The BET Bromodomain Inhibitor OTX015 Affects Pathogenetic Pathways in Preclinical B-cell Tumor Models and Synergizes with Targeted Drugs

Michela Boi¹, Eugenio Gaudio¹, Paola Bonetti¹, Ivo Kwee².³, Elena Bernasconi¹, Chiara Tarantelli¹, Andrea Rinaldi¹, Monica Testoni¹, Luciano Cascione¹.⁴, Maurilio Ponzoni⁵, Afua Adjeiwa Mensah¹, Anastasios Stathis⁴, Georg Stussi⁴, Maria Eugenia Riveiro⁶, Patrice Herait⁷, Giorgio Inghirami⁸.⁹.¹⁰, Esteban Cvitkovic⁶.⁷, Emanuele Zucca⁴, and Francesco Bertoni¹.⁴

Abstract

Purpose: In cancer cells, the epigenome is often deregulated, and inhibition of the bromodomain and extra-terminal (BET) family of bromodomain-containing proteins is a novel epigenetic therapeutic approach. Preliminary results of an ongoing phase I trial have reported promising activity and tolerability with the new BET bromodomain inhibitor OTX015.

Experimental Design: We assessed the preclinical activity of OTX015 as single agent and in combination in mature B-cell lymphoma models and performed in vitro and in vivo experiments to identify the mechanism of action and the genetic features associated with sensitivity to the compound.

Results: OTX015 showed antiproliferative activity in a large panel of cell lines derived from mature B-cell lymphoid tumors with median IC50 of 240 nmol/L, without significant differences among the different histotypes. In vitro and in vivo experiments showed that OTX015 targeted NFKB/TLR/JAK/STAT signaling pathways, MYC- and E2F1-regulated genes, cell-cycle regulation, and chromatin structure. OTX015 presented in vitro synergism with several anticancer agents, especially with mTOR and BTK inhibitors. Gene expression signatures associated with different degrees of sensitivity to OTX015 were identified. Although OTX015 was mostly cytostatic, the compound induced apoptosis in a genetically defined subgroup of cells, derived from activated B-cell–like diffuse large B-cell lymphoma, bearing wtMYD88 mutations in MYD88, and CD79B or CARD11.

Conclusions: Together with the data coming from the ongoing phase I study, the in vitro and in vivo data presented here provide the basis for further clinical investigation of OTX015 as single agent and in combination therapies.

Clinical Cancer Res; 21(7); 1–11. © 2015 AACR.

Introduction

The epigenome is in a highly dynamic condition due to precise temporal and spatial chromatin modifications, and proper chromatin regulation is fundamental in controlling gene expression and critical for fundamental cellular processes, including self-renewal, differentiation, and proliferation (1, 2). In cancer cells, the epigenome is very often deregulated due to aberrant changes in histone modifications, DNA methylation, and chromatin structure. OTX015 presented in vitro synergism with several anticancer agents, especially with mTOR and BTK inhibitors. Gene expression signatures associated with different degrees of sensitivity to OTX015 were identified. Although OTX015 was mostly cytostatic, the compound induced apoptosis in a genetically defined subgroup of cells, derived from activated B-cell–like diffuse large B-cell lymphoma, bearing wtMYD88 mutations in MYD88, and CD79B or CARD11. The contribution to the assembly and the positioning of the transcriptional machinery represents one of the most important functions of chromatin remodeling that is largely mediated by a variety of histone-modifying enzymes that write and read the "histone code" (1, 2). Epigenetic writers are enzymes that chemically modify DNA or histones, erasers remove such chemical modifications, and, finally, readers recognize specific histone acetylated lysine residues and facilitate transcriptional activation by recruiting transcription factors and other elements of the transcription machinery. Importantly, chromatin modifications can be manipulated and reversed (3), providing the rational to pharmacologically target the epigenome. In the lymphoma field, the epigenetic erasers histone deacetylases (HDAC) represent the currently most explored therapeutic targets (3), with HDAC inhibitors (HDACI), vorinostat and romidepsin, approved by the FDA for cutaneous T-cell lymphomas.
Inhibition of the bromodomain and extra-terminal (BET) family of bromodomain-containing proteins in cancer. OTX015, a new oral BET bromodomain inhibitor that is now in its early clinical development, shows a wide preclinical activity in lymphoma models and it affects important biologic pathways, such as MYC, NFkB, TLR, and IAK/STAT pathways. The observed synergism with different compounds provides the basis for the future clinical development of OTX015 in combination.

**Translational Relevance**
In cancer cells, the epigenome is often deregulated, and inhibition of the bromodomain and extra-terminal (BET) family of bromodomain-containing proteins is a novel epigenetic therapeutic approach. OTX015, a new oral BET bromodomain inhibitor that is now in its early clinical development, shows a wide preclinical activity in lymphoma models and it affects important biologic pathways, such as MYC, NFkB, TLR, and IAK/STAT pathways. The observed synergism with different compounds provides the basis for the future clinical development of OTX015 in combination.

**Cell proliferation, cell death, cell cycle**
The effect on cell proliferation, cell growth, apoptosis, and cell cycle were assessed as previously described (23, 24).

**Senescence**
Cells were treated with DMSO or different doses of OTX015 and stained using a β-Galactosidase Staining Kit (Calbiochem). Cells were fixed with 4% formalin and the nuclei stained with fast-red dye. Images were acquired using a Zeiss light microscope. Senescence-associated β-galactosidase (SA-βgal) activity was also assessed by a fluorescence-based assay using flow cytometry. Cells were seeded and treated with OTX015 or the equivalent amount of DMSO and, after the treatment, were incubated with 100 nmol/L Bafilomycin A1 (Sigma-Aldrich) for 1 hour to alkalize the lysosomes. Cells were then incubated for 2 hours with 33 μL of 2 mmol/L 5-dodecanoylaminofluorescein-di-β-D-galactopyranoside (C12FDG; Invitrogen), washed thoroughly and analyzed with a FACScan flow cytometer (Becton Dickinson AG). Data were analyzed with FlowJo 7.6.3 software (Tree Star).

**Western blotting analysis**
Protein extraction, separation, and immunoblotting were performed as previously described (23). The following antibodies were used: anti-BRD2 (ab37633), anti-BRD3 (ab56342), anti-BRD4 (ab75898, AbCam), anti-MYC (9E10, Becton Dickinson AG or Cell Signaling Technology), anti-α-GPDH (MAB374, Millipore), anti-STAT3 (9139), anti-phospho-STAT3 (Ser727; 9134), and anti-phospho-STAT3 (Tyr705; 9131; Cell Signaling Technology).

**Immunohistochemistry**
Monoclonal antibodies against phospho-STAT, p50 (Cell Signaling Technology) were applied. In all instances, antigen retrieval was performed with Tris-EDTA at pH 9, 30 minutes at 98°C. Reactions were developed with Ultravision Quanto Detection System (TL-125-QHL; Thermo Scientific).

**Real-time PCR**
RNA was extracted using the RNeasy Kit (Qiagen AG). Real-time PCR was performed as previously described (ref. 23; sequences available upon request).

**Gene expression profiling**
Gene expression profiling (GEP) was done using the HumanHT-12-v4 Expression BeadChip (Illumina). Data processing and statistical analysis was performed using R/Bioconductor (25). Transcript mapping was based on HG19 using manufacturer supplied annotation. Data were quantile normalized and subsequently batch corrected using ComBat (26). Differential expression analysis was performed using LIMMA (27). Functional annotation was performed using the Gene set enrichment analysis (28) and MetaCore (Thomson Reuters) tools. The top significantly differentially expressed genes between treated and untreated cells were analyzed in MetaCore by building a network consisting of the shortest paths (that is, having the smallest possible number of directed one-step interactions) between pairs of the transcripts in each direction, using standard Dijkstra shortest paths algorithm, maximum path length.

**Materials and Methods**

**Cell lines and molecules**
Established human cell lines derived from DLBCL, mantle cell lymphomas (MCL), multiple myeloma (MM), splenic marginal zone lymphoma (splenic MZL), and prolymphocytic leukemia (PLL) were cultured in the culture media listed in Supplementary Table S1. The cell lines were not authenticated independently. OTX015 was provided by Oncoethix SA. Other compounds used were everolimus, doxorubicin, ibrutinib, lenalidomide, benda-mustine, decitabine, idelalisib, vorinostat, romidepsin (Selleckchem), and rituximab (Roche).

**OF2** Clin Cancer Res; 21(7) April 1, 2015

Clinical Cancer Research

Published OnlineFirst January 26, 2015; DOI: 10.1158/1078-0432.CCR-14-1561

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length of two, and filtering by limiting the interactions type to transcription regulation, influence on expression, and miRNA binding. Raw data will be available at the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) database.

Evaluation of interleukins production

Cells treated with OTX015 or DMSO were harvested, washed, and resuspended in fix-perm buffer (Becton Dickinson AG) and left for 30 minutes at 4°C. After a wash with perm buffer (Becton Dickinson AG), cells were resuspended in perm buffer and the antibodies anti-IL4-PE and anti-IL10-PE (eBioscience). Samples were left for 30 minutes in the dark. After the incubation, cells were washed in PBS, resuspended in PBS, analyzed using the FACScan flow cytometer. Data were analyzed with FlowJo 7.6.3 software.

Chromatin immunoprecipitation followed by high-throughput DNA sequencing

We analyzed the publicly available chromatin immunoprecipitation followed by high-throughput DNA sequencing (ChiP-Seq) dataset from the Short Read Archive under accession number SRP043524 (20). The 36-bp sequence reads were aligned to human reference genome build GRCh37 (hg19) using Bowtie (29). Redundant reads were removed and reads uniquely mapping to reference genome were used for further analysis. A maximum of one mismatch was allowed for each read. The detection of genomic regions enriched by chromatin immunoprecipitation (ChiP) versus the negative control immunoprecipitation (IP) experiment done with an anti-Flag antibody was carried out using HOMER v2.6 (30). Specific peaks were defined as having at least a 4-fold difference in enrichment within a 200-bp region between the two SRP043524 experimental conditions (DMSO vs. IQ1) and applying the HOMER default thresholds for statistical significance (FDR = 0.001 and Poisson P cutoff = 1e–04). All discovered putative peaks were ranked by their normalized tag counts (number of tags found at the peak, normalized to 10 million total mapped tags) and annotated with annotatePeaks.pl using the GCRH37 (hg19) dataset.

Chromatin immunoprecipitation

Cells were cross-linked with 1% formaldehyde. Cross-linking was quenched with 125 mmol/L glycine. Cells were washed with ice-cold PBS containing 1× HALT protease inhibitor (Thermo Scientific) and resuspended in SDS lysis buffer (ChIP Assay Kit, Millipore) before sonication. For each immunoprecipitation reaction, 1×10^6 chromatin cell equivalents were incubated overnight with 5 μg anti-BRD4 antibody (catalog no. A301-985A; Bethyl Laboratories) or 3 μg of the negative control antibody, anti-IgG (Millipore). Immune complexes were collected by incubation with 20 μL magnetic protein G beads (4°C, 1.5 hours). Protein G-bound complexes were sequentially washed with low salt wash buffer, high salt wash buffer, LiCl wash buffer, and twice with TE Buffer (ChIP Assay Kit; Millipore). Elution of protein/DNA complexes was performed using 1% SDS and 0.1 mol/L NaHCO3. Following reversal of cross-links (65°C for 4 hours), samples were treated with RNase A and then Proteinase K. DNA samples purification was performed with QIAquick PCR Purification Kit (Qiagen). Chromatin samples to which no antibody had been added were processed in parallel as input references. Quantitative real-time PCR of ChIP-DNA was performed using primers specific for a locus of BRD4 binding in the upstream regulatory region of MYD88 (chr3:38179682-38179840) as determined by analyzing the ChiP-Seq dataset SRP043524 (20); primers specific for human alpha-satellite were used as a negative control (sequences available upon request).

In vivo experiments

At day 1, tumors were established by injecting SU-DHL-2 lymphoma cells (200 μL of PBS, 15 × 10^6 cells/mouse) into the left flanks of female NOD-SCID mice (approximately 20 g of body weight; The Harlan Laboratory, S. Pietro al Natisone, Udine, Italy). Tumor size was measured on regular basis. Treatments were conducted daily, twice a day. Mice maintenance and animal experiments were performed with study protocols approved by the local Cantonal Veterinary Authority (No. 5/2011). Differences in tumor volumes were calculated using the Wilcoxon rank-sum test (Stata/SE 12.1 for Mac, Stata Corporation). The P value for significance was <0.05.

Drug combinations and evaluation of synergism

Cells were seeded in 96-well plates at 10^4 cells per well. Molecules were serially diluted in tissue culture media and added to cells (in five replicates) and incubated for 72 hours at 37°C, 5% CO2. MTT assay was then performed as described (31). The combinations were evaluated using the Chou–Talalay Combination Index (CI), calculated with the Synergy R package (32). The effect of the combinations was defined synergistic (CI < 0.9), additive (CI, 0–9–1.1) or antagonist (CI > 1.1).

Results

OTX015 has antiproliferative activity in in vitro models of B-cell lymphomas

We first evaluated the antiproliferative activity of the BET bromodomain inhibitor OTX015 in a panel of 33 cell lines derived from mature B-cell lymphoid tumors. As assessed by MTT assays performed after 72 hours of drug exposure, OTX015 was active in a dose-dependent manner in almost all the cell lines (Fig. 1A), at concentrations achievable in the clinical setting (33). The median IC50 value for the whole series was 240 nmol/L (range, 70 nmol/L–15 μmol/L). The median values for the individual lymphoma entities were 195 nmol/L (70 nmol/L–1.5 μmol/L) in DLBCL, 470 nmol/L (340 nmol/L–1.5 μmol/L) in MCL, 170 nmol/L (105–240 nmol/L) in splenic MZL, 450 nmol/L (60–700 nmol/L) in MM, and 90 nmol/L in the PLL cell line. The antiproliferative effect of OTX015 did not significantly differ among the different the histotypes, or between germinal-center type (GCB) DLBCL (190 nmol/L; 80 nmol/L–1.5 μmol/L) and activated B-cell–like (ABC) DLBCL (200 nmol/L; 70 nmol/L–2.28 μmol/L).

In accordance with the observed antiproliferative activity, OTX015 treatment of DLBCL cells inhibited cell growth and induced cell-cycle arrest with G1 accumulation and decreased S-phase (Fig. 1B and Supplementary Fig. S1). OTX015 also induced a time-dependent increase of β-Gal–positive cells (Fig. 1C).

OTX015 induces apoptosis in a genetically defined subgroup of DLBCL

We then assessed the induction of apoptosis after OTX015 exposure in DLBCL cells. Dose- and time-dependent apoptosis was observed in 3 of 22 (14%) cell lines treated for 72 hours with a
Figure 1.
OTX015 has antiproliferative activity in \textit{in vitro} models of B-cell lymphomas. A, IC\textsubscript{50} values calculated through MTT assay in 33 mature B-cell lymphoid tumor cell lines after 72 hours of drug exposure (experiments done in triplicate). Green, PLL; red, ABC-DLBCL; blue, GCB-DLBCL; yellow, MCL; black, MM; orange, splenic MZL. B, representative flow cytometry profiles showing cell-cycle alterations induced by OTX015 (500 nmol/L) in the SU-DHL-6 cell line. C, representative image showing the appearance of β-Gal-positive cells after exposure of the DoHH2 cell line to OTX015. D, percentage of apoptotic cells before and after 72 hours of OTX015 (500 nmol/L) or DMSO (NT).
A massive apoptotic induction was obtained in SU-DHL-2 and TMD8, whereas OCI-Ly3 had a lower, although significant, induction of apoptosis. All these three cell lines presented common genetic and biologic features: derivation from ABC-DLBCL, mutated MYD88 gene, wild-type TP53, mutations in CD79B (SU-DHL-2, TMD8), or in CARD11 (OCI-Ly3). The presence of mutations in genes coding for MYD88 and for components of the BCR signaling was significantly associated with apoptosis induction ($P = 0.027$).

**Transcriptional signature of OTX015 in DLBCL cell lines**

To obtain a global view of the transcriptional changes after OTX015 treatment, we performed GEP on two sensitive cell lines, one derived from GCB-DLBCL (SU-DHL-6) and one from ABC-DLBCL (SU-DHL-2), treated with DMSO or with OTX015 (500 nmol/L) for 1, 2, 4, 8, and 12 hours. OTX015 affected, in a time-dependent manner, important biologic processes: NF-κB, Toll-like receptor (TLR), Janus kinase (JAK)/STAT signaling pathways, MYC- and E2F1-regulated genes, cell-cycle regulation, and chromatin structure (Fig. 2 and Supplementary Figs. S3 and S4; $FDR<0.001$, $NES=-2.50$).

**Figure 2.** OTX015 induces MYC downregulation in B-cell lymphomas. A, GSEA plot illustrating the enrichment of the GSEA M2711 MYC oncogenic signature in SU-DHL-2 and SU-DHL-6 cells treated with OTX015 (500 nmol/L). FDR, false discovery rate; NES, normalized enrichment score. B, MYC protein levels are downregulated after OTX015 treatment. C, MYC and MYC targets CAD and NUC mRNA are downregulated after OTX015 treatment for 8 hours in a dose-dependent manner. Y axis, fold change versus GAPDH and DMSO-treated cells. D, MYC mRNA levels are downregulated in a time-dependent manner by OTX015 and restored after removal of the compound from the media after 2 hours of treatment. Y axis, fold change versus GAPDH and DMSO-treated cells.
those upregulated by HDACi, while the downregulated transcripts represented by transcripts coding for histones, overlapping with Supplementary Table S2). The upregulated genes were mainly comprised MYC and E2F1 targets or genes involved in NFκB/TLR/JAK/STAT pathways. Supplementary Table S3 lists the top most differentially expressed probes (adjusted \( P < 0.01 \) and absolute fold change >1.5), with MYC as the most downregulated transcript. The OTX015 GEP signature appeared similar to that reported following exposure to another BET bromodomain inhibitor, JQ1, in different tumor models (4, 5, 14, 15, 21) (Supplementary Fig. S5). OTX015 appeared similar to JQ1 also in terms of activity on cell viability and induction of apoptosis in SU-DHL-2 and DOHH2 DLBCL cell lines (data not shown), as also recently reported by Chapuy and colleagues (10).

**OTX015 induces MYC downregulation in DLBCL**

Similar to that reported for other BET bromodomain inhibitors (4, 5, 21), treatment with OTX015 led to an important negative regulation of MYC and its target genes. MYC was the central hub connecting almost all the most significantly OTX015-regulated genes (Supplementary Fig. S4), and MYC target genes were significantly enriched among OTX015-regulated transcripts (Fig. 2A). MYC changes were validated at RNA and protein level (Fig. 2B and C). The effect of OTX015 on MYC and its target genes was both time and dose dependent (Fig. 2C and D). The effect on MYC appeared reversible, as the mRNA levels started to be restored in a time-dependent manner, albeit with different kinetics among the individual DLBCL cell lines, after replacing drug-containing medium with fresh medium (Fig. 2D). The level and the kinetics of MYC downregulation after drug exposure did not appear associated with the sensitivity to OTX015 (data not shown).

**OTX015 affects the NFκB, TLR, and JAK/STAT signaling pathways in DLBCL cells**

OTX015 negatively regulated transcripts encoding members of the NF-κB, TLR, and JAK/STAT signaling pathways, such as MYD88, IRAK1, TLR6, TNFRSF17, IL6, and IRF4, in both GCB- and ABC-DLBCL cells (Fig. 3). The inhibitory effect of OTX015 on the pathways was further confirmed at protein level. Both immunoblotting and immunohistochemistry showed a reduction of the transcriptionally active phospho-STAT3 in SU-DHL-2 and TMD8, two ABC-DLBCL cell lines, as well a reduction of the nuclear localization of p50 (NFκB1), indicating a clear inhibitory effect of OTX015 on the canonical NF-κB pathway (Fig. 3B–D). In ABC-DLBCL cell lines, we observed a reduced production of IL10 and IL4 after 24 hours of OTX015 treatment (Supplementary Fig. S6) and a dose-dependent downregulation of additional transcripts suggestive of NF-κB activation (BIRC3, TNAIP3; data not shown).

On the basis of the hypothesis that MYD88 may play a central role in the mechanism of action of OTX015, we sought to understand whether BET bromodomain inhibitors had a direct effect on the gene regulation. We first analyzed the publicly available Chip-Seq data obtained for HBL1 ABC-DLBCL cells treated with the BET bromodomain inhibitor JQ1 (20). Treatment with JQ1 (500 nmol/L) for 3 hours reduced the binding of BRD4 to the upstream regulatory region of MYD88 (FDR < 0.001; Supplementary Fig. S7A). We then performed a ChIP experiment in SU-DHL-2 ABC-DLBCL cells exposed to DMSO or to OTX015 (500 nmol/L) for 3 hours. The drug appeared to reduce the binding of BRD4 to the upstream regulatory regions of the MYD88 gene (Supplementary Fig. S7B). These data suggest that BET bromodomain inhibitors dislocate BRD4 from MYD88 regulatory regions.

**OTX015 has biologic activity in a DLBCL in vivo model**

We then assessed the ability of OTX015 to downregulate MYC, the NF-κB, TLR, and JAK/STAT pathway also in an in vivo model. We treated SU-DHL-2 xenografts grown subcutaneously in NOD-SCID mice with OTX015 (50 mg/kg, orally, twice a day; \( n = 4 \) mice) or with control (vehicle orally, twice a day; \( n = 4 \) mice) for three days, starting when the tumors had reached the volume of 500 mm³. No body weight losses were registered during the three days of treatment. Real-time PCR showed that there was a significant downregulation of MYC, IL6, TLR6, TNFRSF17 and, although not reaching the statistical significance, of IRAK1, IRF4, and STAT3 (Fig. 4A).

We then assessed the in vivo antilymphoma activity of OTX015. NOD-SCID mice were treated with control vehicle (per os, twice a day; \( n = 7 \) mice) or with OTX015 (25 mg/kg, orally, twice a day; \( n = 8 \) mice), starting 5 days after the subcutaneous injection of SU-DHL-2 cells and then each day for 25 days. No loss in body weight was observed. OTX015 induced a reduced growth of the lymphoma xenografts at each analyzed time point (days 7, 10, 14, 17, 21, and 25; Fig. 4B). At the end of the experiment (day 25), the median tumor volumes for the control and for the experimental arm were 600 mm³ (95% CI, 550–684) and 239 mm³ (95% CI, 0–582), respectively (\( P = 0.001 \)).

**OTX015 shows in vitro synergism with several anticancer agents**

We evaluated the combination of OTX015 with a series of conventional and targeted antilymphoma agents in a panel of five DLBCL cell lines (Fig. 5). Strong synergism was observed, in all the cell lines, when OTX015 was combined with the mTOR inhibitor everolimus (median CI, 0.11; range, 0.1–0.17) and, in ABC-cells, with the BTK inhibitor ibrutinib (CI, 0.04; 0.02–0.1). Synergism was also observed with OTX015 plus the PI3K-delta inhibitor idelalisib (CI, 0.5; 0.04–2.4), the class I and II HDACi vorinostat (CI, 0.5; 0.3–0.6), anti-CD20 mAb rituximab (CI, 0.5; 0.4–0.5), the hypomethylating agent decitabine (CI, 0.6; 0.6–0.7), the immunomodulant lenalidomide (CI, 0.7; 0.6–0.7), OTX015 combinations with the class I HDACi romidepsin (CI, 1.08; 1–1.22) and with the chemotherapy agents bendamustine (CI, 0.63; 0.1–3.97) and doxorubicin (CI, 0.83; 0.71–0.96) presented a moderate additive effect. GCB- and ABC-DLBCL cells showed a different sensitivity to the combinations: a stronger synergism was observed in ABC than in GCB DLBCL cells for ibrutinib (\( P < 0.0001 \)), for idelalisib (\( P < 0.0001 \)), lenalidomide (\( P = 0.0001 \)), and rituximab (\( P = 0.007 \)).

**Baseline gene expression profile is associated with response to OTX015**

To identify genes and pathways that might predict sensitivity to OTX015 in DLBCL we integrated the sensitivity data with the baseline gene expression profile in 14 cell lines with an IC₅₀ lower than 500 nmol/L and eight with a higher IC₅₀.

Transcripts positively associated with OTX015 sensitivity were significantly enriched of genes involved in IFN, IL6 and IL10 signaling genes, TLR and JAK/STAT signaling, STAT3 targets, genes involved in glucose metabolism, and hypoxia-regulated genes (Fig. 6A and Supplementary Table S4A). The leading edge genes (the top differentially ranked transcripts based on sensitivity to
OTX015 comprised STAT1, STAT3, STAT4, STAT6, IL6, JAK1, JAK2, TNF, IRAK1, TLR9, MYD88, TLR8, TRAF3, and AKT1.

Transcripts associated with lower sensitivity to OTX015 were significantly enriched of E2F target genes, genes involved in cell-cycle regulation, DNA repair, P53 signaling, chromatin structure, and apoptosis (Fig. 6B; Supplementary Table S4B). BCL2L1, HDAC1, HDAC2, HDAC5, HDAC8, CHEK1, CHEK2, TP53, ATM, BRCAl, CDKN1A, CDKN2A, BIRC5, MGMT were among the leading edge genes. MYC or MYC targets did not appear associated with sensitivity to OTX015.

**Discussion**

We have evaluated the activity and the mechanism of action of a new BET bromodomain inhibitor, OTX015, in preclinical models of mature B-cell lymphoid tumors. We have shown that: (i) OTX015 has *in vitro* and *in vivo* antiproliferative activity; (ii) OTX015 effect is largely cytostatic, with induction of apoptosis in only a genetically defined subset of cell lines; (iii) OTX015 inhibits MYC, NF-κB, TLR, and JAK/STAT pathways; (iv) OTX015 shows synergistic or additive effects when combined with several
Antitumor activity of OTX015 was validated in an in vivo model of ABC-DLBCL with demonstration of a reduced tumor growth. The mTOR is central to a signaling cascade leading to cell growth inhibition and apoptosis. OTX015 had an antiproliferative activity in the most of the lymphoma cell lines tested, with no differences in sensitivity among the histologic subtypes. The effect was largely cytostatic, with apoptosis limited to a few cell lines. OTX015 appeared to induce a senescence-like response in those cell lines. This was a remarkable achievement since MYC is a difficult therapeutic target and it plays a major role in the pathogenesis and progression of lymphomas, as exemplified by the very poor outcome of DLBCL cases over-expressing both MYC and BCL2 proteins and of "double hit" lymphomas usually bearing concomitant translocations of MYC and BCL2 oncogenes.

An important biologic effect we observed with OTX015, both in the in vitro and in vivo setting, was the downregulation of the NF-kB, TLR, and JAK/STAT3 signaling pathways, important in both the pathogenesis and the chemoresistance of lymphomas, particularly of ABC-DLBCL. This observation is in accordance with the strong suppression of the TLR signaling-mediated lipopolysaccharide-induced inflammatory response, reported with BET bromodomain inhibitors (37). A similar effect has been reported for JQ1 in different DLBCL cell lines (10). Additional observations indicated that the downregulation of the TLR and JAK/STAT3 signaling pathways is a relevant mechanism of action of OTX015 in DLBCL. First, high expression of genes involved in interferon, TLR and JAK/STAT signaling and STAT3 targets were highly associated with the sensitivity to the compound. Second, OTX015-induced apoptosis was limited to cell lines, bearing a functional TP53, derived from ABC-DLBCL with somatic mutations activating both BCR signaling and MYD88. Mutations activating the BCR and TLR/MYD88 signaling are common in ABC-DLBCL, in which they represent driver events (38), and their prevalence is especially high in two aggressive extranodal forms of DLBCL, primary central nervous system lymphoma and primary testis DLBCL (39, 40). CD79A and CD79B are upstream of BTK. BTK binds to MYD88, especially if the latter is encoded by the L265P somatic variant (41). OTX015 downregulated the expression of MYD88 and of additional members of the TLR pathway, and both OTX015 and JQ1 reduced the BRD4 binding to MYD88 upstream regulatory region. These events could inhibit both BCR and TLR signaling, particularly affecting cells dependent on these pathways. Similarly, the BTK inhibitor ibrutinib is more active in ABC-DLBCL cases with both CD79B/CD79A and MYD88 mutated genes (38, 42). Importantly, the combination of OTX015 and ibrutinib was strongly synergistic in ABC-DLBCL cells. The downregulation of IRF4 might contribute to the synergism, as reported for the combination of ibrutinib and lenalidomide (43). Notably, OTX015 was active also in ABC-DLBCL cells carrying somatic mutations of CARD11, coding for a protein downstream of BTK, and, accordingly, representing a marker of resistance to ibrutinib (38, 42).

Besides ibrutinib, additional antilymphoma agents presented an increased activity when combined with OTX015. The strongest synergism was obtained with the combination with the mTOR inhibitor everolimus at doses that can be reached in patients (44). The mTOR is central to a signaling cascade leading to cell growth and proliferation, and mTOR inhibitors are approved for treatment of relapsed MCL (45). OTX015 was also synergistic with the PI3K-delta inhibitor idelalisib, which has shown promising clinical responses in B-cell lymphomas (46), and with the lenalidomide, as also recently reported for another BET bromodomain inhibitor in MCL (47).

The synergism observed for OTX015 combined with clinically achievable doses of rituximab is reinforced by recent reports with another BET bromodomain inhibitor (13). The downregulation of CCDC86, coding for the nuclear factor cyclon, by both JQ1 (13) and OTX015, might represent the mechanism of action of the synergism. The combination appeared more active in ABC-DLBCL cell lines, maybe due the common targeting of the IL10 and STAT3 pathway by both OTX015 and rituximab (48).
OTX015 presented synergism with the demethylating agent decitabine and the HDACi vorinostat at concentrations pharmacologically achievable in clinical use (49, 50), in accordance with the similarities here observed at the level of gene expression signatures, and also with published data obtained with other BET bromodomain inhibitors (11). HDACs of both classes I and II were associated with a lower sensitivity to OTX015 as single agent and, indeed the synergism appeared stronger with the class I and IIa/b HDACi vorinostat than that seen with the class I HDACi romidepsin suggesting that the synergism might be class dependent.

The integration of baseline GEP and sensitivity in a large panel of DLBCL cell lines allowed the identification of functional pathways that might predict the response to the BET bromodomain inhibitor. The cells with the highest sensitivity to OTX015 had not only high expression levels of genes involved in the TLR/JAK/STAT pathway, but also of transcripts that code for proteins implicated in glucose metabolism and in hypoxia. Although the latter two features can be associated with an active MYC program, it is important to underline that we did not detect any association between expression of MYC or MYC targets and sensitivity to OTX015. On the basis of our data, it will be important to correlate the demonstration of an active TLR/JAK/STAT pathway (for example detecting pSTAT3) and/or the presence of somatic mutations of MYD88, CD79A/B, CARD11, and TP53 with the response to BET bromodomain inhibitors in the ongoing clinical trials.

A strong E2F gene expression signature was associated with lower sensitivity to OTX015, although not with a clear resistance to the compound as all our cell lines were much more sensitive to the BET bromodomain inhibitor than other large series of cancer cell lines comprising solid tumors models (5, 14, 15). In accordance with the cell-cycle arrest observed at 24 to 48 hours, E2F1 target genes, but not E2F1 itself, are downregulated by both OTX015 and JQ1 (10), at a later time point than the effect on MYC or on TLR/JAK/STAT pathways. The high synergism reported in leukemia preclinical models with the combination of the BET bromodomain inhibitor PFI-1 with a pan-aurora kinase inhibitor (12) suggests that the combination of BET bromodomain inhibitors with drugs targeting the cell cycle might overcome the observed lower sensitivity.

The gene expression signatures associated with a lower sensitivity to OTX015 comprised transcripts (BCL2L1, BIRC5, MGMT, CHEK1, CHEK2), which represent potential molecules to be inhibited in combination with BET bromodomain inhibitors. There are now different BET bromodomain inhibitors under development with some of them, including OTX015, already in phase 1 clinical studies. On the basis of our data of an OTX015 gene expression signature highly overlapping with that reported with JQ1, and on the literature (10, 12, 16, 51) all the molecules, so far all pan-BET bromodomain inhibitors, have similar preclinical activity data and mechanism of action. Alongside the different drug delivery modalities (for example, OTX015 and GS525762 are given orally, TEN-010 subcutaneously), the toxicity and pharmacokinetics data that from the ongoing clinical studies will be most relevant for the further clinical development of this class of compounds.

In conclusion, the BET bromodomain inhibitor OTX015 appears as a promising new antilymphoma agent with anti-proliferative activity in the vast majority of the examined preclinical models, capable of downregulating important signaling pathways and of synergizing with other anticancer molecules. Particular genetic lesions and gene expression signatures are associated with high sensitivity to OTX015 antitumor activity. Additional studies are needed to elucidate the mechanism of action of OTX015, particularly when combined with other targeted agents. Together with the early report of clinical responses in both leukemia and lymphoma patients treated with OTX015 in the absence of major toxicities (22), the data presented here provide the basis for further clinical investigation of OTX015 in combination therapies.
Disclosure of Potential Conflicts of Interest

P. Herait has ownership interest (including patents) in Oncoethix. F. Bertoni reports receiving commercial research grants from Oncoethix and is a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E. Gaudio, P. Bonetti, G. Inghirami, E. Zucca, F. Bertoni
Development of methodology: E. Gaudio, P. Bonetti, C. Tarantelli, G. Inghirami
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Rinaldi, M. Ponzoni, G. Stussi, G. Inghirami
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Boi, P. Bonetti, C. Tarantelli, G. Inghirami, E. Zucca, F. Bertoni
Writing, review, and/or revision of the manuscript: M. Boi, E. Gaudio, P. Bonetti, M. Ponzoni, A. Stathis, G. Stussi, M.E. Riveiro, G. Inghirami, E. Cvitkovic, E. Zucca, F. Bertoni
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Bernasconi, M. Testoni, P. Herait, F. Bertoni

Study supervision: M.E. Riveiro, G. Inghirami, E. Cvitkovic, F. Bertoni
Other (performed experiments): A.A. Mensah

Acknowledgments

The authors thank Diletta Di Mitri for her help in the evaluation of interleukins production.

Grant Support

This work was supported by research funds from Oncoethix (to F. Bertoni), Nelia et Amadeo Barletta Foundation (to F. Bertoni), and AIRC 5x1000 No. 10007 (to G. Inghirami).

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Received June 19, 2014; revised January 9, 2015; accepted January 19, 2015; published OnlineFirst January 26, 2015.

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Michela Boi, Eugenio Gaudio, Paola Bonetti, et al.

Clin Cancer Res  Published OnlineFirst January 26, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-1561

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/01/27/1078-0432.CCR-14-1561.DC1

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