OTX015, a novel pan BET-BRD inhibitor is active in non-small-cell lung cancer cell (NSCLC) lines bearing the fusion protein EML4–ALK

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BACKGROUND

Members of the bromodomain and extra terminal domain (BET) family of proteins (BRD2, BRD3, BRD4, and BRDT) function as important reader molecules that associate with acetylated histones and govern the assembly of chromatin complexes and transcription activators at specific promoter sites. Recently, it has been described that BET-inhibition is a promising therapeutic strategy for KRAS-mutant NSCLC with wild-type LKB1 (*Shimamura, Clin Cancer Res 2013*). The chimeric oncogene EML4-ALK identified a distinct entity of NSCLC exhibiting a high rate of therapeutic activity to ALK inhibitors, but most patients acquire resistance within a few months. We report the preclinical findings obtained with OTX015 (Fig 1), a novel oral pan-BET-BRD inhibitor in a panel of NSCLC cell lines harboring different oncogenic mutations, such as KRAS, MYC amplifications and the fusion protein EML4–ALK.

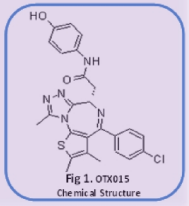

Fig 1. OTX015 Chemical Structure

Material and Methods

Five established NSCLC cell lines, HOP62, HOP92, A549 H2228, and H3122 harboring different oncogenic mutations for KRAS, LKB1, TP53 and ALK (Table 1) were selected to evaluate OTX015 in this diseases. Cell lines were exposed to increasing doses of OTX015 for 72 h (OncoEthix SA, Switzerland) and cell proliferation evaluated by MTT assays. GI50 and Emax values were calculated with the equation for sigmoidal dose response using Prism 5.00 for Windows. Results represent the mean ± 95%CI of at least 3 independent experiments performed in triplicate. Protein levels were analyzed by Western Blot using commercial antibodies. For cell cycle analysis cells were stained with PI and analyzed using a FACScan flow cytometer after 24h of treatment. RT- PCR was performed using Fast SYBR Green Master Mix on a StepOnePlus Real-Time PCR System. Cell cycle, apoptosis induction and qRT-PCR results represent the mean ± SD of at least 3 independent experiments, where *p<0.05 versus control cells (0.1% DMSO), employing Anova followed by Dunnett's Multiple Comparison Test. OTX015 combination studies were performed in cell lines exposed for 48 h to increasing doses of OTX015 alone or in combination with crizotinib. Drug combination effects were assessed using the Chou & Talalay method. Results represent the median and range of 2 independent experiments performed in triplicate.

RESULTS

OTX015 (**Fig. 1**) displays cytostatic effects in 4 out of 5 NSCLC cell lines, two of which harbor the fusion protein EML4-ALK+. In A549 cells, the concurrent mutations in KRAS and LKB1 genes abrogates OTX015 effects, as previously described for JQ1 an OTX015 analog (Shimamura, Clin Cancer Res 2013). In our cell line panel, we observed GI50 values between 0.1-0.7 µM after 72h-exposure to OTX015 (**Table 1**). OTX015 treatment also resulted in a reduction in the percentage of cells in the S phase (**Fig. 1**). Cell signaling pathway assessment showed that OTX015 induced a transient upregulation of active p-STAT3 with subsequent downregulation after 24h and for up to 72h exposure, this pathway being the key downstream effector of ALK, which is frequently upregulated in crizotinib-resistant cell lines (**Fig. 2B**). Additive effects were observed with the concomitant combination of OTX015 and crizotinib (median CI=0.98) in H2228 cells (**Fig. 2C**). The expression of BRD2/3/4, C-MYC, N-MYC, BCL-2 or P21 and CyclinD1 was characterized at the protein and mRNA levels in all cell lines. Both OTX015-sensitive and -resistant lines exhibited similar basal expression levels for all these proteins (**Fig. 3**). In OTX015-sensistive cell lines, we observed a rapid downregulation of C-MYC mRNA and protein levels in HOP92 cells (**Fig. 4A, 4C**) and in N-MYC mRNA levels in HOP62 and H3122 cells (**Fig. 4B**).

OTX015 is broadly active in EML4-ALK (+) and (-) NSCLC cells

Cell line	OTX015		Characterization of the mutational status of key proteins in NSCLC cell lines					
	GI50 [µM] (95%IC) 72h	Emax (at 6 µM)	EML4-ALK fusion protein	KRAS Exon2	LKB1 (*)	TP53 (*)	RB1 (*)	MYC Status (*)
HOP62	0.11 (0.08-0.17)	54	Negative	Heterozygous mutation c.34G>T	WT	Homozygous mutation c673-2A>G	WT	No MYC Amplification
HOP92	0.10 (0.06-0.16)	58	Negative	WT	WT	Homozygous mutation Substitution Missense C128G>T	WT	MYC Amplification
H2228	0.63 (0.42-0.95)	35	Positive Variant 3	WT	WT	Homozygous mutation 991C>T	Heterozygous deletion frameshit c610delG	NE
H3122	0.70 (0.52-0.93)	41	Positive Variant 1	NE	NE	NE	NE	NE
A549	>6	82	Negative	Heterozygous mutation c.34G>A	Mutation Substitution nonsense c.109 C>T	WT	WT	No MYC Amplification

Table 1. OTX015 anti-proliferative effects after 72h in NSCLC cell lines and characterization of common mutations found in NSCLC cells. Cell lines harboring the EML4-ALK fusion protein are sensitive to OTX015 (*) http://www.sanger.ac.uk/perl/genetics/CGP/cosmic. NE= no evaluated.

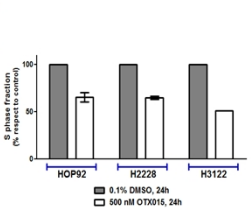

Fig 1. OTX015 reduced S phase cell fraction after 24 h exposure.

OTX015 induces ultimate downregulation of p-STAT3 and shows *in vitro* additive activity in combination with crizotinib in NSCLC-ALK+ cells

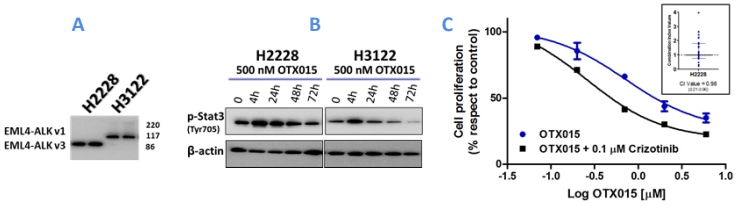

Fig 2. (A) EML4-ALK fusion proteins expression in H2228 and H3122 cells. (B) OTX015 induced a transient upregulation of p-STAT3 with subsequent downregulation after 24 h in NSCLC-ALK+ cell lines. (C) Additive effects were observed with the concomitant combination of OTX015 with crizotinib in H2228 cells. Insert. CI values of OTX015 combined with crizotinib, determined by the Chou-Talalay method.

OTX015 sensitivity is not correlated to basal levels of BRDs, C-MYC, N-MYC, BCL2 or P21 in NSCLC cells

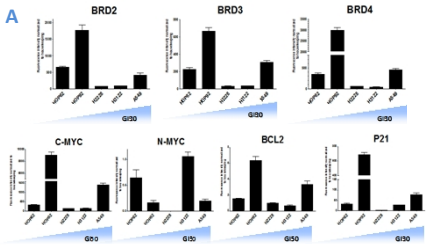

Fig 3. (A) BRDs, C-MYC, N-MYC, BCL2 P21 mRNA levels in NSCLC cells classified according to OTX015 sensitivity. (B) Basal protein expression of BRD4, -2, C-MYC, BCL2 and cyclin D1 in NSCLC cells. There was no correlation with basal mRNA or protein levels and OTX015 sensitivity.

In sensitive NSCLC models, OTX015 treatment results in a rapid and sustained downregulation of C-MYC or N-MYC

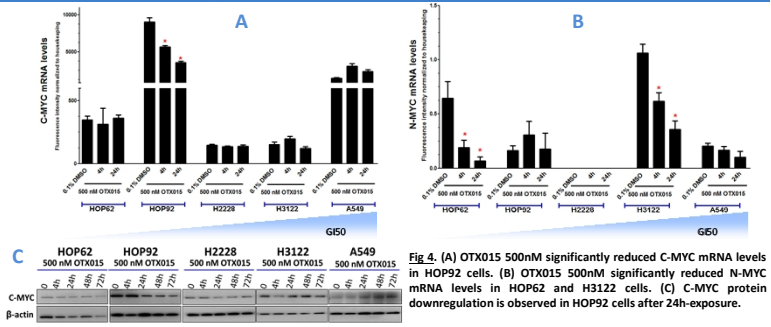

Fig 4. (A) OTX015 500nM significantly reduced C-MYC mRNA levels in HOP92 cells. (B) OTX015 500nM significantly reduced N-MYC mRNA levels in HOP62 and H3122 cells. (C) C-MYC protein downregulation is observed in HOP92 cells after 24h-exposure.

CONCLUSIONS

Our data indicates that OTX015 displays anti-proliferative effects in NSCLC cells, including the subset of NSCLC cells harboring the EML4-ALK fusion gene, supporting its further clinical development in NSCLC patients.

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