 and cleavage of PARP1 and caspases 3 and 8 increased, suggesting induction of chromatin remodeling and activation of apoptosis (Fig. 7). These results suggest a synergism of [Flu+Clo+Bu+Rom] in cells derived from patients with aggressive T-cell malignancies involving mechanisms similar to those seen in cultured cell lines.

4. Discussion

We recently reported possible mechanisms of Rom cytotoxicity in malignant T-cells [14]. In the present study, we examined the efficacy of Rom in combination with Flu, Clo and Bu and determined the possibility of using this combination as part of pre-transplant regimens for patients with T-cell malignancies. A strong synergism among these drugs was observed which may be attributed to drug-induced histone modifications and DNA damage, decreased levels of GSH and increased ROS production, down regulation of the drug transporter MRP1, and inhibition of multiple survival signaling pathways (Fig. 8).

Romidepsin inhibits histone deacetylases, resulting in increased levels of acetylated histones [7, 8]. [Flu+Clo] also induced histone modifications in AML cells [31]. It is conceivable that low concentrations of Flu, Clo and Rom may provide the initial histone acetylation needed for chromatin remodeling (Fig. 8A). Such chromatin relaxation may result in enhanced exposure of the DNA to Bu-mediated alkylation. The resulting DNA adducts may provide signals for additional histone acetylation to further open up chromatin and make the damaged DNA more accessible to DNA repair machinery. These effects are presumably enhanced when Flu and Clo are incorporated into growing nascent DNA strands during

replication via the same mechanism. This futile cycle of histone acetylation, DNA alkylation and nucleoside analog incorporation into DNA exacerbates DNA damage and results in apoptosis, as shown in Figs. 1–3.

The observed cell death in the presence of [Flu+Clo+Bu+Rom] may also have been caused in part by excessive ROS production as shown in Fig. 4, which may in turn be due to activated stress signaling pathways and/or decreased levels of GSH (Fig. 4F), an anti-oxidant that neutralizes ROS. In the event of decreased GSH concentration, the level of ROS should further increase, which may have contributed to or caused the observed damage to the mitochondrial membrane (Fig. 4B) and triggered a cascade of events leading to activation of caspases and eventual apoptosis.

A possible increase in free intracellular Bu concentration may also in part explain the observed synergism. Flu and Rom decreased the level of MRP1 (Fig. 6A), a drug transporter responsible for the efflux of GSH-conjugated Bu. A more dramatic effect was observed when these drugs were combined with Clo and Bu (Fig. 6B). With this down-regulation of MRP1, more free Bu would be available to alkylate DNA, resulting in an enhanced DDR (Fig. 8C).

[Flu+Clo+Bu+Rom] inhibits several pro-survival signaling pathways including the PI3K/AKT- mTOR (Fig. 5A), NF κ B (Fig. 5B), Raf-MEK-ERK (Fig. 5C), JAK-STAT (Fig. 5D), and Wnt/ β -catenin (Fig. S1A, Supplementary data) pathways. It should be noted that the 4-drug exposure was associated with a more dramatic decrease in the levels and/or phosphorylation of many survival-signaling proteins than for any other treatment groups, whereas this differential was less pronounced for some molecular events (e.g., compare the phosphorylation status of A-Raf versus STAT3 in Figs. 5C, 5D). These differential effects on individual survival-signaling components likely reflect some of these changes reflecting primary/causative events versus secondary/collateral responses; a detailed examination of these pathways in different cell types should provide insight into the identity of the major causative effects.

We recently reported the inhibitory effect of Rom on Wnt/ β -catenin signaling via up-regulation of its inhibitor SFRP1 through Rom-mediated hypomethylation of the *SFRP1* gene promoter [14]. In the present study, a combination of Rom with Flu, Clo and Bu showed even greater inhibition of the Wnt/ β -catenin pathway as suggested by the cleavage of β -catenin and down-regulation of its target gene *c-MYC* (Fig. S1A, Supplementary data). The inhibition of these pro-survival pathways (Fig. 8D), plus the activation of the SAPK/JNK stress signaling pathway (Fig. S1B, Supplementary data), may all contribute to the observed synergistic cytotoxicity of [Flu+Clo+Bu+Rom] in malignant T-cells.

In summary, our data provide evidence of interrelated mechanisms that commit malignant T cells to apoptosis following complex genotoxic insult. Since similar synergistic cytotoxicity of [Flu+Clo+Bu+Rom] was observed in established T-cell lines and patient samples, our results may be used to design a clinical trial to evaluate these drugs as part of pre-transplant conditioning therapy for patients with T-cell malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (CCSG Core CA16672), and the Stephen L. and Lavinia Boyd Fund for Leukemia Research, and by funds donated by grateful patients.

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Highlights

Romidepsin is synergistic with fludarabine, clofarabine and busulfan; exposure of cells to these drugs activated apoptosis.

Apoptosis correlated with histone modifications and DNA-damage response.

ROS increased while GSH and mitochondrial membrane potential decreased.

Some pro-survival pathways were inhibited.

Drug transporter MRP1 was down-regulated.

(A) Cytotoxicity and apoptosis: PEER cells **(C) Cytotoxicity and apoptosis: SUPT1 cells** **(B) Synergism of Flu, Clo, Bu and Rom: PEER cells** **(D) Synergism of Flu, Clo, Bu and Rom: SUPT1 cells** **Fig. 1.**

Synergistic cytotoxicity of [Flu+Clo+Bu+Rom] in PEER and SUPT1 cells. Cells were exposed continuously to the indicated drugs (alone or in combination) for 48 hrs prior to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Annexin V (Ann V) assays (A and C) to determine effects of drug(s) on proliferation and apoptosis. Synergism of Flu, Clo, Bu and Rom in PEER (B) and SUPT1 (D) cells was determined using the CalcuSyn software. Combination index (CI) of less than 1 indicates synergism at the indicated fraction affected (Fa). Flu: fludarabine; Clo: clofarabine; Bu: busulfan; Rom: romidepsin.

Fig. 2.

Western blot analysis showing activation of the DNA-damage response (A) and changes in the status of histone and α-tubulin modifications and level of modifying enzymes (B). PEER cells were exposed to the indicated drug(s) for 24 hrs (A) or 48 hrs (B) prior to analysis. Abbreviations are the same as those in Fig. 1 other than Ac (acetyl), Me (methyl) and H3 (histone H3).

Fig. 3.

Changes in the status of proteins involved in apoptosis and cell survival (A) and determination of the relevance of caspases (B and C). (A) PEER cells were exposed to the indicated drug(s) for 48 hrs prior to Western blot analysis. (B and C) SUPT1 cells were exposed to solvent (Control) or 4-drug combination in the absence or presence of the pan-caspase inhibitor Z-VAD-FMK for 48 hrs prior to Annexin V assay (B) and Western blot (C). Abbreviations are the same as those in Figure 1 other than Casp (caspase).

Fig. 4.

Analysis of reactive oxygen species (ROS) production as a cause of cell death. PEER cells were exposed to the indicated drug(s) for 48 hrs and analyzed for production of ROS using CM-H₂DCFDA (A), changes in mitochondrial membrane potential using JC-1 (B), enzymatic activity of caspase 3 (C), apoptosis (D) and apoptosis-associated protein cleavage by Western blot (E). To inhibit the production of ROS, cells were pre-exposed to 4 mM N-acetylcysteine (NAC) for 30 min prior to exposure to [Flu+Clo+Bu] or [Flu+Clo+Bu+Rom]. (F) The level of glutathione (GSH) was also analyzed relative to the control using Ellman's reagent. Bars above zero suggest increased GSH concentration and bars below zero show decreased GSH concentration. Abbreviations are the same as those in Figs. 1 and 3.

Fig. 5. Inhibitory effects of the [Flu+Clo+Bu+Rom] drug combination on the PI3K-AKT-mTOR (A), NF κ B (B), Raf-MEK-ERK (C) and JAK-STAT pro-survival pathways. Cells were exposed to drug(s) for 48 hrs and total protein extracts were analyzed by Western blotting.

Fig. 6. Effects of [Flu±Clo±Bu±Rom] combination on the expression of drug transporters. Cells were exposed continuously to relatively high concentrations of individual drugs (A) or low drug concentrations individually and in combination (B) for 48 hrs and analyzed by Western blotting.

Fig. 7.

Effects of [Flu±Clo±Bu±Rom] combinations on patient-derived T-cells. Mononuclear cells were isolated from peripheral blood of patients with T-cell malignancies and exposed to the indicated drugs for 48 hrs prior to Western blot analysis. Patient J-01 had relapsed Sezary syndrome, an aggressive variant of CTCL. He received multiple lines of chemotherapy, radiation, and allogeneic SCT. His circulating Sezary cells were isolated during an aggressive stage and ultimately fatal relapse after his transplantation. Patient L-02 blood sample was obtained upon diagnosis with an aggressive leukemic variant of PTCL and prior to treatment. Patient B-03 had T-PLL with a past medical history of Li-Fraumeni syndrome, rhabdomyosarcoma, and vestibular schwannoma. His blood sample was obtained at relapse after multiple lines of intensive chemotherapy.

A) Drug-induced histone acetylation and DNA damage **(C) Drug-induced down regulation of drug transporter MRP1** **B) Decreased [GSH] and increased [ROS]** **(D) Inhibition of survival pathways** **Fig. 8.**

Suggested mechanisms of the synergistic cytotoxicity of [Flu+Clo+Bu+Rom] in malignant T-cells. Each mechanism is further described under “Discussion”. Abbreviations are the same as those in other Figures other than NAs (nucleoside analogs), GS-SG (oxidized glutathione) and GST (glutathione-S-transferase).