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BET bromodomain inhibition rescues erythropoietin differentiation of human erythroleukemia cell line UT7

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ABSTRACT

Malignant transformation is a multistep process requiring oncogenic activation, promoting cellular proliferation, frequently coupled to inhibition of terminal differentiation. Consequently, forcing the reengagement of terminal differentiation of transformed cells coupled or not with an inhibition of their proliferation is a putative therapeutic approach to counteracting tumorigenicity. UT7 is a human leukemic cell line able to grow in the presence of IL3, GM-CSF and Epo. This cell line has been widely used to study Epo-R/Epo signaling pathways but is a poor model for erythroid differentiation. We used the BET bromodomain inhibition drug JQ1 to target gene expression, including that of c-Myc. We have shown that only 2 days of JQ1 treatment was required to transitory inhibit Epo-induced UT7 proliferation and to restore terminal erythroid differentiation. This study highlights the importance of a cellular erythroid cycle break mediated by c-Myc inhibition before initiation of the erythropoiesis program and describes a new model for BET bromodomain inhibitor drug application.

1. Introduction

Mouse and human erythroleukemia cell lines have been widely used to investigate the molecular mechanisms that regulate proliferative and anti-apoptotic responses triggered by Epo (erythropoietin) stimulation [1]. However, most of these cell lines either do not differentiate or differentiate poorly after Epo induction. The mouse cell line G1E (GATA-$1^{-/-}$) is a more suitable model for studying the erythroid differentiation program [2]. The erythroleukemic human cell line UT7/Epo (referred to as UT7 in this paper) is strictly growth factor-dependent for its proliferation but erythropoietin is unable to engage terminal erythroid differentiation [3]. Thus, Epo is required for its growth and survival, but a minority of cells is able to engage in erythroid differentiation. These cells are different from UT7/GM cells that can differentiate moderately in the presence of Epo [4].

Various events lead to erythroleukemia [1]. We previously described a putative oncogenic role for the erythropoietin receptor (Epo-R) in murine and human erythroleukemia cells [5]. In all cases, large numbers of Epo-R are expressed. In murine Friend virus-induced erythroleukemia cell lines, Epo-R overexpression is the result of the LTR of this spleen focus-forming virus inserting into the first exon of the Epo-R gene [5]. Human erythroleukemia TF1 cells express large amounts of an abnormal truncated Epo-R. TF1 growth requires GM-CSF or IL3, and Epo induces an erythroid differentiation program. We have shown that the expression of a truncated Epo-R impairs activation of the transcription factor STAT5 and this correlates with the capacity of TF1 to engage in erythroid differentiation [6]. Expression of a normal Epo-R in TF1 restores Epo-induced STAT5 activity, cellular proliferation and inhibition of terminal differentiation [6]. In the case of the cell line UT7, Epo-R overexpression is associated with gene amplification and 3′ gene rearrangement where c-Myc locus may map close to the Epo-R locus [5].

GATA-1 is a master erythroid transcription factor involved in the transcriptional inhibition of c-Myc [7]. The cell cycle break at G1/S phase, induced by the sequestration of E2F into the GATA-1/pRb complex [8] and the dependence of c-Myc expression by E2F [9,10] may inter-connect erythroid differentiation and down-regulation of c-Myc expression [11]. Moreover, children with DS-AMKL and DS-TL carry mutations leading to truncation of the N terminus of the GATA-1 protein [12]; this mutant fails to bind

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pRb [8], to inhibit cell proliferation [8] and to repress expression of E2F target genes including c-Myc [13]. JQ1 is a bromodomain specific inhibitor, and has been reported to inhibit c-Myc expression [14]. Here, we demonstrate that JQ1 can restore erythroid differentiation of UT7 only by a transitory treatment, whereas, permanent treatment inhibits terminal differentiation indicating a biphasic function of c-Myc expression.

2. Materials and methods

2.1. Cell line culture

UT7 cells were maintained in modified Eagle medium (IMEM) containing 10% fetal calf serum and 2 U/ml Epo. JQ1 protocol treatments: On day 0, G1E-ER cells were grown in the presence of Epo and were incubated for 24 h with estradiol (E), then on day 1, for (+)-JQ1 or (−)-JQ1 at 500 nM. On day 3, cells were washed (w) and cultured with fresh medium containing Epo and E with and without JQ1. (+)-JQ1 and (−)-JQ1 were synthesized by Sigma–Aldrich, France (HPLC purity: 99.4% at 220 nm). G1E-ER [11] cells were cultured as described elsewhere [7].

2.2. Flow cytometry

APC-conjugated anti-human CD71 (CD71-APC, Miltenyi, cat no. 130-091-727), phycoerythrin-conjugated anti-human c-Kit (CD117-PE, eBioscience, cat no. 12-1178) and FITC-conjugated anti-human glycophorin A (CD235-FITC, eBioscience, cat no. 11-9987) were used for surface labeling of UT7 cells. Phycoerythrin-conjugated anti-mouse TER119 (TER119-PE, eBioscience, cat no. 12-5921-82) and APC-conjugated anti-mouse CD117 (c-Kit-APC, eBioscience, cat no. 17-1171-82) were used for surface labeling of G1E-ER cells. A FACS Calibur was used for flow cytometry and data were analyzed with the Cell Quest Pro software.

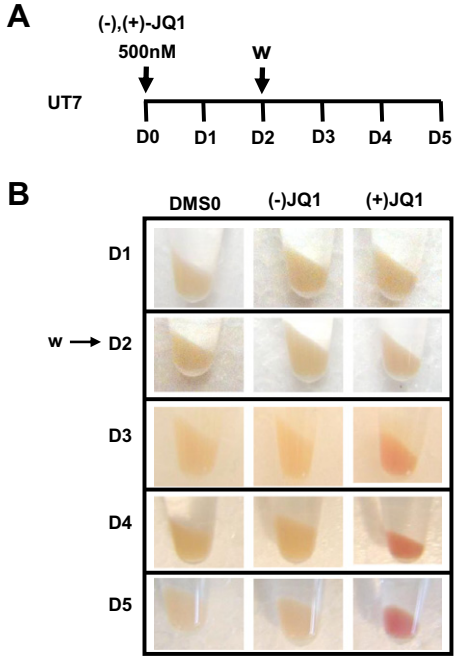

Fig. 1. The bromodomain inhibitor (+)-JQ1 induces differentiation of the erythroid cell line UT7. (A) Schematic representation of the experimental culture protocol. On day 0, UT7 cells growing in the presence of Epo were incubated for 48 h with (+)-JQ1 or (−)-JQ1 at 500 nM. On day 2, cells were washed (w) and cultured with fresh medium containing Epo (see also Supplemental Fig. 1). (B) Visualization of hemoglobinized cell pellets on each day (D).

2.3. Reverse transcription and real-time quantitative PCR

Total RNA was extracted with PureLink RNA kit reagents and treated with DNase I (Invitrogen). RT-qPCR was performed using Superscript III (Invitrogen). The c-Myc primers used were TaqMan gene expression assay (Applied Biosystems) probes specific for human mRNA (c-Myc: Hs00905030/m1). Results were quantified: an ABI Prism 7300 system with Taqman FAM dye was used for real-time PCR (qPCR) and qPCR results were normalized to those obtained for 18S RNA with specific primers (18S: Hs99999901/S1).

3. Results and discussion

3.1. (+)-JQ1 induces UT7 erythroid differentiation

To investigate the role of c-Myc inhibition in erythroid differentiation, we used the bromodomain inhibitor, (+)-JQ1 [14], to down-regulate c-Myc transcription in the cell line UT7. First, we performed permanent (+)-JQ1 treatment; UT7 cells engaged their erythroid differentiation at day 3 but at day 4, UT7 culture contained 95% cell death. To avoid this effect, UT7 cells were grown for 2 days in Epo with (+)-JQ1 or its enantiomer (−)-JQ1 as negative control; the cells were then washed, diluted and grown with only Epo for 3 days (Fig. 1A). To determine optimal conditions, we tested various (+)-JQ1 concentrations and assessed their physiological effects (erythroid differentiation, proliferation, survival and cell death): (+)-JQ1 at 500 nM was found to be optimal (Supplemental Fig. 1). To visualize hemoglobinization, aliquots of 5×10^6 cells grown in these conditions were collected daily and centrifuged. The cell pellets were red only for the (+)-JQ1 condition on days 3–5 (Fig. 1B).[2] The presence of hemoglobin was confirmed by benzidine-staining assays (Fig. 2A) and by qRT-PCR assays testing for human α and ε globin mRNA expression (Fig. 2B). Concomitantly, the growth of UT7 cells was inhibited by (+)-JQ1 (Fig. 2C) without cell death. FACS analysis indicated a decrease of c-Kit expression and an increase of c-Kit$^-$-GpA$^+$ populations in (+)-JQ1-treated samples (Fig. 2D and Supplemental Fig. 2A and B). This finding indicates that transient treatment by (+)-JQ1 may reinitialize an erythroid differentiation program in the UT7 cell line. (+)-JQ1 inhibits the c-Myc transcription program and breaks the cell cycle at G1 phase without affecting the production of E2F factor mRNA or protein [14,15]. However, (+)-JQ1 may affect the expression of numerous E2F-dependent genes [14]. Various inhibitor molecules of the BET family have been described, for example GSK1210151A, that seem to be more effective than JQ1 and may inhibit transcription elongation of the c-Myc gene, but also BCL2 and CDK6 genes [16]. They may have a larger spectrum of action, acting on more than one target, although c-Myc is the most frequently described. In the case of UT7, the transient (+)-JQ1-induced caused a large decline of c-Myc transcriptional elongation during the first 2 days of treatment and correlated well with the induction of erythroid differentiation at day 3 before the peak of globin expressions (Fig. 2E). This c-Myc inhibition was abrogated after cell wash and declined again at day 5, later after (+)-JQ1 treatment. This biphasic c-Myc expression could be the issue of the reactivation of c-Myc expression caused by the Epo and SCF stimulation on the residual c-Kit$^+$ cell population.

3.2. Permanent treatment by (+)-JQ1 inhibits terminal erythroid differentiation

We have shown that permanent treatment by (+)-JQ1 induced UT7 partial terminal erythroid differentiation but also cell death.

[2] For interpretation of color in Figs. 1 and 3, the reader is referred to the web version of this article.

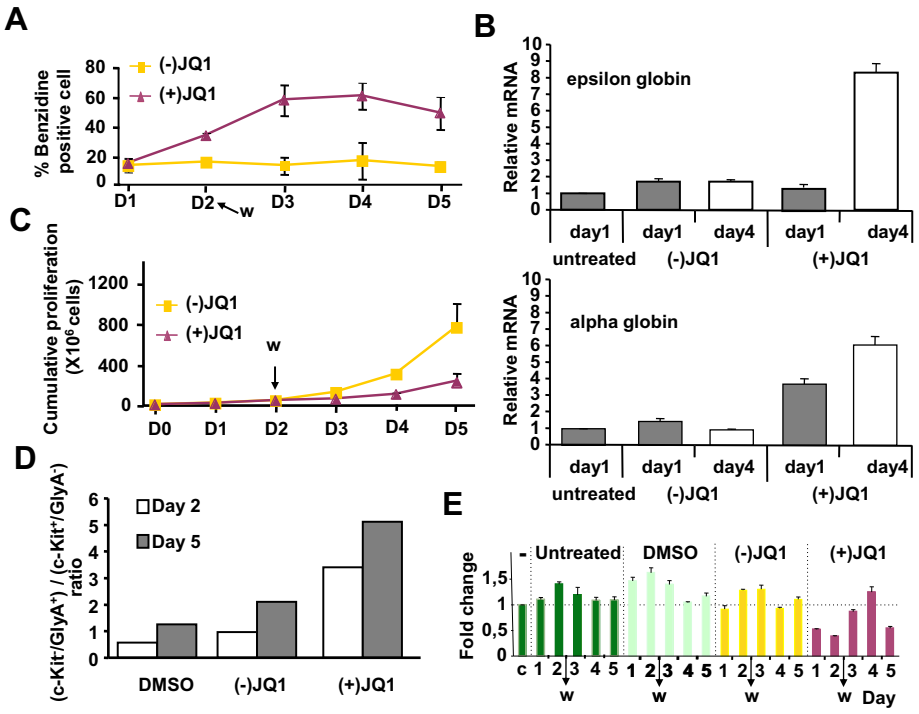

Fig. 2. (A) Benzidine-positive UT7 cells analysis ($n = 4$) after (+)-JQ1 or (−)-JQ1 at 500 nM treatment. (B) qRT-PCR analysis of human α and ε globin expression. *18S* rRNA was used to normalize qRT-PCR results and a value 1 was assigned to normal donor for relative mRNA calculations ($n = 3$). (C) Analysis of cell proliferation by counting live cells (trypan blue dye exclusion). Cells were plated at a concentration of 10^5 per ml and cumulative cell number, on each day, is indicated. Four independent experiments gave a similar result. (D) c-Kit and GlyA FACS analysis of UT7 erythroid differentiation on days 2 and 5. The ration of c-Kit$^-$/GlyA$^+$ on c-Kit$^+$/GlyA− is indicated. (E) Effect of (+)-JQ1 or (−)-JQ1 treatment on c-Myc expression. qRT-PCR, using TaqMan probe specific for human c-Myc, was performed at days 1–5. Fold change was calculated by the $2^-\Delta\Delta$CT method with the *18S* rRNA used for normalization. Fold change was normalized to c-Myc mRNA in the day 0 sample (−). Three independent experiments were performed and gave similar results.

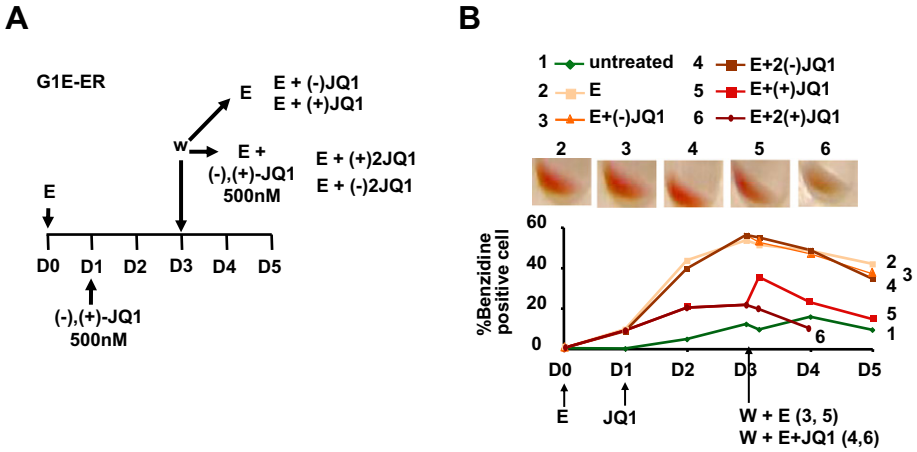

Fig. 3. The bromodomain inhibitor (+)-JQ1 delays G1E-ER cell line differentiation. (A) Schematic representation of the experimental G1E-ER culture protocol. On day 0, G1E-ER cells were growth in the presence of Epo and were incubated for 24 h with estradiol (E), then on day 1, for (+)-JQ1 or (−)-JQ1 at 500 nM. On day 3, cells were washed (w) and cultured with fresh medium containing Epo and either E alone (referenced as E+(+)JQ1 or E+(−)JQ1) or with E and (+)-JQ1 or (−)-JQ1 at 500 nM (referenced as E+(+)2JQ1 or E+(−)2JQ1). (B) Visualization of hemoglobinized cell pellets on day 4 after drug treatment as indicated in (A). Benzidine-positive cell analyses were performed at each day of treatment. Three independent experiments gave similar results.

To investigate the long-term treatment effect of (+)-JQ1 on erythroid differentiation, we have used the G1E-ER GATA-1$^{-/-}$ cell line where erythroid maturation can be induced by estradiol-activation of GATA-1 [11]. Without estradiol stimulation, (+)-JQ1 fails to inhibit murine G1E-GATA-1$^{-/-}$ cell proliferation and to down regulate c-Myc transcription [17]. GW841819X, another BET inhibitor [18], can inhibit the binding of Brd3 to acetylated GATA-1 decreasing erythroid differentiation [19]. These observations seem to be contradictory to our results with UT7. These various BET inhibitors may have different BET targets and consequently different cellular effects, so we tested the effects of (+)-JQ1 on terminal erythroid differentiation of G1E-ER cells after GATA-1ER activation by estrogen. Estradiol-stimulated G1E-ER cells (24 h) were grown with or without (+)-JQ1 or with (−)-JQ1 as control (36 h), washed and cultured in standard conditions with or without (+)-JQ1 (3 days) (Fig. 3A). Hemoglobinization of pellets was observed in

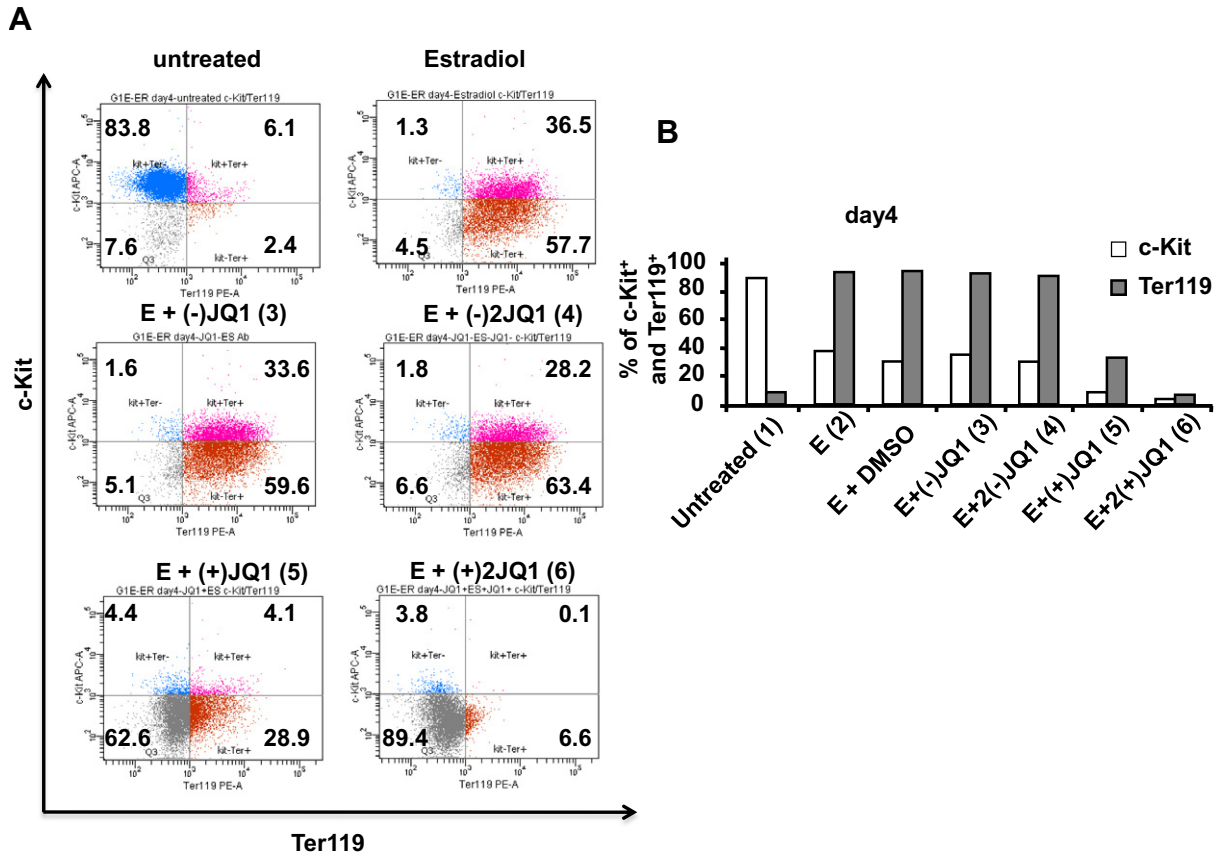

Fig. 4. Transitory and permanent (+)-JQ1 treatments, respectively, delay or inhibit terminal erythroid differentiation of estradiol-induced G1E-ER cells. (A and B) FACS analysis on day 4, with specific mouse c-Kit, and Ter119 antibodies, of treated G1E-ER as indicated. Three independent experiments were performed and gave similar results.

all conditions but when (+)-JQ1 was maintained, the pellets were significantly less red (Fig. 3B). Benzidine analysis revealed two phases (Fig. 3B): (+)-JQ1 inhibited erythroid differentiation until cells were washed and then, 4 h after (+)-JQ1 elimination (E+(+)-JQ1), benzidine-positive cell production accelerated (Fig. 2B) and in one case to above control values (data not shown). However, when (+)-JQ1 was maintained after washing (E+2(+)-JQ1), cell differentiation was significantly lower than in all other conditions (Fig. 3B) confirming the BET inhibitor inhibition of cell differentiation [19]. FACS analysis of c-Kit and Ter119 expression on day 4 confirmed these results (Fig. 4A) and showed total c-Kit inhibition after (+)-JQ1 treatment (Fig. 4B). After estradiol stimulation, the inhibition of cell proliferation and the cell death by (+)-JQ1 were not different to that by (−)-JQ1 or estradiol alone (Supplemental Fig. 3A and B). The findings are consistent with the notion that GATA-1 down regulated c-Myc expression (proliferation break at G1/S phase [8,13]). Thus c-Myc inhibition occurred either, in UT7, where GATA-1 are unable to perform this function, by (+)-JQ1 short treatment or, in estradiol-stimulated G1E-ER, by GATA-1 association to pRb/E2F [8,13] and in both cases this inhibition are required for the initiation of erythroid differentiation. Inhibition of terminal erythroid differentiation by permanent treatment with BET inhibitor indicates the requirement of a biphasic c-Myc expression after c-Kit lost (ours UT7 results).

Therefore, we propose that the bromodomain inhibitor (+)-JQ1 can be used, in appropriate conditions that respect the biphasic function of c-Myc during terminal erythroid differentiation, to rescue the erythroid program of the Epo erythroid differentiated refractory erythroleukemia cell line UT7. The strategy of brief inactivation of oncogenes as therapy has been already described [20] and our analysis illustrates a potential new strategy of bromodomain inhibitor application in erythropoiesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.112.

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