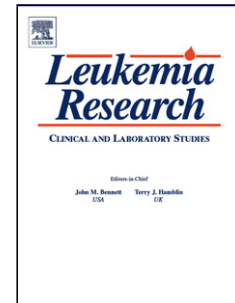


## Accepted Manuscript

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**CPX-351 exhibits hENT-independent uptake and can be potentiated by fludarabine in leukaemic cells lines and primary refractory AML.**

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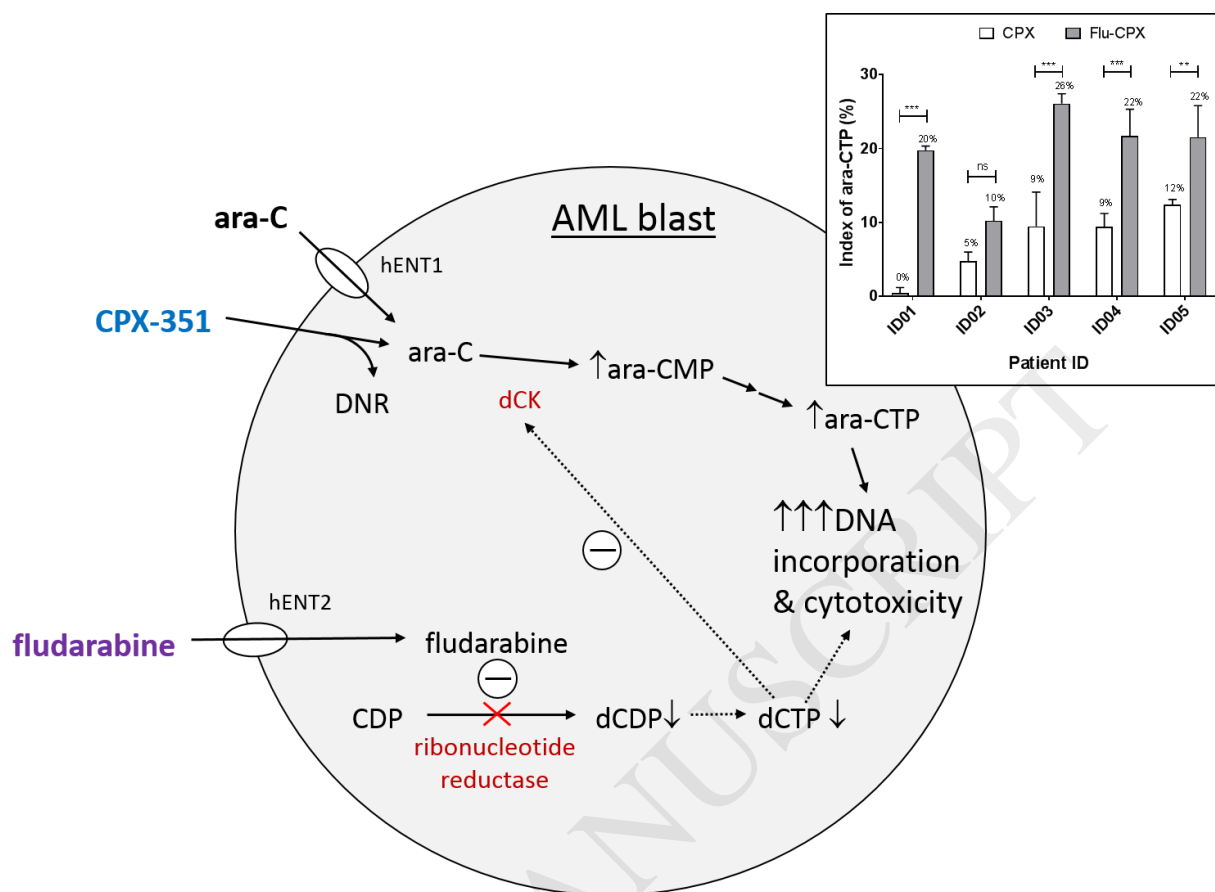
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Running title: Fludarabine potentiates ara-CTP generation from CPX-351

**Graphical Abstract**



### Highlights

- CPX-351 uptake is not dependant on human equilibrative nucleoside transporter 1 (hENT1) expression.
- Fludarabine can potentiate *in vitro* ara-CTP generation and cytotoxicity from CPX-351 in leukaemic cell lines.
- Pre-treatment of *ex vivo* AML blasts with fludarabine potentiates ara-CTP production from CPX-351.

### Abstract

CPX-351, a liposomal formulation co-encapsulating cytarabine and daunorubicin (DNR) in a synergistic 5:1 molar ratio, has shown favourable response in newly diagnosed elderly high-risk AML. This study assessed intracellular ara-CTP levels following *in vitro* exposure of human immortalised leukaemic cell lines and primary AML blasts to CPX-351, and investigated fludarabine potentiation of intracellular ara-CTP formation from CPX-351. Comparison of intracellular handling of CPX-351 to cytarabine in HL-60 cells indicated slower conversion to ara-CTP for CPX-351, but equivalent cytotoxicity to cytarabine and combined DNR/cytarabine (DA) at 48 hours, mostly likely reflecting the need for intracellular liposome

processing to release encapsulated drugs. Further assessment demonstrated cytotoxicity of CPX-351 to be superior to DA at 48 and 72 hours in cytarabine-resistant THP-1 cells ( $p < 0.001$ ), and this effect could not be inhibited upon blockade of human equilibrative nucleoside transporter (hENT) function with dipyridamole. Assessment of Flu-CPX in primary blasts from presentation AML patients ( $n=5$ ) demonstrated a more rapid and pronounced potentiation of ara-CTP from CPX-351 than in immortalised cell lines, with 4/5 patients showing significant increases in ara-CTP, notably for those that went on to fail induction and relapse treatment *in vivo* ( $n=3$ ). This suggests a favourable impact on patient outcome from Flu-CPX.

Abbreviations: ara-C, cytosine arabinoside/cytarabine; DNR, daunorubicin, ara-CTP, cytosine arabinoside triphosphate; dCTP, deoxycytidine triphosphate; dCK, deoxycytidine kinase; hENT, human equilibrative nucleoside transporter; DPM, dipyridamole; CDA, cytidine deaminase; FLT3-ITD, FMS-like tyrosine kinase 3 internal tandem duplication; NPM1, nucleophosmin 1; G-CSF, granulocyte colony stimulating factor; FLAG-Ida, fludarabine, ara-C, G-CSF, idarubicin; FBS, foetal bovine serum; SI%, sensitivity index.

Keywords: CPX-351; fludarabine; ara-CTP; *E. coli* HA1; biosensor

## Introduction

Cytarabine (ara-C) is a well-established agent widely used for treating AML. Conventionally, it is combined with anthracyclines for treating newly diagnosed Acute Myeloid Leukaemia (AML). At higher doses it is combined with the purine analogue fludarabine plus idarubicin and G-CSF (FLAG-Ida) for the treatment of relapsed or refractory AML. Fludarabine is known to potentiate intracellular accumulation of the active cytotoxic metabolite ara-CTP from ara-C via inhibition of ribonucleotide reductase and the normal formation of endogenous dCTP (Gandhi *et al.*, 1993), and removal of the inhibition of deoxycytidine kinase (dCK) by dCTP (Liliemark and Plunkett, 1986; Datta *et al.*, 1989).

CPX-351 is a proprietary liposomal formulation of ara-C and daunorubicin (DNR) encapsulated at a synergistic 5:1 molar ratio found to markedly enhance efficacy in preclinical leukaemia models (Tardi *et al.*, 2009; Lim *et al.*, 2010). CPX-351 exhibits an improved therapeutic index compared to conventional DA therapy in newly diagnosed elderly AML patients (Lancet *et al.*, 2014) as well as in patients with relapsed or resistant to DA therapy (Feldman *et al.*, 2011, Cortes *et al.*, 2015). The rationale behind the improvements observed for CPX-351 lies in the altered drug distribution and metabolism (Feldman *et al.*, 2012) that leads to prolonged circulation time and elevated concentrations within the blood (Tardi *et al.*, 2009), as well as concentration within the bone marrow (Lim *et al.*, 2010) and a selectivity for leukaemic progenitor cells over normal haemopoietic cells (Kim *et al.*, 2011). However, the intracellular kinetics of CPX-351 and metabolism of free intracellular ara-C and DNR resulting from CPX-351 are yet to be determined. In a Phase III trial, CPX was compared to 7+3 ara-C/DNR in newly diagnosed elderly high risk (secondary) AML patients, and showed superior event free survival and overall survival (Brunetti *et al.*, 2017) supporting the use of CPX-351 in these patients which led to its recent approval by FDA. In addition, a recent study evaluating *ex vivo* blasts from sub-populations of AML patients has revealed the potential advantage of treating FLT3-ITD positive AML cells with CPX-351 (Gordon *et al.*, 2017). These and other positive results have led to planned evaluation of CPX-351 on the UK National Cancer Research Institute (NCRI) Phase 3 AML 18 trial for elderly patients with AML and myelodysplastic syndrome (MDS). CPX-351 is also being evaluated versus FLAG-Ida in the UK NCRI AML 19 trial for younger patients with high-risk AML (known adverse cytogenetics). To explore the possibility of exploiting the same benefits of combination fludarabine therapy used in the FLAG-Ida regimen, we evaluated Flu-CPX versus CPX-351 in a range of immortalised cell lines and primary blasts from patients with refractory disease. Intracellular ara-CTP was assessed using biosensor HA1, which has been shown as equivalent to assessment by high performance liquid chromatography (HPLC) ( $R^2$  0.972,  $p=0.0028$ ), with a lower limit of detection (Anderson *et al.*, 2013) and using one fifth the requirement of HPLC for patient blasts (Yamauchi *et al.*, 2009). The effect of human equilibrative nucleoside transporter (hENT) blockade on uptake and metabolism of CPX-351 was assessed in the cytarabine-resistant THP-1 cell line, as down-

regulation of hENT1 has previously been implicated in resistance to cytarabine treatment (Macanas-Pirard *et al.*, 2017).

## Methods

### *Preparation of biosensor*

An overnight culture of *E. coli* HA1 was cultured in RPMI-1640 medium (Invitrogen) supplemented with foetal bovine serum (FBS, Sigma-Aldrich, UK) (10%) and L-glutamine (2 mM, Invitrogen, UK) and the optical density (OD 600 nm) adjusted to OD 1.0 in fresh RPMI 1640 medium (colourless, unsupplemented) pre-warmed to 37°C. The OD 1.0 culture was incubated for 30 minutes at 37°C in an orbital shaker at 200 rpm prior to use.

### *Maintenance and propagation of cell lines*

Cell lines HL-60, KG-1a, K562 and THP-1 were grown in RPMI 1640 supplemented with FBS (10%) and L-glutamine (2 mM) in 75 cm<sup>2</sup> tissue culture flasks (Corning) at 37°C in an atmosphere enriched with 5% CO<sub>2</sub>. Cells were sub-cultured 1:5 into fresh supplemented RPMI 1640 medium twice weekly.

### *Optimisation of CPX-351 dosing interval in HL-60 cells*

Viable immortalised HL-60 cells were enumerated using Trypan Blue staining (Sigma-Aldrich) and an Improved Neubauer haemocytometer, harvested by centrifugation at 300 x *g* for 5 minutes, and re-suspended to a density of 2 x 10<sup>6</sup> cells/mL in RPMI 1640 minimal medium (colourless, unsupplemented). Aliquots (2mL) were dosed with DA (DNR 5 µM, ara-C 25 µM) or CPX-351 (25 µM), and incubated for 1, 2 or 4 hours at 37°C in an atmosphere enriched with 5% CO<sub>2</sub>. The concentration of CPX-351 was calculated to produce the molar equivalents of standard dose ara-C. The concentrations of DNR and ara-C were calculated from clinically relevant doses based on dosing a 70 kg individual (surface area 1.73 m<sup>2</sup>) with standard dose DA.

### *Preparation of cell lysate*

After treatment cells were centrifuged at 300 x *g* for 5 minutes, washed in 4 mL RPMI 1640 medium (unsupplemented, minus phenol red) and re-suspended in 2 mL RPMI 1640 medium (unsupplemented, minus phenol red) containing saponin (0.1%) and EDTA (1.75 mM) as per Alloush *et al.* (2010). Cells were vortexed, and cell debris removed from cell lysate by centrifugation at 2800 x*g* for 5 minutes. Cell lysates were produced and used immediately or stored at -20°C and thawed to room temperature before use.

### *Assay of cell lysate for intracellular ara-C/ara-CTP*

*E. coli* HA1 was prepared as described and diluted ten-fold into cell lysate. Triplicate 200 µL volumes were aseptically transferred to a 96 well black microtitre plate with IPTG (1 mM, Sigma-Aldrich, UK) and calf intestinal alkaline phosphatase (AP, Sigma-Aldrich, UK) (10 U per well) added as appropriate (to discriminate ara-CTP from ara-C), and incubated for 15 hours at 37°C in an Infinite® F200 Pro plate reader (Tecan, UK) with dynamic gain adjustment. Bioluminescence was monitored every 15 minutes.

*Incubation of immortalised leukaemic cells with chemotherapeutic drugs for assessment of potentiation of ara-CTP generation*

Viable immortalised leukaemic cells (HL-60, KG-1a, K562 and THP-1) were enumerated using Trypan Blue staining and an Improved Neubauer haemocytometer, harvested by centrifugation at 300 x g for 5 minutes, and re-suspended to a density of  $2 \times 10^6$  cells/mL in RPMI 1640 medium (un-supplemented, minus phenol red). Aliquots (2mL) were dosed with DMSO (0.1%, Fisher-Scientific, UK) as vehicle control or fludarabine (5  $\mu$ M). Cells were incubated for 4 hours at 37°C in an atmosphere enriched with 5% CO<sub>2</sub>. Subsequently ara-C (10 or 25  $\mu$ M), DNR (5  $\mu$ M) or CPX-351 (10 or 25  $\mu$ M) were added and cells were incubated for a further 4 hours at 37°C in an atmosphere enriched with 5% CO<sub>2</sub>. Lysate was prepared and analysed for ara-CTP generation as described.

*Isolation, cryopreservation and thawing of primary AML cells*

Mononuclear cells were isolated from peripheral blood, collected with informed consent (07/H1109/181) from patients at Bristol Haematology and Oncology Centre (BHOC) by a density gradient technique following manufacturer's instructions (Histopaque 1077, Sigma, UK). Peripheral blood mononuclear cells (PBMC) were cryopreserved at  $\leq 3 \times 10^7$  cells/mL in RPMI 1640 medium (with phenol red) containing FBS (25%), DMSO (5%) and L-glutamine (2 mM).

Cryopreserved PBMC (1 mL) were thawed in RPMI 1640 medium (with phenol red) containing FBS (50%) and L-glutamine (2 mM) (4 mL), pelleted at 300 x g for 5 minutes, and washed in RPMI 1640 medium (with phenol red) containing FBS (20%) and L-glutamine (2 mM) to remove traces of DMSO. Viability post-resuscitation was assessed by trypan blue enumeration with >80% required for further analysis. The cells were finally re-suspended in 10 mL RPMI 1640 medium (with phenol red) with FBS (20%) and L-glutamine (2 mM) and incubated overnight at 37°C in 5% CO<sub>2</sub> before treatment with chemotherapeutic drugs fludarabine (5  $\mu$ M) for 2 hours followed by CPX-351 (25  $\mu$ M) for 1 hour.

*Cytotoxicity assessment*

Viable immortalised leukaemic cells ( $5 \times 10^4$  per well, 100  $\mu$ L final volume) were cultured in the presence or absence of DNR (5  $\mu$ M), cytarabine (25  $\mu$ M) or CPX-351 (25  $\mu$ M) with or without fludarabine (5  $\mu$ M) for 24, 48 and 72 hours and ATP generation by viable cells assessed by CellTiterGlo™ (Promega, UK). ATP levels were measured using an Infinite® F200Pro (Tecan, UK). Light units were used in the calculation of the viable mass (ATP%), with results expressed as percentage of treated over control. Results represent three separate experiments each performed in triplicate and are expressed as mean  $\pm$  standard deviation (n = 3).

*Human equilibrative nucleoside transporter (hENT) inhibition*

Viable immortalised leukaemic cells (THP-1) were enumerated and cell number adjusted to  $5 \times 10^5$ /mL ( $5 \times 10^4$  per well). Cells were dosed with dipyridamole (DPM, Sigma-Aldrich, UK) (2  $\mu$ M) or vehicle control (0.1% DMSO) for 2 hours followed with ara-C (25  $\mu$ M) or CPX-351 (25  $\mu$ M) and luminescence from ATP assessed by CellTiterGlo™ assay (Promega) at 24, 28 and 72 hours (n=3). Results are expressed as %ATP treated/control.

*Statistical Analysis*

Statistical analysis was undertaken where stated using GraphPad Prism 7 software (GraphPad, CA, USA). Sensitivity Index (SI%) values from biosensor HA1 were calculated as described in Alloush *et al.* (2010).

## Results

*Intracellular ara-CTP from CPX-351 detected by biosensor HA1, is slower to generate but reaches comparable levels by 4 hours incubation with equivalent cytotoxic response as DA over 72 hours*

*In vivo* relevant and equivalent concentrations of ara-C and CPX-351 (25  $\mu$ M) were assessed over 1-4 hours incubation with the HL-60 cell line (Fig. 1A), which we have previously shown to be highly sensitive to cytarabine (Anderson *et al.*, 2013). Dosing with free (non-liposomal) ara-C produced an increase in bioluminescence (and hence ara-CTP) at each time-point tested consistent with previously published results (Anderson *et al.*, 2013), achieving 169%, 318% and 497% SI% values at 1, 2 and 4 hours incubation respectively. Incubation of HL-60 cells with CPX-351 required a longer time-period to result in similar levels of ara-CTP, but a dose-dependent increase was noted, with the highest increase (SI=274%) produced using 4-hour incubation of CPX-351 (25  $\mu$ M). Assessment of intracellular ara-C and ara-CTP following dosing with ara-C (Fig. 1C) showed significant generation of ara-CTP by 2 hours ( $p < 0.001$ ), however CPX-351 required 4 hours to achieve a similar level (Fig. 1D). Importantly, no significant difference in HL-60 cytotoxicity was noted between ara-C and CPX-351 at any time-point tested (0-72 hours) (Fig. 1B) except for 48 hours where CPX-351 induced a further 9% decrease in %ATP over ara-C ( $p < 0.01$ ). Additionally combined DA was compared with CPX-351 at each time-point and showed a significant decrease in cytotoxicity at 24 hours compared to both ara-C and CPX-351 ( $p < 0.001$ ), however at 48 and 72 hours no significant difference in cytotoxicity was observed between ara-C and DA, or CPX-351 and DA ( $p > 0.05$ ).

*CPX-351 shows superior or equivalent toxic effects in HL-60, K562 and THP-1 cell lines but not KG-1a*

To extend assessment of CPX-351 cytotoxicity, a panel of cell lines with varying sensitivity to cytarabine were tested with CPX-351 versus DA (Fig. 2). HL-60 cells showed the greatest sensitivity to DA after 72 hours incubation (viability versus control  $7 \pm 0.5\%$ ) with equivalent cytotoxicity observed for CPX-351 ( $11 \pm 0.5\%$ ) at the same time-point ( $p > 0.05$ ) (Fig. 2A). However at 48 hours CPX-351 showed superior cytotoxicity over DA ( $18 \pm 0.9\%$  versus  $26 \pm 0.8\%$ ,  $p < 0.05$ ). K562 (erythroleukaemia) (Lozzio and Lozzio, 1975) and THP-1 cells (M5 AML) (Tsuchiya *et al.*, 1980) represent two of the most cytarabine-resistant immortalised leukaemic lines available. For K562 cells, no significant decrease in viability was observable at any time-point examined for either DA or CPX-351 (Fig. 2C). The maximal cytotoxic effect was noted at 72 hours for DA and CPX-351 ( $90 \pm 2.4\%$  versus  $89 \pm 8.7\%$ ,  $p > 0.05$ ). For THP-1 cells, a significant increase in cytotoxic effect was noted for CPX-351 over DA at 48 hours ( $11 \pm 0.4\%$  versus  $38 \pm 1.8\%$ ,  $p < 0.001$ ) and 72 hours ( $2 \pm 0.2\%$  versus  $16 \pm 0.6\%$ ,  $p < 0.001$ ) (Fig. 2D). KG-1a cells showed less susceptibility to DA and CPX-351 compared to HL-60 cells, and were the only cell line to show increased cytotoxicity in favour of DA at both 48 hours ( $51 \pm 2\%$  versus  $87 \pm 2.1\%$ ,  $p < 0.001$ ) and 72 hours ( $23 \pm 1.3\%$  versus  $58 \pm 2.6\%$ ,  $p < 0.01$ ) (Fig. 2B).



*Intracellular concentration of ara-CTP from CPX-351 is not inhibited by hENT-1 blockade in immortalised THP-1 leukaemic cells*

Fig. 3 shows the cytotoxicity of free ara-C versus CPX-351 on THP-1 cells at 24, 48 and 72 hours in the presence and absence of DPM, an inhibitor of hENT1 and 2 (Wang *et al.*, 2013). Ara-C (25  $\mu$ M) incubation induced an 85% reduction in THP-1 cell viability at 72 hours that was nullified by 2 hour pre-incubation with DPM (2  $\mu$ M), confirming the requirement for hENT expression in the cytotoxicity of free ara-C. As expected given the rationale for liposomal packaging of DA in CPX-351, DPM had no significant effect on the cytotoxicity of CPX-351 (25  $\mu$ M) at the same time-points tested ( $p>0.05$ ), confirming that hENT transporters are not required for cytotoxicity of CPX-351.

*Potentiation of ara-CTP from CPX-351 by pre-treatment with fludarabine is equivalent to cytarabine in immortalised leukaemic cell lines*

Following 4-hour incubation with fludarabine (5  $\mu$ M) or DMSO control, cell suspensions (THP-1, HL-60 and K562) were exposed to DA or CPX-351 and incubated for 4 hours (Fig. 4), in line with optimised dosing results (Fig. 1A). For comparison the SI% values for CPX-351, Flu-CPX, DA and Flu-DA treated samples are included. Fig. 4 shows results from biosensor HA1 for THP-1 (Fig. 4A), HL-60 (Fig. 4B) and K562 (Fig. 4C) cell lines dosed with DA, Flu-DA, CPX-351 or Flu-CPX expressed as intracellular ara-CTP/ara-C ratios. DMSO was used as solvent for fludarabine and was controlled (0.1%) throughout alongside a fludarabine-only control (Fig. 4) and an untreated control (data not shown). Neither DMSO nor fludarabine control showed any increase in SI% over untreated control for any sample (ara-CTP/ara-C ratio of 1).

In all cell lines an increase in ara-CTP accumulation was observed upon fludarabine pre-incubation, however Flu-DA produced a greater accumulation than Flu-CPX. No significant increase in ara-CTP/ara-C ratio was observed in THP-1 cells following four-hour incubation with DA or CPX-351 versus control. However, potentiation of ara-CTP generation from CPX-351 was observed in THP-1 cells (Fig. 4A) with an increase in ara-CTP/ara-C ratio with fludarabine pre-incubation from 0.96 to 1.24, indicating an intracellular shift from ara-C to ara-CTP ( $p=0.014$ ). This translated into an increase in SI% from 0 to 42% with fludarabine pre-incubation. Similarly potentiation was observed for Flu-DA versus DA in this cell line, increasing the ara-CTP/ara-C ratio from 0.95 to 1.94 ( $p=0.0014$ ), and the SI% from 14 to 112%. This represented the largest increase observed in the immortalised cell lines tested, in line with our previous findings for this cell line using biosensor HA1, and corroborated by HPLC (Anderson *et al.*, 2013). In HL60 cells, a trend towards potentiation was observed (Fig. 4C), with Flu-CPX increasing the ara-CTP/ara-C ratio from 1.11 to 1.36 ( $p=0.361$ ) and the SI% from 25 to 50%. Flu-CPX induced a significant increase in ara-CTP/ara-C ratio over fludarabine only control ( $p=0.031$ ). Comparison of DA to Flu-DA in this cell line (Fig. 4D) showed a similar increase in ara-CTP/ara-C ratio (1.33 to 1.65,  $p=0.103$ ) as observed for Flu-CPX, but a significant increase in ara-CTP/ara-C ratio over fludarabine only control ( $p=0.0008$ ). The effect of fludarabine pre-incubation was less pronounced in K562 cells (Fig. 4E & F) showing a trend towards increased SI% from 15 to 34% for CPX-351 ( $p=0.283$ ), and 73 to 86% for Flu-DA ( $p=0.888$ ).

Fig. 5 shows viability of immortalised leukaemic cells lines following incubation with ara-C, FLA, CPX-351 or Flu-CPX over a 72-hour period. Fludarabine alone showed a slight but significant cytotoxic effect on HL-60 (viability 70%) and K562 (viability 89%) cell lines, but not THP-1. HL-60 cells were the most sensitive of the cell lines to both ara-C (25  $\mu$ M) as single agent, and combined FLA, with 7% and 6% viability over control at 72 hours, respectively. CPX-351 (25  $\mu$ M) reduced HL-60 cell viability to 11% at 72 hours that decreased further to 6% viable with fludarabine co-incubation, confirming the trend observed in Fig. 4C. THP-1 cell viability decreased to 15.8% after 72-hour incubation with ara-C, which did not decrease further with fludarabine incubation (14.8%) (Fig. 5B) despite a significant increase in ara-CTP generation ( $p < 0.01$ , Fig. 4B). CPX-351 was again more cytotoxic to THP-1 cells than ara-C alone ( $p = 0.0004$ ) (also observed in Fig. 2D), which was most likely due to daunorubicin within CPX-351 liposomes, however Flu-CPX showed no further decrease in viability after 72 hours incubation (2.1% and 2.4% respectively). K562 cells showed no significant decrease in viability when treated with ara-C or CPX-351 for 72 hours (90% and 89% respectively). The combination of fludarabine with ara-C and CPX-351 reduced the viability to 84% ( $p = 0.019$ ) and 85% ( $p = 0.042$ ) respectively, however no significant difference was noted for either combination versus the effect of fludarabine alone, with fludarabine producing significant toxicity in this cell line ( $p < 0.01$ ).

*Flu-CPX potentiates generation of ara-CTP from CPX-351 in primary refractory AML blasts*

The patient cohort analysed were median age 68 years (range 66 to 72 years) and were analysed as presentation peripheral blood samples collected and cryopreserved within 24 hours of phlebotomy (Table S1). All samples were revived and analysed within 1 year of receipt due to our previous observation of loss of functionality with longer-term cryopreservation (data not shown). Optimisation of the dosing schedule in patient blasts for CPX-351 was initially performed using material from patient ID04 since material was more plentiful for this patient. No detrimental effect on potentiation by fludarabine was observed by reducing the pre-incubation from 4 hours to 2 hours, nor the subsequent CPX-351 incubation to 1 hour (data not shown). Patient ID01 was diagnosed with FLT3- AML with monosomy 7 and due to poor fitness scores was treated with 1 cycle of low-dose ara-C but subsequently showed rising white cell count and died. Fig. 6 indicates that patient ID01 would have produced little ara-CTP from CPX-351 (SI=0.4%), however fludarabine pre-treatment induced a 19% improvement in SI% for this patient ( $p < 0.001$ ). Patient ID02 had AML with normal karyotype that was treated with DA 3+10 but who died 8 days after completing course 1. A similar trend in response was noted as for ID01 although this was not significant, with ID02 showing low conversion of ara-C to ara-CTP from CPX-351 (SI=4.6%) that was improved with fludarabine pre-treatment to 10.1% ( $p > 0.05$ ).

Patient ID03 was diagnosed with NPM1+/FLT3+ AML (normal cytogenetics) and received DA 3+10 (at 50% dose) and was refractory, followed by two cycles of FLAG-Ida showing residual disease, followed by palliation. This patient showed the ability to generate ara-CTP from CPX-351 (SI=9.5%) that was improved by fludarabine pre-treatment to 26.1% ( $p < 0.001$ ) (Fig. 6).

Patient ID04 had NPM1-/FLT3+ AML (normal cytogenetics) and received two cycles of DA (3+10 then 3+8) but was refractory, then received and was refractory to FLAG-Ida. This patient showed an improvement from 9.4% to 21.7% for Flu-CPX over CPX-351 alone. Patient ID05 was diagnosed with NPM1-/FLT3+ M1 AML and received DA 3+10 with Mylotarg followed by FLAG-Ida but was refractory to all. Fig. 6 indicates that this patient's blasts showed the highest response to CPX-351 of the cohort tested, and Flu-CPX improved ara-CTP generation from CPX-351 in this patient's sample, increasing the SI% from 12.4% to 21.6% ( $p < 0.001$ ).

### Discussion

Initial analysis of uptake and metabolism of CPX-351 in the cytarabine-sensitive cell line HL-60 identified an approximate 2-hour lag in ara-CTP generation for CPX-351 compared to DA molar equivalents (Fig. 1A). This delay in intracellular formation of ara-CTP was consistent with prior observations whereby CPX-351 liposomes are taken up intact by leukaemia cells in an energy-dependent process, requiring intracellular processing of the liposomes to release the encapsulated drugs, which subsequently can interact with their nuclear targets (Kim *et al.*, 2011; Lim *et al.*, 2010). Biosensor *E. coli* HA1 is able to monitor both ara-C and ara-CTP by measuring signals in the absence and presence of AP, respectively. Interestingly, a time-dependent increase in the ratio of ara-CTP/ara-C could be noted across the three time-points tested for CPX-351 (0-4 hours post-dosing), whereas when the free drug ara-C was incubated with HL-60 cells the predominant species at all time-points was ara-CTP (Fig. 1C & D). This indicates that the lag could be caused prior to dCK mono-phosphorylation of ara-C, and most likely is due to intracellular release of ara-C from the liposome during the first hour of incubation (Fig. 1A). Of key importance was the observation that cytotoxicity of CPX-351 reached equivalence to cytarabine in HL-60 cells by 24 hours, and DA by 48 hours incubation (Fig. 1B). Further investigation of the intracellular ara-C and DNR handling from CPX-351 over the initial 24 hours post-treatment is required to elucidate the intracellular kinetics.

To compare efficacy of DA versus CPX-351 further, a range of commercially available leukaemic cell lines were incubated with molar equivalents of CPX-351 and ara-C, and viability was assessed (Fig. 2). The cell lines selected represent a range of sensitivities to cytarabine, from highly sensitive (HL-60, KG-1a) to resistant (K562, THP-1) (Anderson *et al.*, 2013). HL-60 and THP-1 cells showed increased susceptibility to CPX-351-induced cytotoxicity compared to DA at 48 hours. This cytotoxic effect reached equivalence for HL-60 cells at 72 hours (Fig. 2A), however for the more resistant cell line THP-1, a sustained superior effect of CPX-351 was observable at 72 hours (Fig. 2D). K562 cells were found to be the most resistant cell line tested, with neither DA nor CPX-351 inducing a significant cytotoxic effect (Fig. 2C). K562 cells have been shown to respond to tetrahydrouridine-induced inhibition of cytidine deaminase (CDA), inducing a two-fold increase in ara-CTP accumulation (Riva *et al.*, 1992), which may provide a possible mechanism for their relative insensitivity to cytarabine and CPX-351. This cell line may be suitable for testing of high-dose equivalents for CPX-351 versus cytarabine, mirroring relapse regimen equivalents of ara-C (1.5-3g/m<sup>2</sup>). KG-1a was the only cell line to show superior cytotoxic effect of DA over CPX-351 (Fig. 2B). KG-1a cells are derived from a male patient with M0 AML

(Koeffler, 1983) and are known to express leukaemic stem cell-like (LSC) properties (CD34+CD38-) rendering them resistant to anthracycline-based chemotherapy (She *et al.*, 2012). This cell line may be a useful tool for further investigation of CPX-351 efficacy in LSC populations and warrants further investigation.

Nucleoside analogues such as fludarabine and cytarabine require active uptake via hENT that accounts for 80% of influx into the cell (Sundaram *et al.*, 2001). Loss of hENT1 is known to induce cytarabine resistance and shorter survival *in vivo* (Cai *et al.*, 2008; Galmarini *et al.*, 2002). The role of hENT-1 in the cytotoxicity of ara-C from CPX-351 versus free ara-C was assessed to illustrate the importance of liposomal uptake independent of hENT-1 transport. One proposed mechanism of cytarabine resistance thought to affect response to AML therapy is low-level expression of hENT1. A study evaluating cytarabine resistance mechanisms in 26 AML patients failed to identify a correlation between hENT1 and ara-CTP generation (Yamauchi *et al.*, 2009). However, no study has evaluated hENT1 expression versus outcome of therapy in AML, but a notable correlation has been observed between hENT1 expression and response to the related compound gemcitabine in patients with resected gastric cancer (Santini *et al.*, 2010). In this study, hENT-inhibition was assessed using DPM, an inhibitor of hENT1 and 2 (Wang *et al.*, 2013). DPM pre-incubation successfully inhibited cytarabine-induced cytotoxicity in THP-1 cells at 48 and 72 hours ( $p < 0.001$ ), however cytotoxicity of CPX-351 was unaffected by hENT blockade ( $p > 0.05$ ) (Fig. 3). It should be noted however that whilst cytarabine requires hENT1 for uptake into cells, fludarabine requires hENT2 and thus any reduction in hENT2 expression could affect the efficacy of Flu-CPX as is known for the use of fludarabine in chronic lymphocytic leukaemia (CLL) (Molina-Arcas *et al.*, 2005). DPM would be a suitable inhibitor to test this effect further in a range of cell lines and patient blasts as it is known to inhibit both hENT1 and hENT2, and could be compared with a hENT1-specific inhibitor such as nitrobenzylthioinosine (NBMPR) (Boleti *et al.*, 1997).

Following optimisation of *in vitro* exposure to CPX-351, potentiation of ara-CTP generation was assessed using biosensor HA1 (Alloush *et al.*, 2010) following 4-hour incubation with fludarabine (5  $\mu\text{M}$ ), a concentration previously shown to increase ara-CTP accumulation *in vivo* (Gandhi *et al.*, 1993) and in susceptible cell lines (Anderson *et al.*, 2013). Cytotoxicity of the combinations was assessed over a further 72 hours (Fig. 5). Bioluminescence from biosensor HA1 was used to assess both ara-CTP and ara-C (Alloush *et al.*, 2010). For ease of comparison the ara-CTP/ara-C ratio was calculated for each treatment, with a ratio  $> 1$  indicating the intracellular predominance of the active metabolite ara-CTP, and  $< 1$  indicating ara-C as the predominant form of the drug. Flu-CPX was assessed versus Flu-DA to control for the presence of DNR within CPX-351 liposomes, despite Flu-DA not being used clinically. Comparison of Flu-DA versus FLA (fludarabine 5  $\mu\text{M}$  and ara-C 25  $\mu\text{M}$ ) showed no significant difference in SI% from biosensor HA1, indicating that the presence of DNR did not impact on ara-CTP generation (data not shown). Clinically, high-dose cytarabine is used at relapse. However, due to previously observed saturation of ara-CTP accumulation in cell lines (Anderson *et al.*, 2013), and following *in vivo* high-dose cytarabine 1-2 hours

post-administration (Plunkett *et al.*, 1987), standard-dose DA equivalents were tested herein.

Potential of ara-CTP from CPX-351 by fludarabine pre-treatment was most pronounced in THP-1 cells (Fig. 4A), causing a significant shift in conversion to ara-CTP versus CPX-351 as single agent ( $p < 0.05$ ). Interestingly, potentiation of ara-CTP generation after treatment with Flu-DA was also notable in THP-1 cells ( $p < 0.01$ ) (Fig. 4B), however cytotoxicity was substantially higher for CPX-351 versus ara-C, and importantly Flu-CPX versus FLA at both 48 and 72 hours post-dosing (Fig. 5B). In contrast, THP-1 cells showed no significant increase in ara-CTP accumulation following four-hour incubation with CPX-351 or DA without fludarabine versus control (Fig. 4A & B), despite significant cytotoxicity by ara-C and CPX-351 from 24 and 48 hours respectively. Reduced sensitivity to ara-C through decreased ara-CTP generation have been observed previously in this cell line (Anderson *et al.*, 2013; Ge *et al.*, 2004) and is attributable to increased CDA function (Ge *et al.*, 2004). As the cytotoxicity of ara-C is not restricted to formation of ara-CTP, other mechanisms such as induction of apoptosis through membrane raft-induced ceramide formation (Grazide *et al.*, 2002) mitochondrial swelling (Xue *et al.*, 2002), and NF- $\kappa$ B down-regulation (Sreenivasan *et al.*, 2003) may be active in this cell line. This phenomenon warrants further investigation to determine whether this lack of concordance between ara-CTP accumulation at 4 hours and 72 hour cytotoxicity is due to the previously observed altered kinetics of CPX-351 versus standard DA (Lim *et al.*, 2010), or direct toxicity from parent ara-C formed from CPX-351.

No significant shift in ara-CTP/ara-C ratio was observable for either HL-60 or K562 cells treated with Flu-CPX versus CPX-351 alone (Fig. 4C & E respectively), however the same was also true for Flu-DA versus DA (Fig. 4D & F respectively). HL-60 are known to be highly sensitive to cytarabine-based chemotherapy, with  $IC_{50}$  values of 200-350 nM (Anderson *et al.*, 2013; Yamauchi *et al.*, 2014). Due to the high sensitivity of this cell line to ara-C (Anderson *et al.*, 2013, Yamauchi *et al.*, 2014), no significant reduction in viability was noted with fludarabine potentiation (Fig. 5A), mirroring the finding that no significant difference in ara-CTP generation was observed after fludarabine pre-treatment (Fig. 4D). K562 cells were resistant to both ara-C and CPX-351 during the initial 72 hours post-dosing, with fludarabine potentiation of either CPX-351 or ara-C showing no further cytotoxic effect over fludarabine alone ( $p > 0.05$ ) (Fig. 5C). With K562 anthracycline-resistant phenotypes having been developed and investigated (Damiano *et al.*, 2001; Peng *et al.*, 2011), this cell line may be useful in modelling intracellular handling of CPX-351 in resistant disease.

Following observation of increased ara-CTP accumulation and cytotoxicity of Flu-CPX in immortalised cell lines, the combination was tested in *ex vivo* blasts from a cohort of presentation AML patient samples ( $n=5$ ). All patients were subsequently refractory to frontline chemotherapy with DA or low-dose ara-C (ID01), and 3/5 had subsequently failed to respond to FLAG-Ida (ID03, ID04, ID05) (Fig. 6). Flu-CPX induced a significant increase in accumulation of ara-CTP over CPX-351 alone in 4/5 patient samples tested. The most extensive improvement in ara-CTP accumulation from Flu-CPX was observable for patient ID01, who suffered poor fitness scores and was treated with low-dose cytarabine. This predicted improvement indicates

that patient ID01 could potentially have benefited from the combination given the improved tolerability reported previously for CPX-351 (Lancet *et al.*, 2014). Three patients with mutations in FLT3-ITD were analysed as part of the cohort. CPX-351 has previously shown favourable response in AML blasts harbouring the FLT3-ITD mutation, due to increased uptake and cytotoxic effect (Gordon *et al.*, 2017). A significant potentiation of ara-CTP was observable in blasts from all 3/3 FLT3-ITD positive patients analysed ( $p < 0.01$ ) (ID03, ID04 and ID05), and this fludarabine-mediated potentiation of ara-CTP from CPX-351 may indicate a rationale for this combination in FLT3-ITD positive disease.

This study has demonstrated the ability of fludarabine to potentiate formation of ara-CTP upon exposure of immortalised (THP-1) and primary leukaemia cells to CPX-351, suggesting a favourable impact on efficacy for this combination. Interestingly, CPX-351 appeared to circumvent the impact of hENT1 inhibition on cytotoxicity in immortalised leukaemia cells, and showed a greater propensity for fludarabine to potentiate ara-CTP generation from CPX-351 in patient blasts, despite the cohort showing lower basal sensitivity to CPX-351 compared to the cell lines tested. Biosensor HA1 is able to detect intracellular ara-CTP from CPX-351 and should be evaluated further in a larger cohort of patient samples with known outcome, for use as a treatment decision-making tool. Flu-CPX is being examined in the current UK NCRI AML19 Trial. Further studies are required to elucidate intracellular drug handling of CPX-351, and evaluate cytotoxicity and intracellular ara-CTP and DNR accumulation from CPX-351 in models of resistant AML.

#### **Authorship contributions**

EA, GR, DR & VS designed the research. EA, BR, JH & HB performed the research. EA, PM & LM analysed the data. EA wrote the paper. LM contributed essential reagents (CPX-351).

#### **Conflicts of interest**

This work was partly funded by Celator Pharma with scholarly time for EA, GR, DR and VS funded by the University of the West of England. LM is an employee of Jazz Pharmaceuticals.

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## Figure Captions

**Fig. 1: Generation of ara-CTP from free drug ara-C and CPX-351 (A) and viability of CPX-351 versus free ara-C and DA on HL-60 cells after 0-72 hour incubation (B), and kinetics of ara-C conversion to ara-CTP for ara-C-treated (C) and CPX-351-treated (D) HL-60 cells.** (A) Sensitivity Index (SI%) from biosensor HA1 incubated with lysate from HL-60 cells either untreated, or treated with DA (DNR 5  $\mu$ M, ara-C 25  $\mu$ M) or CPX-352 (25  $\mu$ M) for 1 hour, 2 hours or 4 hours. (B) HL-60 cells seeded at  $5 \times 10^5$ /mL were dosed with DA (5  $\mu$ M and ara-C 25  $\mu$ M), CPX-351 (25  $\mu$ M) or DA (5  $\mu$ M DNR and 25  $\mu$ M ara-C) and luminescence from ATP assessed by CellTiterGlo™ assay (Promega) at 24, 28 and 72 hours. Raw relative light unit (RLU) values are indicated above controls. Intracellular ara-C & ara-CTP levels for HL-60 cells treated with (C) ara-C (25  $\mu$ M) or (D) CPX-351 (25  $\mu$ M) assessed as relative light units from biosensor HA1. Significance calculated for each species at each time-point. SI% values calculated as per Alloush *et al.* (2010). CellTiterGlo™ results are expressed as %ATP treated/control. RLU, relative light units; AP, alkaline phosphatase. Error bars represent standard deviation (n=3) with one-way ANOVA with Tukey's post-hoc test, ns, not significant, \*\*p<0.01, \*\*\*p<0.001.

## Figr-2

**Fig. 2: Cytotoxicity of CPX-351 versus DA on (A) HL-60, (B) KG-1a, (C) K562 and (D) THP-1 cells after 24, 48 and 72 hours incubation.** Leukaemic cells seeded at  $5 \times 10^5$ /mL were dosed with ara-C (25  $\mu$ M) or CPX-351 (25  $\mu$ M) and luminescence from ATP assessed by CellTiterGlo™ assay (Promega) at 24, 28 and 72 hours. Results are expressed as %ATP treated/control with raw relative light unit (RLU) values indicated above controls. Error bars represent standard deviation (n=3) with one-way ANOVA with Tukey's post-hoc test, ns, not significant, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

## Figr-3

**Fig. 3: Effect of hENT inhibition on cytotoxicity of CPX-351 versus free ara-C on THP-1 cells after 24, 48 and 72 hours incubation.** THP-1 cells seeded at  $5 \times 10^5$ /mL were dosed in the presence and absence of DPM pre-treatment for 2 hours (2  $\mu$ M) with ara-C (25  $\mu$ M) or CPX-351 (25  $\mu$ M) and luminescence from ATP assessed by CellTiterGlo™ assay (Promega) at 24, 28 and 72 hours. Results are expressed as %ATP treated/control. Error bars represent standard deviation (n=3) with one-way ANOVA with Tukey's post-hoc test, ns, not significant; \*\*p<0.01; \*\*\*p<0.001.

## Figr-4

**Fig. 4: Effect of fludarabine pre-treatment on potentiation of ara-CTP from CPX-351 in leukaemic cell lines (THP-1, HL-60 and K562).** Cells ( $2 \times 10^6$ /mL) were treated vehicle control (DMSO 0.1% v/v) or fludarabine (5  $\mu$ M) for 4

hours and then CPX-351 (25  $\mu$ M) or DA (DNR 5  $\mu$ M and ara-C 25  $\mu$ M) for 4 hours and lysates produced and analysed as per Anderson *et al.* (2013). Three experimental repeats are shown ( $n = 3$ )  $\pm$  SD. One-way ANOVA with Tukey's post-hoc test was performed versus appropriate control (i.e. Flu-CPX versus fludarabine), with bars indicating comparison  $\pm$  fludarabine pre-treatment (i.e. Flu-CPX versus CPX-351). ns, not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Flu, fludarabine; FLA, fludarabine plus ara-C.

Figr-5

**Fig. 5: Cytotoxicity of combined Flu-CPX versus FLA on (A) HL60, (B) THP-1 and (C) K562 cells after 24, 48 and 72 hours incubation.** Immortalised leukaemic cells seeded at  $5 \times 10^5$ /mL were dosed with vehicle control (0.1% v/v DMSO), fludarabine (5 $\mu$ M), ara-C (25  $\mu$ M), FLA (fludarabine at 5 $\mu$ M and ara-C at 25  $\mu$ M), CPX-351 (25  $\mu$ M) or Flu-CPX (fludarabine at 5 $\mu$ M and CPX-351 at 25  $\mu$ M) and luminescence from ATP assessed by CellTiterGlo™ assay (Promega) at 24, 28 and 72 hours. Results are expressed as  $\log_{10}$  %ATP treated/control, with raw relative light unit (RLU) values indicated above controls. Error bars represent standard deviation ( $n=3$ ) with one-way ANOVA with Sidak's post-hoc test, ns, not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Figr-6

**Fig. 6: Effect of fludarabine pre-treatment on potentiation of ara-CTP from CPX-351 in primary refractory AML blasts.** Peak luminescence from biosensor HA1 incubated with lysate prepared from cryopreserved (<1 year) peripheral blood mononuclear extract from presentation AML patients that failed to achieve remission on standard induction and relapse regimens. Cells were treated for 2 hours with vehicle control (0.1% DMSO v/v) or fludarabine (5  $\mu$ M) followed by 1 hour incubation with CPX-351 (25  $\mu$ M). Error bars represent standard deviation ( $n=3$ ). AP, alkaline phosphatase, SI%, sensitivity Index (SI%) values calculated as per Alloush *et al.* (2010). One-way ANOVA with Tukey's post-hoc test: ns, not significant; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Fig. 1

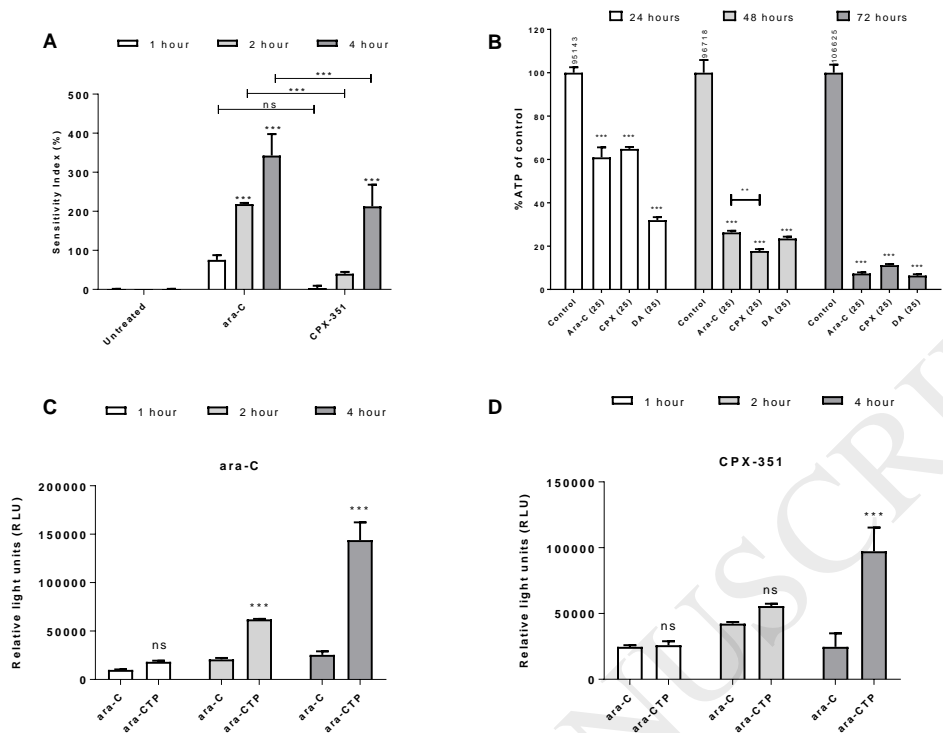


Fig. 2

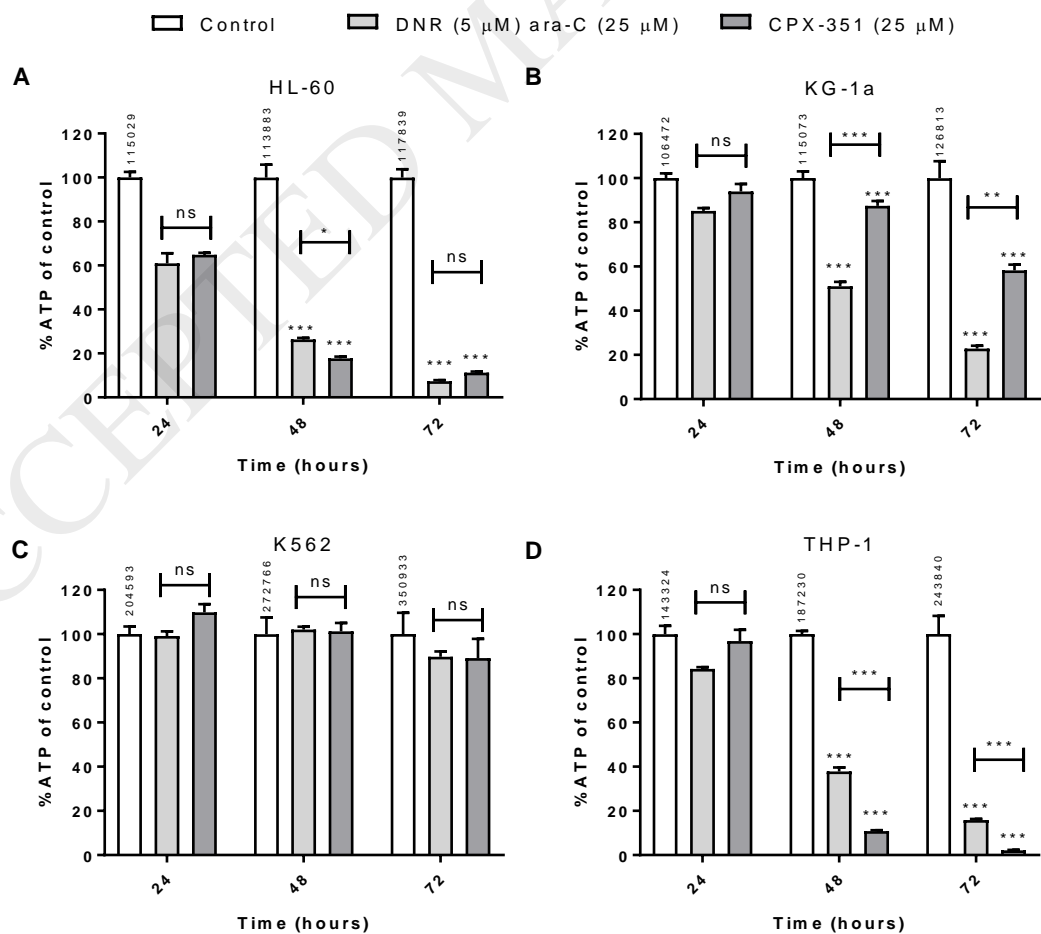


Fig. 3

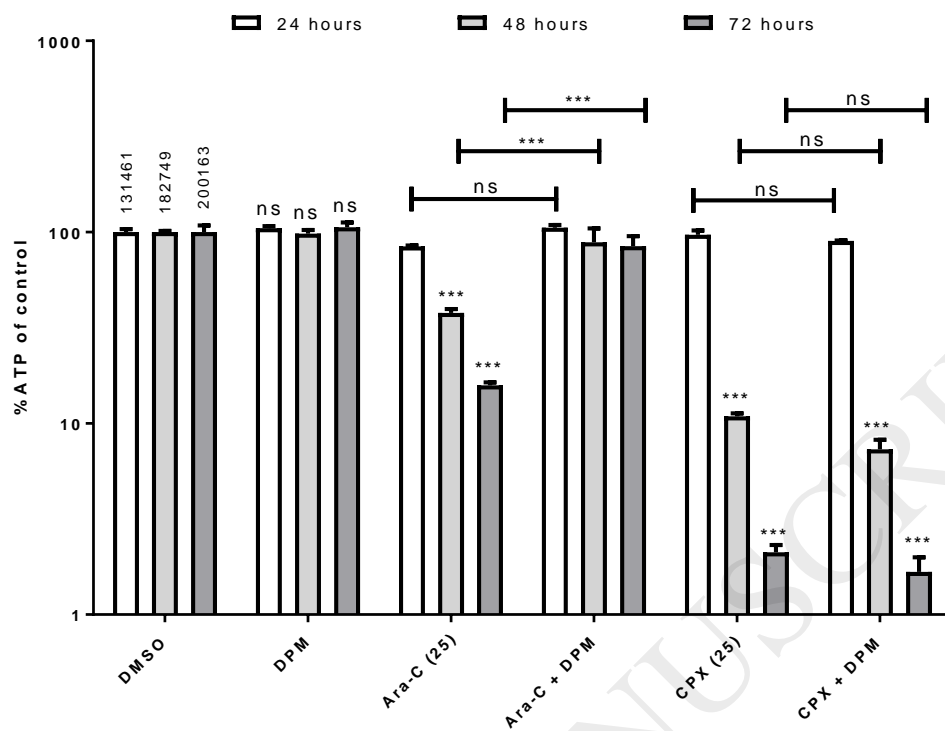


Fig. 4

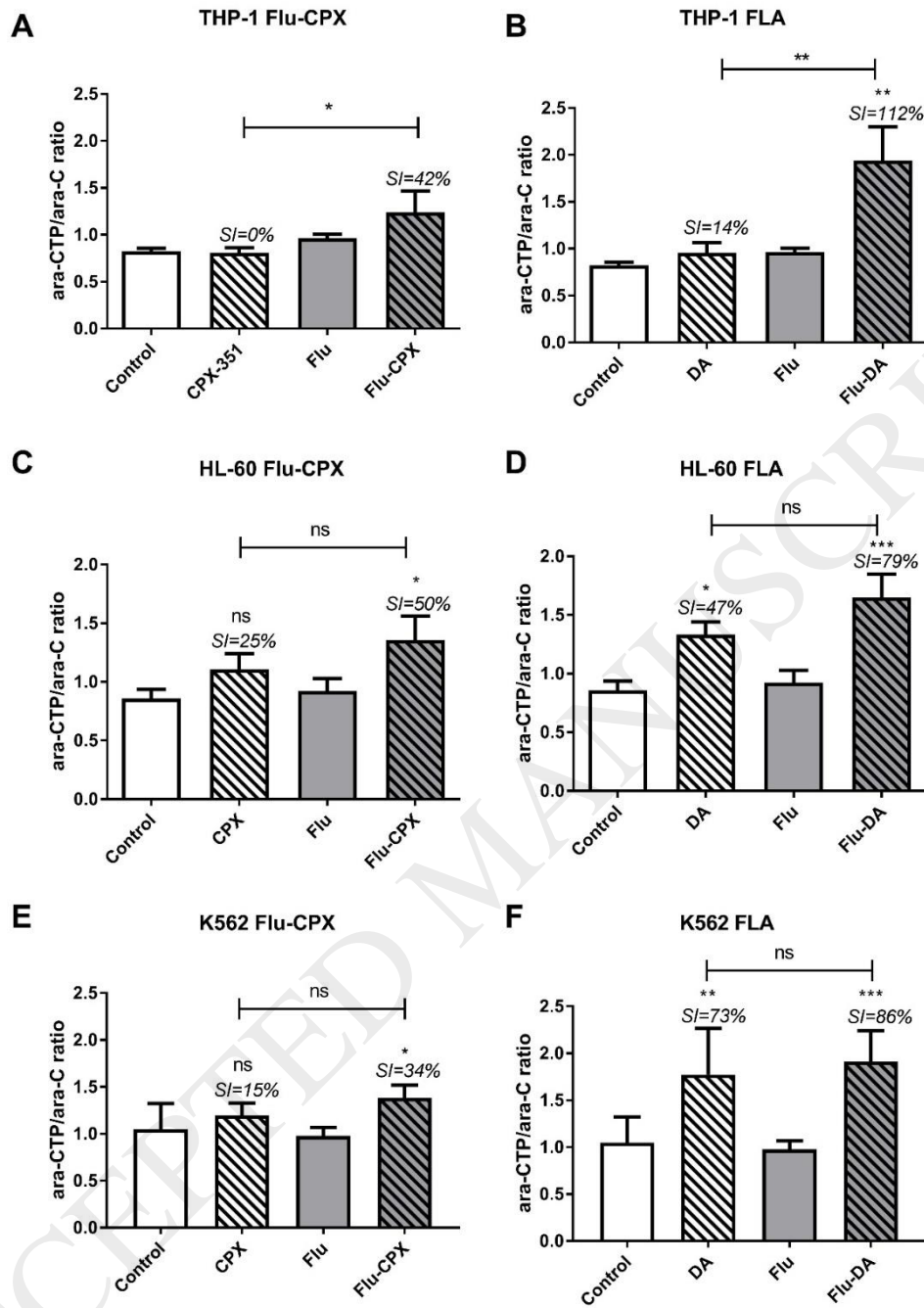


Fig. 5

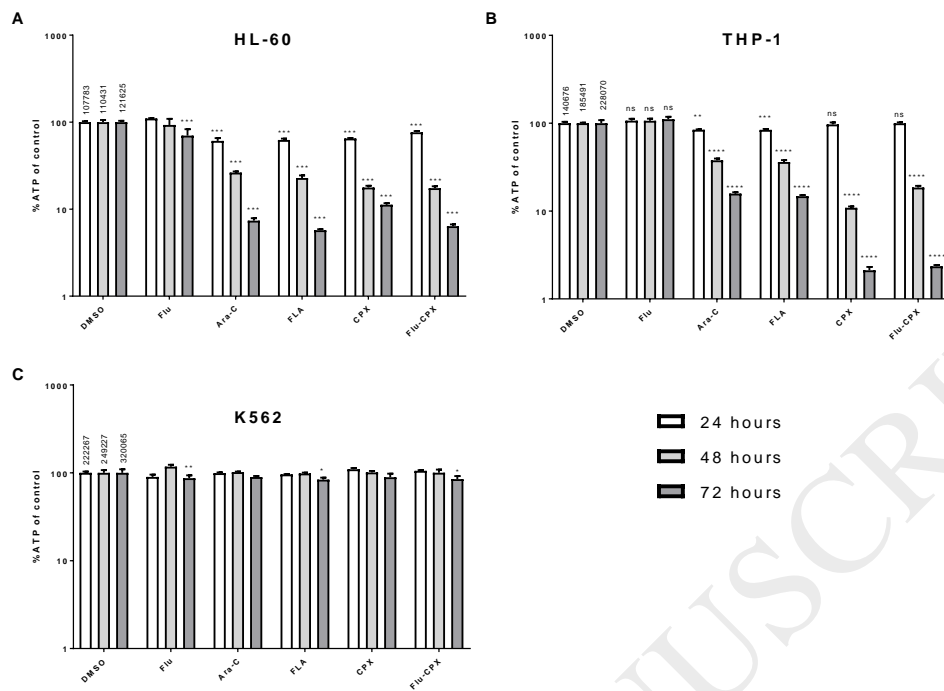


Fig. 6

