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Epigenetic Regulation of Apoptosis in Cutaneous T-Cell Lymphoma: Implications for Therapy with Methotrexate, Jak Inhibitors, and Resveratrol

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TO THE EDITOR

Previously, we showed that deficiencies in apoptotic factors occur in cutaneous T-cell lymphoma (CTCL) and can be enhanced through epigenetic mechanisms (Stutz et al., 2012; Wu and Wood, 2011; Wu et al., 2009). Now, we have further explored the epigenetic regulation of apoptosis in CTCL with special attention to the DNA methyltransferase (DNMT)/signal transducer and activator of transcription (STAT) 3 promoter methylation complex responsible for silencing several tumor suppressor genes, including those involved in apoptosis. We report that the combination of DNA methylation inhibitor (methotrexate [MTX]), Jak inhibitor (fedratinib [FED]), and lysine acetylase inhibitor (resveratrol [RES]) can induce robust cell death in CTCL lines and in leukemic Sézary cells. Their combined

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AUTHOR CONTRIBUTIONS

Conceptualization: GSW; Data Curation: MN, JW, CJS, JD, LG, AHR, GSW; Formal Analysis: MN, JW, GSW; Funding Acquisition: GSW; Investigation: MN, JW, CJS, JD; Methodology: MN, JW, GSW; Resources: JC, LG, AHR, GSW; Supervision: JC, LG, AHR, GSW; Validation: GSW; Visualization: MN, JW, GSW; Writing - Original Draft Preparation: MN, JW, GSW; Writing - Review and Editing: MN, JW, CJS, JD, LG, AHR, GSW

Ethics statement

Human samples from patients with Sézary syndrome and non-neoplastic controls were obtained after written informed consent under the approval of the Institutional Review Boards of the Medical Schools at the University of Wisconsin (Madison, WI), Columbia University (New York, NY), University of Pennsylvania (Philadelphia, PA), and Northwestern University (Evanston, IL). All methods were performed in accordance with the relevant guidelines and regulations.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.06.034.

effects on derepressing tumor suppressor genes and disrupting Jak/STAT activation in CTCL cells are summarized in Figure 1.

Five CTCL lines were used in this study: HH, SZ4, Hut78, MyLa, and SeAx (Wu et al., 2009). Methods are detailed in the Supplementary Materials and Methods. We showed earlier that FAS can be repressed by promoter methylation (Wu and Wood, 2011). In Figure 1, we show that the same is true for FASL. MTX as well as the knockdown of *DNMT1* and/or *DNMT3A* increased FASL expression. The greatest effects occurred in FAS methylation-high HH and SZ4 (Wu and Wood, 2011). Furthermore, MTX enhanced FASL upregulation induced by knockdown of either *DNMT1* or *DNMT3*. These effects were associated with decreased promoter methylation (Supplementary Figure S1). MTX also increased caspases 3/8/9 and apoptotic cell death in HH, SZ4, Hut78, and MyLa (Supplementary Figure S2).

MTX can inhibit Jak/STAT pathways (Thomas et al., 2015). Jak2 is important for STAT3 activation by phosphorylation at Tyr705 (Leonard and O'Shea, 1998). STAT3 induces the transcription of *DNMT1* in malignant T cells (Zhang et al., 2006) and in renal cell carcinoma (Quan et al., 2017). Inhibition of STAT3 might explain why the Jak2 inhibitor, FED, modestly reduced DNMT1 expression in our CTCL lines (Supplementary Figure S3). Furthermore, using anti-DNMT1 pull-down experiments, we identified STAT3 as a DNMT1-binding partner in CTCL lines (Supplementary Figure S4a). Therefore, FED has the potential to inhibit both the expression of DNMT1 and its function as part of the STAT3/DNMT1 methylation complex.

FED, a selective Jak2 inhibitor, was more effective than other mixed Jak inhibitors in reducing CTCL viability (Supplementary Figure S4b). At 1 μM doses, FED reduced CTCL viability as well or better than tofacitinib or ruxolitinib (predominantly Jak1/3 and Jak1/2 inhibitors, respectively) (Zhang et al., 2014). These effects were seen in all CTCL lines tested, including Hut78, despite the fact that it has Jak1/3–activating mutations (Pérez et al., 2015). FED also decreased spheroid formation (Supplementary Figure S4c) and STAT3 Tyr705 phosphorylation (Supplementary Figure S4d). MyLa was the least affected by any of the Jak inhibitors and is the only CTCL line with intact p53.

Compared with MTX alone, MTX + FED enhanced apoptosis in HH and SZ4 in association with increased caspase 8 (Supplementary Figure S5). However, FED did not enhance MTX-induced increases in FAS/FASL (Supplementary Figure S6), suggesting that another caspase 8−related extrinsic apoptotic pathway was responsible for the enhanced killing of MTX + FED compared with that of MTX alone. In this regard, we found that TNF–related apoptosis-inducing ligand expression was enhanced by MTX + FED compared with that by either agent alone (Supplementary Figure S7).

The optimal functioning of STAT3 also involves acetylation (Dasgupta et al., 2014; Sommer et al., 2004). Therefore, we tested the efficacy of the antioxidant and histone acetyltransferase inhibitor, RES, as an anti-CTCL agent alone and in combination with MTX and/or FED. RES also reduces *DNMT* mRNA level and activity (Fernandes et al., 2017). CTCL lines were tested with multiple drug combinations at different doses, for example,

in Supplementary Figure S8. Supplementary Table S1 shows that as a single agent, MTX was dominant in MyLa, SeAx, and SZ4, whereas FED was dominant in HH and Hut78. Although FED and RES alone had widely variable impacts on CTCL apoptosis dependent on different concentrations and different cell lines, both were capable of enhancing MTX-induced cell death.

Although single and double drug combinations matched the killing by triple-drug treatment at some doses and time points, the killing ability of 1 μM MTX + 0.5 μM FED + 5 μM RES was more uniform across all CTCL lines and time points. There was 70–95% CTCL death at 3 days and ≥95% CTCL death at 6–7 days (Supplementary Table S1 and Figure 2a). Furthermore, triple therapy significantly reduced STAT3 Tyr705 phosphorylation and lysine 685 acetylation, generally as well or better than single agents (Figure 2b).

Sézary blood specimens (Willemze et al., 2019) were enriched for cells expressing the tumor phenotype and treated with two dose levels of MTX/FED/RES for 3 days (Supplementary Materials and Methods). Apoptosis was usually greater with higher drug doses. Tumor subset apoptosis was robust (65–100%) in cases 4–8 and less prominent (<50%) in cases 1–3 (Figure 2c). We treated five non-neoplastic human PBMC controls with various dose combinations of MTX, FED, and RES. Annexin V/propidium iodide analysis showed only mild toxicity mostly in the 5–10% range, far less than observed among Sézary samples (Figure 2d).

Advanced CTCL exhibits mutations concentrated in pathways such as apoptosis, Jak/STAT, epigenetic regulation, and genome integrity (Choi et al., 2015). The drugs studied in this report (MTX, FED, RES) significantly impact these pathways. Furthermore, combination therapies are likely to be superior to single or sequential treatments because they reduce the risk of cross-resistant tumor cell mutations (Bozic et al., 2013). Our therapeutic focus has been to enhance CTCL death by derepressing tumor suppressor genes rather than by slowing CTCL proliferation by targeting driver mutations. Killing tumor cells eliminates their chance to mutate further and thereby promote clinical relapse or progression. We derepressed tumor suppressors by depleting DNA methylation donors with MTX and inhibiting STAT3/DNMT complexes. We used FED to block the STAT3 activator, Jak2, rather than using inhibitors of Jak1 or Jak3. Although Jak1/3 sometimes bears driver mutations in CTCL (Pérez et al., 2015), Jak2 inhibition reduced CTCL viability better overall. RES impeded the post-translational acetylation of STAT3 and has other anticancer effects as an anti-oxidant. A combination of MTX/FED/RES appears well-tolerated by normal PBMCs; yet, it can induce considerable apoptosis in CTCL lines and leukemic Sézary cells. All the three agents can be delivered orally and might be an effective combination therapy for advanced CTCL in the ambulatory setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

No data sets were generated or analyzed during this study.

Abbreviations:

CTCL	cutaneous T-cell lymphoma
DNMT	DNA methyltransferase
FED	fedratinib
MTX	methotrexate
RES	resveratrol
STAT	signal transducer and activator of transcription

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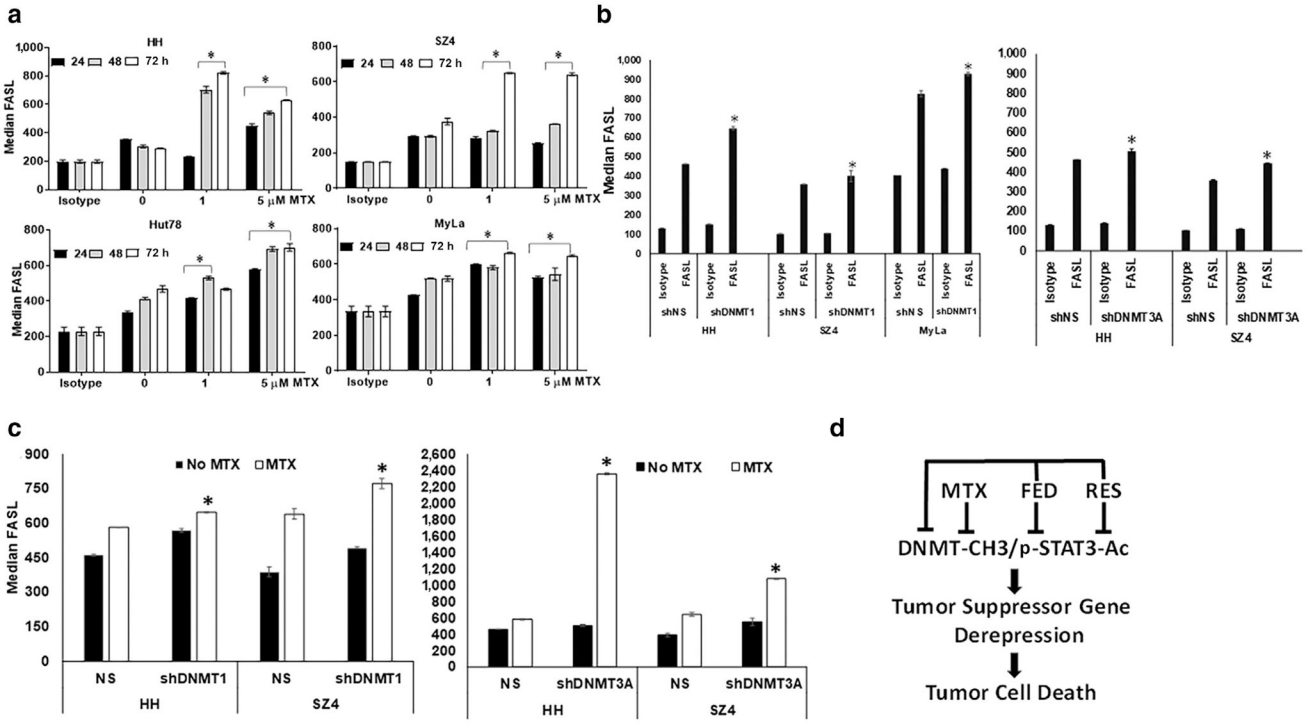

Figure 1. MTX and *DNMT* knockdown increases FASL expression in CTCL.

MTX/FED/RES derepress tumor suppressor genes by inhibiting the DNMT/STAT3 complex. (**a**) Effect of MTX on FASL assessed by flow cytometry at doses of 1 or 5 μM MTX; *$P < 0.01$ for MTX compared with no treatment. (**b**) *DNMT* knockdown increases FASL expression in CTCL at 48 h after transduction. *$P < 0.01$ relative to NS control. (**c**) MTX enhances FASL expression in *DNMT* knockdown cells. A total of 1 μM MTX dose for 48 h. *$P < 0.01$ relative to NS control. Student's *t*-test for **a**–**c**. (**d**) MTX depletes S-adenosylmethionine (methyl donor for DNMTs). MTX and FED reduce the phosphorylation of Jak1/2 and Jak 2, respectively, and thereby STAT3 phosphorylation. RES inhibits STAT3 acetylation (required for optimal functioning) and reduces *DNMT* mRNA and enzymatic activity. Ac, acetylated; CTCL, cutaneous T-cell lymphoma; DNMT, DNA methyltransferase; FED, fedratinib; h, hour; MTX, methotrexate; NS, nonsense; p-STAT3, phosphorylated signal transducer and activator of transcription 3; RES, resveratrol; sh, short hairpin.

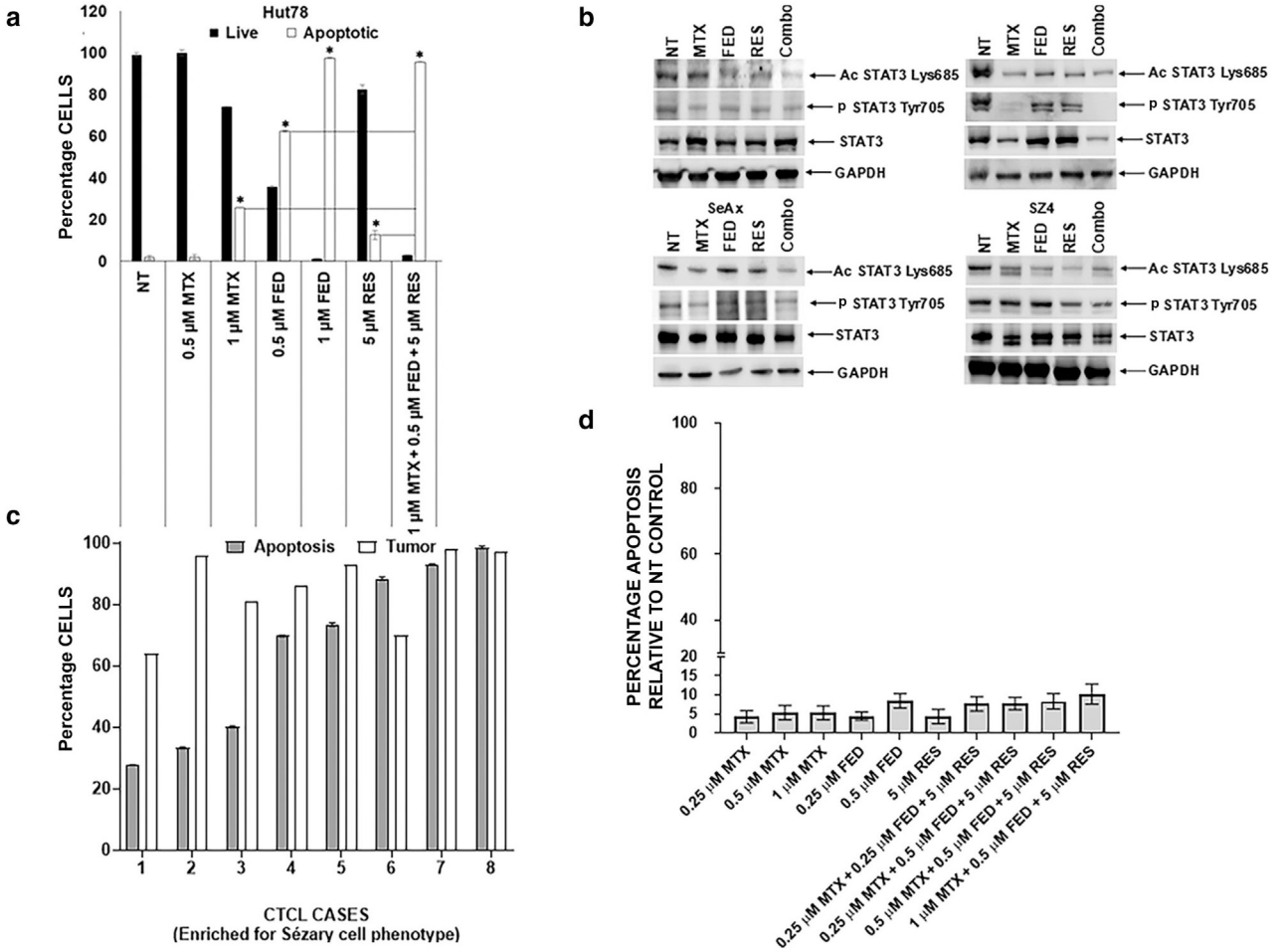

Figure 2. MTX/FED/RES kil CTCL cells and alter STAT3 activation with minimal effects on PBMCs.

(**a**) Triple treatment regimen caused more apoptotic cell death than single treatments. * $P < 0.01$ for treatments versus controls. Student's t-test. (**b**) Immunoblot analysis shows a reduction in p-STAT3 Tyr705 and lysine 685 acetylation in CTCL cells after 72 h of treatment with MTX/FED/RES. GAPDH and STAT3 are the loading controls. (**c**) A total of 1 μM MTX/0.5 μM FED/5 μM RES can induce apoptosis (gray bars) of blood samples enriched for Sézary cell phenotype (white bars). (**d**) MTX/FED/RES induces minimal apoptosis of PBMCs derived from four healthy donors and one patient with a drug rash. (**c, d**) Histograms show the mean levels (±SEM) of combined early and late apoptosis relative to NT controls assessed by Annexin V/PI flow cytometry at 72 h. Ac, acetylated; CTCL, cutaneous T-cell lymphoma; FED, fedratinib; h, hour; MTX, methotrexate; NT, no treatment; p, phosphorylated; PI, propidium iodide; RES, resveratrol; STAT3, signal transducer and activator of transcription 3.