

Improved Selectivity against Acute Myeloid Leukemia (AML) Blasts Over Normal Hematopoietic Progenitors for Cytarabine:Daunorubicin Delivered as CPX-351 Liposome Injection

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Introduction

Combinations of cytarabine (Ara-C) and daunorubicin (DNR) have been the mainstay of induction chemotherapy for AML for three decades. Although many patients achieve a complete remission (CR), at least 20% have leukemia that is refractory (NR) to this treatment and the majority of the remainder eventually relapse with chemotherapy resistant disease. One of the main mechanisms of multidrug resistance in AML is over-expression of ATP-binding cassette (ABC) transporters such as MDR-1 and BCRP-1. CPX-351 is a liposomal formulation of Ara-C and DNR in which the ratio of the two drugs encapsulated (5:1, mol:mol) has been designed to maximize synergistic interactions. In addition, preclinical studies demonstrated a prolonged half-life of 24 to 36h and markedly improved in vivo efficacy against animal leukemia models for CPX-351 (Tardi et al., Leukemia Res. 2009, 33 129–139). More recently, encouraging Phase 1 trial results in patients with advanced acute leukemia have resulted in continued clinical development of this drug in a Phase 2 setting (Feldman et al., 2008 American Society of Hematology (ASH) Annual Meeting, Abstract #2984). The purpose of this study was to compare the relative sensitivity of colony forming cells (CFC) from AML patient blast samples and normal peripheral blood (NPB) and normal bone marrow (NBM) to CPX-351 and the same concentrations of Ara-C and DNR as a free drug (FD) treatment. Investigations of the relative sensitivity of AML blasts from CR and NR patients to CPX-351 as well as the intracellular accumulation and sub-cellular localization of the liposomal drugs were undertaken in an attempt to elucidate the basis for the putative improved efficacy observed with CPX-351.

Key Observations

Comparison of the IC50 values obtained against AML-CFC for CPX-351 and FD demonstrated no significant difference in progenitor kill despite the fact that drug release from CPX-351 liposomes in media was negligible under exposure conditions, suggesting direct uptake of CPX-351 liposomes by the leukemia cells. However, CPX-351 was approximately 3-fold less cytotoxic against normal PB and BM CFC than FD. CPX-351 was also 3-9-fold more potent against AML-CFC than against normal CFC while the same comparison for FD showed less than a 3-fold difference. HPLC demonstrated accumulation of CPX-351 in AML blasts and confocal microscopy showed the presence of liposomes in the cytoplasm and daunorubicin within the cytoplasm and nucleus of these cells. AML blasts from NR patients showed expression of MDR-1 and/or BCRP-1 which was 3.5 to >200-fold higher than that shown in blasts from CR patients. There was no consistent difference in qualitative induction of AML blast cell apoptosis between CPX-351 and FD with either the CR or the NR samples. However, the addition of Cyclosporin A (CA), which had no effect on the CR sample treated with either CPX-351 or FD, rendered the NR samples more susceptible to both CPX-351 and FD. Interestingly, the CA-induced increase in cell kill was greater with CPX-351 than with FD.

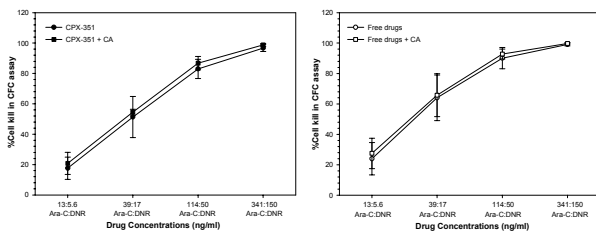
Methods

A panel of AML patient blast samples (including CR and NR to induction chemotherapy) as well as normal PB and BM samples were assessed for drug sensitivity by incubating cells for 24h in various concentrations of CPX-351 or the same concentrations of FD prior to plating in CFC assays with or without ABC transporter inhibition by CA. Quantitative RT-PCR was used to measure expression of the ABC transporter proteins MDR-1 and BCRP-1. HPLC and confocal microscopy were used to study the cellular drug uptake and subcellular distribution of CPX-351, respectively.

Results

1 CPX-351 exhibits the equivalent in vitro cell kill as the free drug cocktail to AML blast progenitors from CR patient samples.

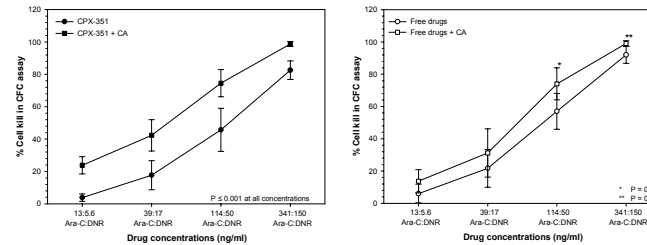
- Equal potency of CPX-351 with the free drugs was unexpected since drug release from liposomes under these experimental conditions is minimal.
- Cytotoxicity of free drugs or CPX-351 was unaffected by Cyclosporin A.



Methods: The AML-CFC progenitor assay was used for assessing cell kill. The assay was performed by incubating cells with the indicated concentrations of drugs for 24-hours. Cells were then plated in a semi-solid methylcellulose-based medium and the numbers of colonies were counted 14 days later. Plotted values represent the means and standard deviations from individual experiments with 7 different CR AML patient blast samples.

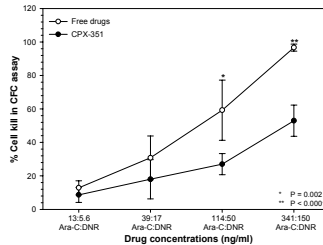
2 CPX-351 exhibits the equivalent cell kill as free drug cocktail to AML blast progenitors from non-responding patients (NR).

- Addition of Cyclosporin A to block PGP transporter function in NR cells increased the potency of CPX-351 efficacy to a greater degree than for free drug cocktail.



Methods: The AML-CFC progenitor assay was used for assessing cell kill. The assay was performed by incubating cells with the indicated concentrations of drugs for 24-hours. Where indicated cyclosporin A was also added at 3 μM for 24-hours. Cells were then plated in a semi-solid methylcellulose-based medium and the numbers of colonies were counted 14 days later. Plotted values represent the means and standard deviations from individual experiments with 6 different NR AML patient blast samples.

3 CPX-351 exhibits reduced cytotoxicity against normal peripheral blood (NPB) and normal bone marrow (NBM) progenitors as well as increased leukemia cell selectivity compared to free drug cocktail.

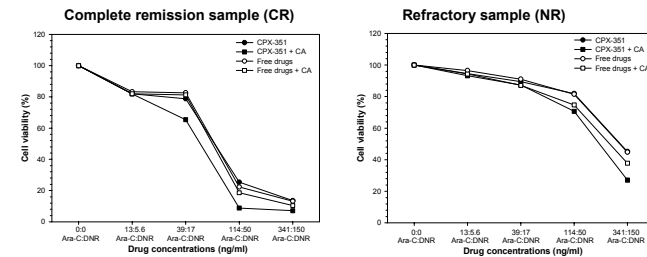


Cell Kill Ratio*: AML Blast/Normal Progenitor	
CPX-351	3.4
Free drug cocktail	1.4

*Determined for the 114.50 ng/ml Ara-C:DNR concentration

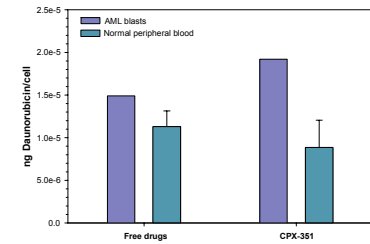
Methods: The AML-CFC progenitor assay was used for assessing cell kill. The assay was performed by incubating cells with the indicated concentrations of drugs for 24-hours. Cells were then plated in a semi-solid methylcellulose-based medium and the numbers of colonies were counted 14 days later. Plotted values represent the means and standard deviations from individual experiments with 7 different normal progenitor cell samples.

4 No difference in qualitative induction of apoptosis is observed in AML blasts between CPX-351 and free drug cocktail in representative CR or NR populations.



Methods: Cells were incubated for 24 hours in the presence of drug and then quantified for Annexin V and propidium iodide staining by flow cytometry. The percent cell kill was assessed in a representative complete remission cell line (85-19) and a refractory cell line (92-01). QRT-PCR from blast relative to GAPDH set at 10,000 assessments for MDR1 were 254 and 56,984, respectively, and were 76 and 694, respectively, for BCRP1.

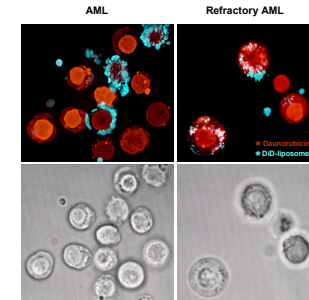
5 HPLC analysis demonstrates greater AML blast-selective drug accumulation for CPX-351 compared to free drugs at equivalent concentrations.



Methods: After completion of a 24-hour drug incubation with 341 ng Ara-C and 150 ng DNR as either free drugs or CPX-351 the cells were isolated for the AML-CFC assay. Remaining cells, ~1x10⁶, were evaluated for the presence of daunorubicin using HPLC. Daunorubicin was quantified using a reverse phase Phenomenex Luna C18(2) column, with 6.7:32.5 (v/v) 25 mM ammonium acetate (pH 4.8):acetonitrile as mobile phase at 1 mL/min and fluorescence detection (Ex = 480 nm, Em 560 nm).

6 Confocal microscopy indicates direct uptake of CPX-351 liposomes in both AML and refractory AML cells.

- Liposomes (blue fluorescence) were observed in the cytoplasm.
- Daunorubicin (red fluorescence) was observed in both the cytoplasm and the nucleus.
- Note: encapsulated daunorubicin is quenched and does not generate a visible fluorescent signal.



Methods: AML or refractory AML cells were incubated with CPX-351 containing 0.5 mM daunorubicin. CPX-351 was prepared with the carbocyanine membrane probe, DID (DiC₁₂) as a marker for liposomes. After 2 hours of drug incubation samples were evaluated for the presence of fluorescent daunorubicin and the fluorescent DID-lipid marker using confocal microscopy and the images were merged (top). Differential interference contrast (DIC) images were also obtained (bottom). Representative images shown.

Conclusions

- CPX-351 exhibited increased cytotoxicity (via CFC assay) against AML blasts than against NPB and NBM progenitors.
- A 2.4-fold increase in leukemia-selective cytotoxicity was observed for CPX-351 compared to free drugs.
- The increased AML blast selectivity observed for CPX-351 was associated with increased drug uptake in blast cells over normal progenitor cells.
- CPX-351 liposomes are taken up by primary human AML blast cells and subsequently release their contents intracellularly.
- Increased AML selectivity by CPX-351 may account for its high level of therapeutic activity observed clinically.