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AUTHOR'S VIEWS

Dynamic induction of drug resistance through a stress-responsive enhancer in acute myeloid leukemia

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ABSTRACT

The drug efflux pump ABCB1 (ATP binding cassette subfamily B member 1) confers chemotherapy resistance in acute myeloid leukemia (AML). We recently identified an *ABCB1* enhancer that is activated in response to a range of cellular stressors, including anthracycline chemotherapy. Stress drives increased *ABCB1* expression and allows leukemia cells to escape from targeted third-generation ABCB1 inhibition.

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Repeated studies have shown *ABCB1* (ATP binding cassette subfamily B member 1) expression to be a powerful independent predictor of treatment failure in acute myeloid leukemia (AML), but targeted ABCB1 inhibitors were not effective in clinical trials.[1] Activating *ABCB1* promoter translocations have recently been identified in recurrent ovarian and breast cancer, providing compelling additional evidence for a direct role of ABCB1 in clinical chemoresistance.[2] The transcriptional regulation of *ABCB1* appears to be cell-type specific, but the mechanisms that establish and maintain *ABCB1* expression in AML are unknown.[3] We sought to identify drivers of *ABCB1* expression in AML by characterizing the enhancers that regulate its transcription.

We first generated multiple drug-resistant leukemia cell lines (K562 cells) by propagating them in escalating concentrations of daunorubicin. Daunorubicin is a member of the anthracycline class of chemotherapy drugs and is standard-of-care in induction therapy of AML. We found that each line acquired drug resistance through the same mechanism: induction of *ABCB1*.[4] Using bioinformatics techniques we also observed that drug-resistant cells upregulated in parallel a transcriptional programme that was similar to one expressed in cells stressed by amino acid deprivation or hypoxia. The integrated stress response (ISR) represents a common adaptive pathway for such stressors and its output is coordinated by the transcription factor (TF) ATF4 (activating transcription factor 4).[5] We found that the TF genes most upregulated in resistant K562 included *ATF4* as well as several of its transcriptional targets (*ATF3*, activating transcription factor 3; *CEBPB*, CCAAT enhancer-binding protein beta; *DDIT3*, DNA damage-inducible transcript 3) and those coding for its binding partners (*JUN*, Jun proto-oncogene; *JUNB*, JunB proto-oncogene; *CEBPG*, CCAAT enhancer-binding protein gamma; *CEBPB, ATF3* and *DDIT3*). These expression data suggested a link between cellular stress, ATF4 and upregulation of *ABCB1*.

To identify candidate *ABCB1* enhancers, we performed chromatin immunoprecipitation (ChIP) with next generating sequencing for histone H3 lysine 27 acetylation (H3K27Ac), a histone modification that marks active enhancers.[6] We identified four putative enhancers within intronic regions of *ABCB1* that were acetylated in drug-resistant but not drug-sensitive leukemia cells. 4C sequencing was then used to confirm contact between these elements and the promoter. The functional role of each enhancer in the regulation of *ABCB1* expression was confirmed by silencing each region using a CRISPR-dCas9-KRAB (CRISPR, Clustered regularly interspaced short palindromic repeats; dCas9, dead CRISPR-associated protein 9; KRAB, Krüppel associated box) system, which induces heterochromatin formation at the target sequence. These data identified one enhancer (designated E3) that was strongly acetylated, in close contact with the promoter and whichcontributed significantly to *ABCB1* expression. E3 contained a DNAse1 hypersensitive site, and motif analysis of this accessible region revealed consensus binding sites for many of the TFs upregulated in resistant cells. ChIP-qPCR (quantitative polymerase chain reaction) confirmed significant increases in the binding of ATF4, ATF3, CEBPB, JUND (JunD proto-oncogene) and JUN to E3 in resistant versus sensitive leukemia cells. Together these data demonstrated that daunorubicin resistance was associated with the activation of an ATF4-bound enhancer in intron 4 of *ABCB1* (Figure 1).

The binding of stress-response TFs to an active enhancer implied that *ABCB1* expression would respond to other forms of cellular stress. Indeed, we found that *ABCB1* and other targets of ATF4 were readily induced by exposure to tosedostat (which causes intracellular amino acid deprivation), thapsigargin (an endoplasmic reticulum stressor) or simply by culturing cells at high density. *ABCB1* was more responsive to rapid upregulation in drug-resistant cells because prolonged daunorubicin exposure had primed them by remodeling the E3 enhancer, providing an epigenetic memory of prior stress. When daunorubicin was withdrawn from drug-resistant leukemia cells, *ABCB1* expression

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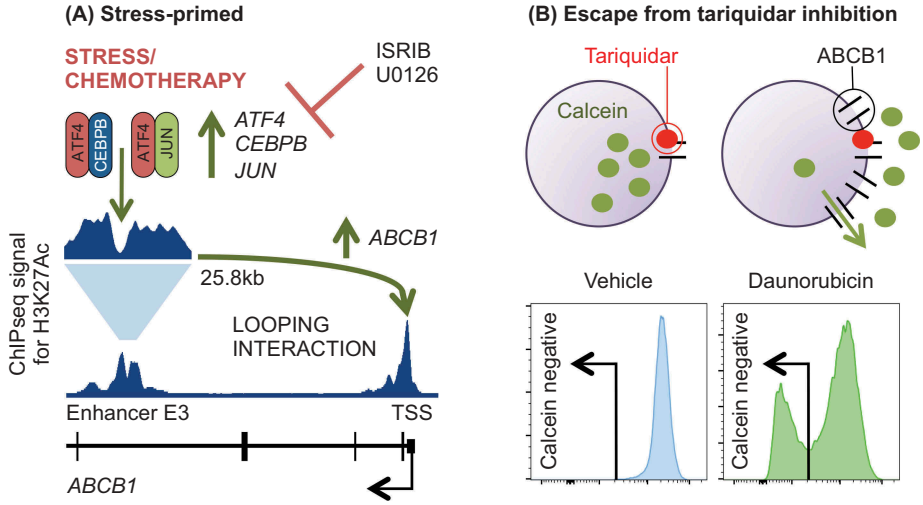

Figure 1. Stress induces *ABCB1* (ATP binding cassette subfamily B member 1) expression in leukemic blasts and permits escape from targeted ABCB1 inhibition. (a) Enhancer 3 (E3) is primed by chronic stress. Recurrent stress or chemotherapy induces an ISR-like transcriptional program that includes the transcription factor (TF) genes *ATF4* (activating transcription factor 4), *CEBPB* (CCAAT enhancer-binding protein beta) and *JUN* (Jun proto-oncogene). The TF proteins bind E3 and drive increased *ABCB1* transcription. Stress-induced upregulation can be mitigated by inhibitors of stress signaling (U0126 and ISRIB, integrated stress response inhibitor). (b) Upregulation of *ABCB1* increases cell surface protein density and allows drug efflux to occur in the presence of pharmacologic doses of tariquidar (red dot). A calcein-negative population is observed following daunorubicin exposure despite concomitant tariquidar treatment (bottom panel). H3K27Ac, histone H3 lysine 27 acetylation; TSS, transcription start site.

diminished but could be promptly reinduced by repeated drug exposure. *ABCB1* reinduction was attenuated when E3 was silenced with dCas9-KRAB.

We next examined *ABCB1* enhancer accessibility and usage in primary AML, and observed that E3 was accessible and active in a proportion of cases. To determine whether primary AML cells responded to stress in a similar manner to drug-resistant K562 leukemia cells, we exposed fresh bulk primary AML blasts to daunorubicin. We observed dose-dependent induction of ATF4 target genes, including *ABCB1, DDIT3, DDIT4* (DNA damage-inducible transcript 4), *CEBPB* and *JUN*. Interestingly, this was not observed in cryopreserved blasts, which expressed higher baseline levels of *ATF4* and *DDIT3*, suggesting that the freeze-thaw process had activated stress pathways and obscured the response to daunorubicin. ChIP-qPCR confirmed increased acetylation of the E3 enhancer in fresh primary AML following daunorubicin treatment.

In clinical trials, the dosing of tariquidar, a targeted ABCB1 inhibitor, had been based on maximal inhibition of rhodamine 123 efflux in $CD56^+$ natural killer cells, which express relatively high, stable levels of *ABCB1*.[7] Having found that *ABCB1* expression in AML was dynamic and stress-responsive we questioned whether the dose of ABCB1 inhibitors used in steady-state cells might be ineffective under conditions of stress. We assessed ABCB1-mediated efflux in the presence of pharmacologic doses of tariquidar in resistant K562 leukemia cells that had been exposed to daunorubicin, tosedostat or vehicle. Under conditions of stress, a proportion of cells continued to demonstrate active efflux; this effect was due to increased *ABCB1* expression alone and could be overcome by increasing the tariquidar concentration ten-fold. Thus, stress-induced *ABCB1* upregulation can overcome pharmacologic inhibition of ABCB1 leading to leukemia cell survival. We also found that *ABCB1* induction could be mitigated by concomitant treatment with inhibitors of stress signaling (U0126 and ISRIB, integrated stress-response inhibitor).

For many solid tumors total disease burden predicts response to therapy, whilst for AML the blast count at presentation continues to predict outcome.[8,9] Tumor hypoxia has also been consistently associated with treatment failure.[10] The cells of fast growing tumors experience hypoxia, acidosis, amino acid, and glucose deprivation. Our data identify *ABCB1* as a target of pro-survival ISR signaling and suggest that chemoresistance is a by-product of adaptation to chronic stress. Patient specimens used for research are typically collected at diagnosis or relapse and adaptive processes occurring during therapy are therefore likely to be have been missed. Whilst steady-state *ABCB1* expression characterizes only a subset of AML cases we found that dynamic upregulation following daunorubicin exposure occurred in all fresh primary samples tested. Our study suggests an explanation for the failure of clinical trials of ABCB1 inhibitors and gives a mechanistic account of the relationship between stress and chemotherapy resistance in cancer.

Disclosure of potential conflicts of interest

No potential conflicts of interest to disclose.

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