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BET-ting on Nrf2: How Nrf2 signaling can influence the therapeutic activities of BET protein inhibitors:

Nrf2 signaling, which is suppressed by BET proteins, can affect the treatment of inflammatory diseases and cancer with BET inhibitors

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Summary

BET proteins such as Brd3 and Brd4 are chromatin-associated factors, which control gene expression programs that promote inflammation and cancer. The Nrf2 transcription factor is a master regulator of genes that protect the organism against xenobiotic attack and oxidative stress. Nrf2 has demonstrated anti-inflammatory activity and can support cancer cell malignancy. This review describes the discovery, mechanism and biomedical implications of the regulatory interplay between Nrf2 and BET proteins. Both Nrf2 and BET proteins are established drug targets. Small molecules that either activate or suppress these proteins are currently tested in clinical trials. The crosstalk between Nrf2 and BET proteins may have important, and until now overlooked, implications for the therapeutic effects of these drugs. Based on the information covered in this review, it should be possible to design combinatorial treatment strategies for cancer and inflammatory diseases, which may improve the efficacy of targeting a Nrf2 or BET proteins individually.

Keywords

Oxidative stress; inflammation; cancer; Nrf2 signaling; BET proteins; COPD; AML

1. Introduction

Nrf2 is a master regulator of the oxidative stress response:

Oxidative stress damages biomolecules, causes or exacerbates different pathological conditions and contributes to age-associated functional decline. The Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) transcription factor is a principal regulator of antioxidant defenses and is important for health and longevity [1–3]. Nrf2 controls the expression of a

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Competing interests

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battery of antioxidant and detoxification genes and thereby protects cells and organisms from oxidative and chemical injury. In unstressed conditions, Nrf2 interacts with its cytoplasmic inhibitor Keap1 (Kelch-like ECH-associated protein 1), which targets it for proteasomal degradation. Oxidative stress prevents Keap1-mediated degradation of Nrf2, resulting in its nuclear translocation. In the nucleus, Nrf2 dimerizes with a small Maf (Musculoaponeurotic fibrosarcoma) protein, binds to 'Antioxidant Response Elements' (ARE) and induces protective gene expression programs [4, 5].

BET proteins inhibit Nrf2 signaling:

BET proteins are characterized by the presence of two bromodomains and an extra-terminal domain. The bromodomains can interact with acetylated lysine residues in histone and non-histone proteins. There are four BET protein-coding genes in mammals, *Brd2* (*Bromodomain-containing protein 2*), *Brd3*, *Brd4* and *BrdT* (*Bromodomain testis-specific protein*). They have complex functions in chromosome organization and in the control of gene expression [6–9]. Multiple studies have recently discovered that BET proteins can inhibit antioxidant Nrf2 signaling both in mammals and in *Drosophila* [10–12].

Chatterjee et al. identified *fs(1)h* (*female sterile (1) homeotic*), the only BET protein-coding gene in *Drosophila*, as a suppressor of Nrf2 signaling in a large-scale *Drosophila* cell-based dsRNA screen [12–14]. The *fs(1)h* locus encodes a 120 kD (Fs(1)h-S) and a 210 kD (Fs(1)h-L) peptide. The Fs(1)h-L protein physically interacts with CncC (Cap-n-collar isoform C), the ortholog of mammalian Nrf2 in *Drosophila* and thereby regulates oxidative stress responses. Moreover, it was shown that BET proteins inhibit Nrf2 signaling independently of Keap1 both in *Drosophila* and mammalian cells, and that combination of a BET inhibitor with a Keap1 inhibitor leads to a synergistic activation of Nrf2 target gene expression [12].

Similarly, BET proteins were reported to regulate Nrf2 target gene expression in primary human airway smooth muscle cells and a human monocytic cell line [10]. Moreover, Michaeloudes et al. showed that mammalian BET proteins physically interact with Nrf2 and that the BET protein inhibitor, JQ1, prevented H₂O₂-induced intracellular reactive oxygen species (ROS) production. Likewise, Hussong et al. discovered that the inhibition of the mammalian BET protein Brd4 induces Nrf2 signaling and protects against H₂O₂-induced cell death [11]. Therefore, BET proteins act as negative regulators of Nrf2 in multiple species, a finding that expands our understanding of the complex network that controls Nrf2 signaling.

More complexity was added to the Nrf2-BET protein interaction when Liu et al. reported that JQ1 can suppress nanaomycin-mediated induction of Nrf2 signaling in neuroblastoma cell lines. Consistent with this finding, JQ1 treatment makes these cells more susceptible to the toxic effect of nanaomycin therapy [15]. Interestingly, Wu et al. reported that the interaction between Brd4 and P53 is affected by the phosphorylation of Brd4 by CK2 and that it also influences P53-mediated target gene expression [16]. It will be important to investigate whether cell line-specific post-translational modifications affect Nrf2-BET protein interaction in neuroblastoma cell lines.

Nrf2 and BET proteins are implicated in multiple diseases:

Nrf2 signaling is compromised in different disease conditions including diabetes, neurodegenerative conditions and cancer, and therefore is an attractive drug target [1]. In addition to well-characterized compounds that increase Nrf2 activity by targeting Keap1, BET inhibitors may provide a separate route for activation of Nrf2 signaling as a treatment of these diseases. Consistent with that assumption, Liang et al. recently showed that treatment with JQ1 reverses the hyperglycemia-induced suppression of Nrf2 activity in the hippocampus. It also alleviates the morphological changes in neurons and the cognitive dysfunctions that are associated with diabetes [17]. The synergism between BET protein inhibitors and Keap1 inhibitors in the activation of Nrf2 signaling might be exploited to achieve stronger and more specific induction of Nrf2 signaling as an advantageous therapeutic approach.

BET proteins have been implicated in several cancers and inflammatory diseases [18]. The therapeutic potential of BET protein inhibitors for the treatment of these and other conditions is currently being examined in multiple pre-clinical and clinical studies [18–21]. The discovery of the regulation of Nrf2 by BET proteins adds a new perspective to the etiology and possible treatment of these diseases.

2. Can the BET/Nrf2 interaction be exploited in the treatment of inflammatory diseases?

BET proteins promote inflammation:

Multiple BET proteins have been implicated in inflammation. Brd4 acts as a co-activator of the transcription factor NF- κ B (Nuclear factor-kappa B) by interacting with an acetylated lysine residue on the RelA subunit to trigger the NF- κ B-mediated expression of pro-inflammatory genes such as IL1 (Interleukin 1), IL6 and TNF α (Tumor necrosis factor alpha) [22]. Similar to Brd4, Brd2 also localizes to the promoters of inflammatory cytokine genes in macrophages that are key players in the cellular immune response. Cytokine production during inflammation is suppressed by JQ1, a compound that inhibits BET proteins by preventing the binding of their bromodomains to acetylated lysines on histone and non-histone proteins [23, 24].

Nrf2 signaling protects against inflammation:

Nrf2 signaling has anti-inflammatory effects in various disease conditions such as inflammation-associated neurodegenerative diseases and lung inflammation [1, 25]. Mice lacking Nrf2 are hypersensitive to neuroinflammation caused by lipopolysaccharide (LPS). Sulforaphane, an Nrf2-inducing drug, protects wild type mice against LPS-induced neuroinflammation [26]. Nrf2^{-/-} mice are also more susceptible to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced neuroinflammation, a mouse model for Parkinson's disease [27], and to dextran sulfate sodium-induced colitis, a mouse model for inflammatory bowel disease [28].

It would be interesting to investigate the contribution of Nrf2 suppression to the pro-inflammatory effects of BET proteins in different diseases and to examine whether BET

protein inhibition coupled with a strong Nrf2 induction would yield better therapeutic outcomes in inflammatory diseases. Here we discuss how the BET/Nrf2 interaction might affect the treatment of chronic obstructive pulmonary disease (COPD) and multiple sclerosis (MS).

2.1. Nrf2 and Bet proteins affect the symptoms of chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is characterized by inflammation in the lung parenchyma and peripheral airways. This condition leads to progressive narrowing of airways and loss of lung parenchyma [29]. Oxidative stress caused by cigarette smoke or other pollutants plays a major role in the development of COPD. The pulmonary inflammation in COPD patients is resistant to corticosteroid treatment and there is a dearth of effective anti-inflammatory treatments for COPD [30].

BET proteins are implicated in COPD: Several lines of evidence indicate that BET proteins contribute to the chronic inflammation seen in COPD. Durham and colleagues showed that the expression of the secreted pro-inflammatory Wnt4 protein in bronchial epithelial cells (BEAS-2B) in response to oxidative stress (H_2O_2) is dependent on Brd4 [31]. Similarly, Khan and colleagues reported that Brd4 is essential for inflammation induced by combined treatment of an oxidative stressor (H_2O_2) and an inflammatory cytokine (IL-1 β) in human airway epithelial cells (NHBE) and in BEAS-2B cells [32].

Nrf2 signaling is suppressed in COPD: COPD is characterized by a suppression of Nrf2 signaling. Suzuki et al. showed Nrf2 expression to be suppressed in the pulmonary macrophages of COPD patients [33]. As a consequence, the expression of different antioxidant and detoxification genes regulated by Nrf2 such as HO-1 (Heme oxygenase 1), GPx2 (Glutathione peroxidase 2) and NQO1 (NAD(P)H quinone dehydrogenase 1) was decreased [34]. The resulting lack of antioxidant defenses exacerbates the damage caused by oxidative insults like cigarette smoke, air pollutants or hyperoxia [29]. On the other hand, pharmacological activation of Nrf2 has been shown to be beneficial in COPD [35, 36].

BET-Nrf2 interactions may aggravate COPD: The mechanism by which Nrf2 signaling is suppressed in COPD patients is poorly understood. It is worth examining whether Brd4-mediated inhibition of Nrf2 might make lung tissue more susceptible to oxidative stress and to pro-inflammatory stimuli. COPD patients are resistant to corticosteroids because they cannot recruit HDAC2 (Histone deacetylase 2) to suppress active inflammatory genes [37]. COPD causes levels of HDAC2 to decrease because phosphorylation after PI3K/Akt signaling targets it for degradation. Interestingly, inhibition of HDAC2 and the resulting increase in Nrf2 acetylation can suppress Nrf2-mediated target gene induction and antioxidant defense in COPD [38, 39]. It was recently shown that inhibition of CncC, the fly ortholog of Nrf2, by the Drosophila BET protein Fs(1)h depends on the acetylation of CncC [12]. It is, therefore, possible that reduced HDAC2 activity in COPD causes an increase in Nrf2 acetylation that renders it more susceptible to BET protein-mediated inhibition. This, in turn, would reduce its inducibility in response to oxidative stress, exacerbating the effect of oxidative stress and inflammation in COPD.

Based on these speculations, it would be interesting to test whether the combination of BET inhibitors with widely used Keap1 inhibitors, such as sulforaphane in the treatment of COPD, might yield beneficial results by suppressing inflammation and achieving stronger activation of Nrf2.

2.2. BET proteins and Nrf2 have opposite effects on Multiple sclerosis (MS)

Multiple sclerosis (MS) is a neurological disease characterized by demyelination of neurons in the central nervous system (CNS). The injury to myelin is caused by autoreactive T cells. Both CD4⁺-Th1 and CD4⁺-Th17 cells are implicated in this process. IL12, which is increased in demyelinating plaques, is required for Th1 differentiation [40]. IL23, on the other hand, drives the infiltration of Th17 cells [41]. The autoreactive T cells that invade the CNS secrete pro-inflammatory cytokines such as IFN γ (Interferon gamma), TNF α , IL17, and IL2 [42]. These cytokines either directly damage the myelin-producing oligodendrocytes or activate microglia and macrophages, which then break down myelin. In addition, CD8⁺ T cells can also injure myelin.

BET proteins contribute to multiple sclerosis: In addition to their role in the transcriptional regulation of inflammatory cytokines implicated in multiple sclerosis, BET proteins are critical for the differentiation and activation of CD4⁺-Th17 cells that are critically involved in the development of multiple sclerosis [41]. Brd2 and Brd4 associate with the IL17 locus in Th17 cells and regulate the expression of cytokines such as IL17, IL21 and GM-CSF (Granulocyte-macrophage colony-stimulating factor) in experimental autoimmune encephalitis (EAE), a mouse model for MS [43]. These results indicate that BET proteins may promote multiple aspects of MS pathogenesis and therefore, that BET inhibitors might be effective in its treatment.

Nrf2 activators are used in the treatment of multiple sclerosis: Multiple sclerosis is characterized by massive oxidative damage to brain tissue caused by reactive oxygen and nitrogen species (ROS and RNS) that are generated in response to inflammation. Nrf2 and its target genes are found to be induced in MS lesions [44]. Nrf2-deficient mice are more susceptible to experimental autoimmune encephalomyelitis and show higher expression of inflammatory cytokines, including TNF α and IL12, suggesting that Nrf2 suppresses inflammatory responses in MS [45]. In accordance with these observations, dimethyl fumarate (DMF), a compound that induces Nrf2 signaling by depleting glutathione, suppresses macrophage inflammation in EAE and attenuates the severity of the disease [46]. DMF, marketed under the brand name Tecfidera, has recently been approved by the FDA for the treatment of multiple sclerosis [47, 48].

Can we leverage the BET-Nrf2 interaction in the treatment of MS?—BET proteins are required for effective NF κ B-dependent inflammatory cytokine expression. In addition, they play a role in the differentiation of CD4⁺-Th17 cells, which are implicated in autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. Therefore, BET proteins are potential targets for the treatment of inflammatory diseases. Mele et al. have shown that inhibition of BET proteins by JQ1 prevented EAE by blocking Th17 differentiation [43]. I-BET151, a pan-BET protein inhibitor, suppresses the expression of

inflammatory cytokines in rheumatoid arthritis [49]. However, the lack of selectivity of current inhibitors for individual BET proteins and the broad-spectrum physiological functions of BET proteins increase the risk of undesirable side effects caused by the use of BET inhibitors.

Conventional Nrf2 activating drugs have reported off-target effects too [50, 51].

—DMF, the approved medication for MS, elicits side effects that include flushing, abdominal pain, diarrhea and vomiting. A combinatorial treatment with sub-maximal doses of a BET inhibitor and DMF might achieve optimal activation of Nrf2 and sufficient suppression of inflammation with fewer unintended side effects and therefore, may improve the quality of treatment for multiple sclerosis patients.

3. Can the BET/Nrf2 interaction be targeted for cancer treatment?

BET proteins are involved in different types of cancer: BET proteins contribute to different types of cancer by modulating the expression of oncogenic proteins such as cMyc and Bcl2 (B-cell lymphoma 2) [52, 53]. The Brd4-NUT (Nuclear protein in testis) fusion protein, which promotes tumor growth in NUT midline carcinoma, drives the expression of cMyc [54]. Inhibition of BET proteins with drugs like JQ1 downregulates cMyc expression and can be an effective therapeutic strategy in diverse cancers including multiple myeloma and Burkitt's lymphoma [55, 56]. Likewise, BET inhibitor I-BET726 suppresses the expression of N-MYC and Bcl2 to inhibit growth and induce cytotoxicity in neuroblastoma [57].

Nrf2 can promote the survival of cancer cells: Nrf2 signaling, on the other hand, has a dual role in cancer. Due to its cytoprotective activities, it can suppress chemical carcinogenesis in model organisms. However, cancer cells exploit the same cytoprotective properties of Nrf2 for protection against endogenous oxidative stress and against oxidative stress caused by cytostatic drugs or radiation therapy. DeNicola et al. showed that the expression of oncogenic alleles of Kras, Braf and cMyc in primary murine cells increases the expression of Nrf2 and reduces the intracellular levels of ROS [58]. Genetic mutations that disrupt Nrf2-Keap1 interaction and lead to a constitutive activation of Nrf2 signaling are found in >20 % of non-small-cell lung cancers (NSCLC). Interestingly, chemical inhibition of NROB1 (Nuclear Receptor Subfamily 0 Group B Member 1), which is selectively expressed in *keap1*-mutant NSCLC cells and drives their transcriptional output, prevents the anchorage-independent growth of these cells [59]. In addition, Nrf2 also promotes cell proliferation by channeling glucose and glutamate towards purine biosynthesis and by modulating the pentose phosphate pathway in cancer cells [60]. In this way, Nrf2 signaling, despite preventing at least certain types of cancer in normal and premalignant tissues, provides a growth and survival advantage to malignant cells [61, 62]. Surprisingly, two different groups of Nrf2 activators, namely DMF and oleanane triterpenoids (CDDO-imidazolide and CDDO-methyl ester), have been shown to exert opposite effects on the A/J model of lung cancer. Whereas CDDO-imidazolide and CDDO-methyl ester reduced the average number of tumors, DMF increased it [63]. Further studies are required to ascertain

whether this could be attributed to the differences in Nrf2-independent gene expression or to the expression of different subsets of Nrf2-dependent genes in response to these compounds.

BET protein inhibitors are already in clinical trials for the treatment of different types of cancer [18]. However, activation of Nrf2 in response to BET inhibitors might also protect the cancer cells thereby diminishing therapeutic benefit. It is, therefore, important to evaluate the contribution of Nrf2 signaling in different cancers where BET proteins play a key role and design the therapy accordingly. Below we discuss acute myeloid leukemia (AML) as a specific example to illustrate how induction of Nrf2 signaling might affect the efficacy of BET inhibitors.

3.1. Both Nrf2 and BET proteins are implicated in acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is characterized by the rapid proliferation of abnormal white blood cells that affects the production of normal blood. It is caused by a wide spectrum of genetic mutations that include chromosomal translocations such as Runx1-ETO (Runt-related transcription factor 1 and Eighty-two-one) and PML-RAR α (Promyelocytic leukemia and Retinoic acid receptor alpha), deletions, for example of Asx11 (Additional sex combs like 1), and point mutations as seen in JAK (Janus kinase), Ras and IDH1 (Isocitrate dehydrogenase 1) [64]. Multiple combinations of such mutations block maturation of the progenitor cells and cause expansion of malignant myeloblasts [65].

BET proteins play an important role in AML: BET proteins have been implicated in different subtypes of AML. Using an RNAi screen, Zuber et al. discovered Brd4 to be important for certain AML subtypes [66]. These authors also showed that the effect of Brd4 on AML maintenance is, at least partially, mediated by increasing the expression of cMyc. Strikingly, Zuber et al. found that the BET inhibitor JQ1 is effective in suppressing a number of AML subtypes, indicating a broad and critical role for Brd4 in at least those subtypes of AML. Dawson et al. found that a core group of genes including *cMyc*, *Bcl2* and *IRF8* (*Interferon regulatory factor 8*) that have roles in myelopoiesis are downregulated by the BET inhibitor I-BET in multiple AML subtypes [67]. A number of these genes are controlled by ‘super-enhancers’, which are sensitive to Brd4 inhibition. In this context, the Brd4 function is inhibited by Nucleophosmin 1 (*NPM1*), which is encoded by one of the most commonly mutated genes in AML. The recognition of BET inhibitors as promising drugs for the treatment of AML has resulted in several ongoing clinical trials [19, 20, 68].

Nrf2 protects AML cells from oxidative stress: Nrf2 signaling is induced in AML cells and provides protection against oxidative stress and chemotherapy. Rushworth et al. showed that AML cells are less sensitive to the proteasome inhibitor bortezomib, which causes cytotoxicity by elevating the intracellular ROS concentration, due to an increased basal level of nuclear Nrf2 [69]. The same authors also found that NF- κ B drives the expression of Nrf2 in AML cells and that long-term microRNA-mediated knockdown of Nrf2 improved the responsiveness of AML cells to chemotherapy [70]. In addition, Nishimoto et al. showed that Nrf2 protects AML cells against cytotoxicity caused by arsenic trioxide (ATO) [71]. All-trans retinoic acid (ATRA) has been reported to suppress Nrf2 signaling through the activation of RAR α [72]. Valenzuela et al. reported that combinatorial

treatment with ATRA and ATO enhanced the cytotoxicity in AML cells [73]. Therapeutic suppression of Nrf2, therefore, may be an effective way of increasing the efficiency of chemotherapeutic drugs in AML.

How BET-Nrf2 interactions might affect AML treatment: Resistance to BET inhibitors has been reported in AML. Rathert et al. found that the suppression of the PRC2 (Polycomb repressive complex 2) complex, which restores the expression of cMyc, confers resistance to a BET inhibitor in an AML mouse model [74]. On the other hand, Jang et al. found that the resistance to the BET inhibitor JQ1 in AML stem cells is caused by AMPK (AMP-activated protein kinase)-ULK1 (Unc-51 like autophagy activating kinase)-mediated autophagy [75]. Similarly, use of BET protein inhibitors in the treatment of AML might inadvertently boost Nrf2 signaling and in this manner, reduce therapeutic efficacy. Simultaneous application of a BET protein inhibitor and an Nrf2 inhibitor might potentially improve outcomes.

ATRA is an effective medication for one AML subtype, acute promyelocytic leukemia (APL), which is characterized by the PML-RAR α fusion that suppresses retinoic acid signaling and blocks cellular differentiation. However, ATRA has little effect on other AML sub-types [76]. Incidentally, recent studies have found that simultaneous application of BET inhibitors and ATRA, an Nrf2 signaling inhibitor, is more cytotoxic to cMyc dysregulated AML cell lines than the application of these drugs individually [77, 78]. It would be interesting to study whether inhibition of Nrf2 signaling by ATRA plays a role in the improvement of the efficacy of BET inhibitors in the treatment of AML.

4. Conclusions and Outlook

BET proteins have been implicated in multiple inflammatory disorders as well as in cancer. BET protein inhibitors, therefore, have been recognized as rational drug candidates for the treatment of these diseases. Oxidative stress plays a major role in the etiology of inflammation and cancer and offers a target for therapeutic intervention. ROS act as signaling molecules during inflammation and also cause injury to the inflamed tissue. Both of these functions may contribute to cancer progression. Increased ROS load can lead to DNA damage, potentially causing carcinogenic mutations. On the other hand, many chemotherapeutic drugs exert their cytotoxicity by increasing ROS levels. Nrf2 protects cancer cells from these chemotherapeutic drugs by inducing a battery of antioxidant and detoxification genes. Nrf2 also rewires the metabolism of cancerous cells to suit their altered metabolic demands. The inhibitory function of BET proteins on Nrf2, therefore, may be a double-edged sword with complex effects on inflammatory diseases and cancer (Fig. 1).

On the other hand, the activation of Nrf2 by BET protein inhibitors would conceivably contribute to their anti-inflammatory effect. Moreover, combining BET inhibitors and traditional Nrf2 activators such as sulforaphane and DMF, that act by disrupting the interaction between Nrf2 and Keap1, can synergistically activate Nrf2 [12]. Such combinatorial treatment regimens would conceivably achieve a strong and specific activation of Nrf2 even with submaximal doses of BET inhibitors and Keap1 inhibitors and therefore, might minimize potential harmful side effects. Conversely, activation of Nrf2 by BET

inhibitors might render cancer cells more tolerant to cytotoxicity and thereby reduce the efficacy of the treatment. In this case, it might be prudent to combine BET inhibitors with Nrf2 inhibitors like ATRA.

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Figure 1. The BET-Nrf2 regulatory network as a target for combination drug treatment.

(A) BET proteins are implicated in cancer and inflammation. BET proteins inhibit Nrf2, the master regulator of the cellular oxidative stress response. Nrf2 signaling has anti-inflammatory effects but can also promote survival of cancer cells. (B) Combination of Keap1 inhibitors and BET protein inhibitors for the treatment of inflammatory pathologies. Synergistic activation of Nrf2 activity suppresses oxidative stress and inflammation while the suppression of BET proteins prevents their proinflammatory effects. (C) Combination of BET protein inhibitors and Nrf2 inhibitors for the treatment of cancer. BET inhibitors cause a loss of tumor-promoting functions of BET proteins while Nrf2 inhibitors prevent the Nrf2-activating “side effect” of BET protein suppression that would otherwise protect cancer cells from oxidative stress due to high metabolic rates or cancer therapeutics.