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Review

The emerging role of BET inhibitors in breast cancer

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ABSTRACT

Bromodomain and extraterminal domain (BET) proteins are epigenetic molecules that regulate the expression of multiple genes involved in carcinogenesis. Breast cancer is an heterogenous disease emerging from aberrant gene expression and epigenetic alteration patterns. Amplification or overexpression of BET proteins has been identified in breast tumors highlighting their clinical significance. Development of BET inhibitors that disrupt BET protein binding to acetylated lysine residues of chromatin and suppress transcription of various oncogenes has shown promising results in breast cancer cells and xenograft models. Currently, Phase I/II clinical trials explore safety and efficacy of BET inhibitors in solid tumors and breast cancer. Treatment-emergent toxicities have been reported, including thrombocytopenia and gastrointestinal disorders. Preliminary results demonstrated greater response rates to BET inhibitors in combination with already approved anticancer agents. Consistently, BET inhibition sensitized breast tumors to chemotherapy drugs, hormone therapy and PI3K inhibitors in vitro. This article aims to review all existing preclinical and clinical evidence regarding BET inhibitors in breast cancer.

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1. Introduction

During carcinogenesis, extensive epigenetic modifications occur, including aberrant acetylation and methylation patterns. These alterations result in dysregulated gene expression and abnormal cell proliferation. Acetylation of histone lysine residues is one of the most essential post-translational processes, that regulates chromatin structure so that it is accessible to DNA and RNA polymerases as well as transcription factors [1]. Lysine acetylation is regulated by the antagonistic action of two types of enzymes, histone acetylases (HATs), that serve as writers of acetylation marks by transferring acetyl groups to the ε-amino group on lysine residues of histones and histone deacetylases (HDACs), the erasers that remove them. Abnormal expression of HDACs has been reported in a broad range of tumors and thus HDAC inhibitors (HDIs) have been developed [2–4]. A third family of proteins, bromodomain and extraterminal domain (BET) proteins contribute to the reading of histone acetylation through recognizing acetylated lysine residues [5]. BET family consists of four proteins, namely BRD2, BRD3, BRD4 and the testis-specific BRDT, which share a common domain architecture of two N-terminal bromodomains (BD1 and BD2), critical for their chromatin binding, and a C-terminal domain [6]. BET proteins accumulate to super-enhancers (SEs) of oncogenes and disease-associated genes and initiate their expression. Super-enhancers are regions of the genome composed of multiple enhancers collectively bound by transcription factors and coactivators, being at higher concentrations in overexpressed genes. BET proteins localize to super-enhancers and act as scaffolds to recruit other proteins and transcription factors [7]. Therefore, BET inhibitors determine the expression of key oncogenes, such as MYC, BCL2, BCL6, PAX5, CDK4 and CDK6 [8]. BRD4 recruits positive transcription elongation factor (PTEF-b) to sites of active transcription and activates it by phosphorylating its CDK9 kinase component [9]. RNA polymerase II is in turn activated via phosphorylation of Ser2 of its carboxy-terminal domain (CTD) by PTEF-b. Therefore, BRD4 is responsible for the initiation of transcription via indirectly activating RNA PoLII. Apart from association with PTEF-b, BRD4 extraterminal domain interacts with other proteins, such as ATAD5, histone methyltransferase NSD3, histone demethylase JMJD6 and CHD4 [10]. BRD2 regulates the expression of E2F-dependent genes that drive cell cycle and differentiation, without interacting with PTEF-b [11]. In addition to this, BRD2 contains a kinase-like domain between the two bromodomains that exhibits mitogen-activated nuclear kinase activity [12]. Finally, BRD3 binds via its BD1 bromodomain to acetylated GATA1 transcription factor and regulates transcription via the E2F–Rb pathway [13,14].

The catalytic role of BET proteins in transcription led to the development of small-molecule inhibitors, BET inhibitors (BETis) that target their functions [6]. JQ1 is the most thoroughly studied BETi that binds competitively to acetylated lysines and thus displaces BRD4 from binding to chromatin [15]. A number of selective and pan-BET inhibitors have been developed since then, including I-BET 762, OTX- 015, ABBV-075, I-BET 151 among others [16–19]. BET inhibitors downregulate the expression of genes important for cell proliferation, such as MYC, BCL2, CDK6 [19–21], induce **G1** cycle arrest and limit tumor self-renewal capacity via interfering with WNT singalling pathway [22]. In addition, BET inhibitors downregulate the carbonic anhydrase 9 (CA9) hypoxia-responsive gene and thus prevent the hypoxia-induced upregulation of genes involved in angiogenesis [23]. Antitumor effects of BET inhibition are quite complex including many different pathways and key genes involved in tumorigenesis, offering a promising field of study. BRD2, BRD3 and BRD4 are expressed in breast cancer, while BRDT is rarely expressed. Regarding different breast cancer subtypes, BRD2 and BRD4 are amplified or overexpressed in about 12.1% and 17% of basal-like breast cancers respectively [24]. Moreover, BRD4 was identified as one of the key genes associated with estrogen-induced breast cancer [25]. These data indicate that BET proteins are upregulated in breast cancer and thus could be potentially targeted.

A series of preclinical and clinical studies are trying to clarify the function and efficacy of BET inhibitors in breast cancer. Preclinical evidence demonstrates a significant potential of BET inhibitors in breast cancer, even in the more aggressive entity of triple negative subtype, which accounts for 15–20% of breast tumors. Inhibition of interacting signaling pathways via synchronous downregulation of multiple genes appeals as a promising therapeutic approach to ER-positive and triple negative breast cancer subgroups. Currently there is no extensive research of BETi efficacy in HER2-positive tumors. In one study, BETi treatment of HER2(+) cell lines proved to be of no benefit, at least at the dose administered [26]. Although luminal HER2(+) cell lines demonstrated sensitivity to BRD4 depletion, many of them retained resistance to BET inhibition [27]. Another study reported some efficacy of BET inhibitors in HER2(+) cell lines [28]. Further studies are needed to address BET inhibition in HER2(+) breast cancer. This paper aims to summarize all preclinical and clinical results regarding BET inhibition in breast cancer.

2. Mechanism of action

2.1. ER (+) breast cancer

Focusing on hormone positive breast cancer, several studies have shown that JQ1 inhibited E2-mediated growth of well-characterized estrogen receptor positive (ER+) breast cancer cell lines in a dose-dependent manner [29–34]. JQ1 treatment resulted in suppression of MYC oncogene expression [21,29,32,35]. Other downregulated genes linked to breast cancer include Breast Carcinoma-Amplified Sequence 1 (BCAS1) genes and PDZ Domain-Containing 1 (PDZK1) [33]. JQ1 reversibly downregulated the expression of three E2-regulated genes, pS2, GREB1 and XBP1 [31]. As already established, estradiol (E2) signaling regulates the elongation activity by activating the suspended RNA PolII, which remains bound at E2-regulated gene promoters [36]. JQ1 blocked RNA PolII advancement from promoters to the coding region and thus the transcription elongation process [29]. Depletion of BRD4 disrupted the E2-dependent transcription, mimicking JQ1 activity [31]. BRD4 accumulation in genome is correlated with active transcription, as indicated by an increase in histone marks H3K27ac and H3K4me3 and RNAPolII. BRD3 and BRD4 synergize with WHSC1 to regulate **estrogen receptor A (ERa)** expression, a gene that encodes histone-lysine N-methyltransferase NSD2 that methylates H3K36, a key event for transcription elongation [10]. BRD3 and BRD4 complex binds to acetylated histone and serves as a scaffold for WHSC1 to access **estrogen receptor gene (ESR1)** promoter. Subsequently, WHSC1 methylates K36 on histone H3 so as to activate the transcription of ESR1, while estrogen binding to ERa stimulates the expression of WHSC1, creating a vicious cycle [32]. JQ1 treatment of tamoxifen resistant cells inhibited the recruitment of WHSC1 and BRD3 and BRD4 to ESR1 promoter, attenuating the transcription of E2-induced genes. As a result, JQ1 efficiently inhibited proliferation of tamoxifen-resistant ER + cell lines as well as that of four long-term estrogen deprived lines (mimicking aromatase inhibitor resistance), mainly by inducing prolonged G1 cell cycle arrest [32]. The effectiveness of BET inhibitors to tamoxifen resistant cells is equivalent to their effect in castration resistant

prostate cancer (CRPC) [37]. Moreover, abundant BRD4 occupancy to MYC results in upregulation of MYC-related genes and drives resistance to mTOR inhibition [35] BET inhibition reverses this BRD4-mediated resistance, sensitizing breast cells to mTOR inhibitors [35] Furthermore, BRD3 is enriched at active ESR1 enhancers leading to significantly higher levels of transcription and E2 responsiveness [30]. Overall, high expression of BRDs seems to correlate with poor overall survival in ER + breast cancer [30]. Increased BRD3 and BRD4 mRNA was associated with a reduced survival in ER + **invasive lobular carcinoma (ILC)** lines [38]. Providing in vivo evidence, JQ1-injected mice exhibited substantial decrease in uterine growth as a sign of reduced estrogen signaling [31]. JQ1 treatment in luminal B breast cancer mouse models not only inhibited tumor growth but also prevented the onset of disease when administered when tumor was undetected [33].

2.2. Triple negative breast cancer (TNBC)

Most basal-like and claudin-low tumors belong to TNBC, with basal-like tumors representing the main subtype [39]. JQ1 inhibited growth of TNBC lines either of the basal-like (HCC1143, MDA-MB-468, HCC70) or of the claudin-low (MDA-MB-231, BT549, HCC38) population in several studies [23,34,40–45]. The same effect was confirmed in vivo in murine models [23,40,43,44]. JQ1 induces cell cycle arrest at G1 phase, as indicated by the elevated p21, p27 and cyclin D1, D3 levels [43,46]. Polyploidy is an early indicator of mitotic catastrophe, a result of abnormal mitosis. Mitotic catastrophe is followed by apoptosis or senescence, a permanent state of cell cycle arrest [47]. Upon JQ1 treatment, cell lines were condemned to either undergo apoptosis or senescence, as indicated by the p21 increase and senescence-associated β-galactosidase (SA-βgal) staining in TNBC cell lines [40,42]. In an attempt to force cells to apoptosis, the factors that define cell fate were thoroughly studied. The mRNA and protein levels of pro-apoptotic and anti-apoptotic members of BCL-2 family were analyzed and only BCL-xL remained highly expressed after BET inhibition in senescence undergoing cell lines [48]. In consistence with these findings, either BCL-xL knockdown or combination of JQ1 and Obatoclax, an inhibitor of Bcl-2 family of proteins, reduced cell viability and increased the number of apoptotic cells [48]. BCL-xL overexpression reduced apoptosis in cells normally apoptotic to BET inhibition, providing a mechanism of intrinsic resistance [48]. Unlike ER + cell lines, MYC response to BET inhibition was variable [27,34,40]. However, JQ1 attenuated the expression of many transcription factors, as Forkhead box M1 (FOXM1), Lim domain only 4 (LM04) and DEP domain containing 1 (DEPDC) [43]. More importantly, LIN9 is one of the key targets of BET inhibition [49]. LIN9 is a mitosis-regulating transcription factor and subunit of MuvB complex, which interacts with B-MYB during S phase and FOXM1 during G phase resulting in the expression of genes required for the completion of mitosis [50]. LIN9 is amplified or overexpressed in 66% of basal-like tumors [24]. Four transcription factors correlated to mitotic catastrophe (FOXM1, E2F8, LIN9, MYBL2) were suppressed by JQ1 treatment via disrupting BRD4 binding to promoter regions. However, LIN9 appears to be the most profound since knockdown of either FOXM1 or MYBL2 did not produce the same effect [49]. Along with LIN9, FoxM1 is a transcription factor involved in DNA repair and cell cycle transition in S and M phase [51,52]. FoxM1 is also significantly downregulated by BETi treatment in basal-like models, resulting in a robust suppression of FoxM1 downstream genes, including AURKB, PLK1, CCNB1 and CCNB2 [53]. Aurora kinase A (AURKA) is a kinase required for correct duplication and separation during mitosis, while aurora kinase B (AURKB) catalyzes the attachment of the mitotic spindle to the centromere, both correlated to tumorigenesis. BET inhibitors dissociate BRD4 from AURKA and AURKB promoters and suppress their expression [40]. An AURKB inhibitor, AZD1152, phenocopied the effects of BET inhibition in TNBC cells, inducing polyploidy followed by apoptosis or senescence. An AURKA inhibitor, MLN8237 (Alisertib) also exhibited the same antiproliferative activity with JQ1 in TNBC cells [40,46]. Another kinase substantially upregulated in basal-like TNBC cells is polo-like kinase 1, which is involved in G2/M transition. Combination treatment with JQ1 and Volasertib, a polo-like kinase inhibitor produced a synergistic activity in TNBC cell lines [46]. Moreover, JQ1 disrupts Twist and BRD4 interaction and Twist/BRD4/P-TEFb/RNA-PolII complex formation at the WNT5 promoter [41]. Twist is a transcriptional factor associated with mesoderm formation and epithelial-mesenchymal transition (EMT) [54]. Twist interacts with BD2 domain of BRD4 and together with RNA-PolII and P-TEFb catalyze the transcription of WNT5 gene. Twist cooperates with Snail transcription factor, which is also regulated by BRD4, to keep EMT activated, a key characteristic of basal-like breast cancer [49]. In the same context, BRD4 is essential for basal epithelial phenotype by promoting the expression of epithelial-specific genes, such as TP63 gene and GRHL3, a transcription factor of the Grainyhead-like family [55]. In addition to this, numerous EMT-related genes contain BRD4-enriched enhancers [55]. These data highlight the function of BRD4 as a regulator of basal-like cell growth through interaction with transcription factors, like FOXO1 [55,56]. Another effect of BET inhibition in TNBC is the establishment of a “BRCA-mutated phenotype” which augments sensitivity to platinum-based therapy and PARP inhibitors [57]. BET inhibition interferes with BRD4 binding to BRCA1 and RAD51 promoters, downregulating their expression and inducing BRCAness in BRCA1 wild-type TNBC cells [57,58]. Homologous recombination (HR) efficiency and formation of single-stranded DNA by BRCA1 upon DNA damage are both impaired by BETi treatment [58] Considering that BRCA1/2 mutated breast cancer patients demonstrated a better overall response rate to carboplatin in clinical trials, BETi treatment could synergize with platinum therapy [59]. Not only BET inhibition leads to a BRCA-mutant like phenotype, but also BRCA1 mutation sensitizes TNBC tumors to BET inhibitors [60]. Epigenetic drug screening defined BET inhibitors as synthetic lethal drugs in a BRCA1-silenced TNBC cell line, along with aurora kinase inhibitors and histone deacetylase inhibitors [60]. BETi treatment downregulated many mitochondria-related genes associated with oxidative phosphorylation and upregulated thioredoxin-interacting protein (TXNIP), causing a significant increase of reactive oxygen species (ROS) levels in BRCA1-deficient cells. Consequently, BET inhibition induced synthetic lethality in BRCA1-deficient TNBC cell lines and murine xenograft models [60]. As already established in prostate cancer, JQ1 disrupted interaction of BRD4 and androgen receptor (AR), providing an effect that could be exploited in AR + TNBC breast cancer treatment [37]. Indeed, JQ1 demonstrated a dose-dependent antitumor efficacy in AR + TNBC cell lines and xenograft model, which was further enhanced by addition of enzalutamide in vitro but not in vivo [40,61]. JQ1 did not directly downregulate AR expression, but rather inhibited interactions between ATAD2, a co-activator of AR, with BRD4 and AR complex, suppressing the transcription of AR-induced genes [61]. Finally, BET inhibition disrupted hypoxia response and angiogenesis in triple negative breast cancer via downregulation of hypoxia-induced genes [23]. JQ1 altered the expression of multiple hypoxia-induced genes, including hypoxia-inducible factor (HIF) genes, carbonic anhydrase 9 (CA9) and vascular endothelial growth factor A (VEGF-A). In vivo xenograft models exhibited reduced expression of the hypoxia-associated factors and decreased angiogenesis as indicated by lower blood vessel marker CD31 levels [23]. As far as metastatic ability is concerned, JQ1 reduced migration of two TNBC

cell lines via impairing Jagged/Notch1 signaling pathway [62]. Jagged 1 is a transmembrane protein which binds to Notch1/3 receptors and activates them, resulting in the translocation of Notch intracellular domain to the nucleus [63]. JAG1 has also an established role in initiation and promotion of oncogenesis, including epithelial–mesenchymal transition (EMT), metastasis, and resistance to therapy in several cancer types [64]. BRD4 regulates JAG1 transcription, the gene encoding Jagged1 protein, and thus defines the migration and invasion process [62]. Indeed, a meta-analysis of 664 BC patients associated higher BRD4 and JAG1 levels with shorter time to metastatic development [62].

3. Combination treatment and resistance

3.1. Chemotherapy agents

BET inhibition efficiently downregulated BRCA1 and RAD51 inducing a BRCA-mutated phenotype in TNBC cells [57,58]. Clinical trials demonstrated an increased response to carboplatin in BRCA1/2 mutated TNBC population (41). In vitro, JQ1 offered an additive effect to platinum-based treatment in TNBC BRCA wild-type cell lines [43,57,58]. In addition to this, JQ1 combination with docetaxel or vinorelbine also exhibited a synergistic effect [43].

3.2. PARP inhibitors

BET inhibition increased sensitivity of BRCA wild-type TNBC cells to treatment with Olaparib both in vitro and in vivo via inducing a BRCA mutant-like phenotype [57,58].

3.3. Tamoxifen

JQ1 attenuated proliferation of tamoxifen-resistant ER + cells at a greater extent comparing to parental cells, by reducing BRD3/4 recruitment to ERa promoter [32]. In addition to this, ENST00000456526 is a long noncoding RNA, namely LOL (lncRNA of luminal), which is overexpressed in luminal breast cancer [65]. ER + tamoxifen-resistant cells demonstrate a higher LOL expression compared to parental cells. LOL downregulation provided sensitivity to tamoxifen treatment in tamoxifen-resistant breast cancer cells and xenograft models (71). Given that LOL constitutes an enhancer-associated lncRNA with great BRD4 occupancy, BET inhibition reversed tamoxifen resistance in ER + cells by reducing LOL expression (71).

3.4. MTOR inhibitors (everolimus)

There is evidence that resistance to everolimus is mediated by MYC upregulation as BRD4 recruitment to MYC tends to be higher in everolimus resistant cell lines [27,35]. Therefore, BET inhibition re-sensitized resistant cells to mTOR inhibitors [35] Combination treatment with everolimus and JQ1 exhibited greater efficacy both in vitro and in vivo compared to either monotherapy. In another study, OTX015, a novel BET inhibitor also synergized with everolimus in vitro and in xenograft models [44]. Finally, mTOR inhibitors rapamycin and Torin increased sensitivity to BET inhibition in JQ1-resistant cells [27].

3.5. Fulvestrant

JQ1 and fulvestrant cotreatment inhibited tumor growth in a tamoxifen-resistant murine xenograft model [32].

3.6. PI3K inhibitors

Resistance to PI3K inhibition is often mediated by feedback activation of alternative tyrosine kinase receptors (RTKs), like AKT and mTOR pathways, despite the initial response [66,67]. This feedback rebound effect can be blocked by BET inhibition, which efficiently dissociates BRD4 from transcriptional sites of multiple RTKs [34]. Combination treatment of PI3K-resistant cells with BET and PI3K inhibitors re-sensitized cells to PI3K inhibition, by inhibiting the reactivation of PI3K/AKT pathway and the re-expression of RTK proteins, such as INSR, IGF1R, HER2 and HER3 (57). These results were further confirmed in vivo [34].

3.7. MEK inhibitors (trametinib)

Similarly to PI3K inhibition, MEK inhibition is often bypassed by rebound upregulation of tyrosine kinase receptors (RTKs), like FGFR2, KIT, IGF1R and DDR1 via de novo formation of BRD4-enriched enhancers [68] BET inhibition synergized with trametinib in growth inhibition of trametinib-sensitive and trametinib resistant breast cancer cell lines [68]. In vivo, I-BET151 BET inhibitor provided an additive effect to trametinib treatment in xenograft models [68].

3.8. HDAC inhibitors

HDACs play a major role in the reversion of chromatin acetylation and, thus, gene expression. HDAC inhibitors attenuate abnormal acetylation and can reactivate the expression of tumor suppressors [69] Combination treatment of ER+ and TNBC cell lines with JQ1 and HDAC inhibitors, **valproic acid (VPA)** and mocetinostat was more effective than either monotherapy therapy, especially in TNBC cell lines [70]. This effect was mainly mediated by upregulation of USP17 deubiquitinating family, which inhibits the activity of Ras/MAPK pathway.

3.9. Lapatinib

FOXO family, a subclass of Forkhead transcription factors, are well-known tumor suppressors. Activation of PI3K/AKT signaling pathway drives FOXO phosphorylation by AKT kinase, resulting in FOXO exclusion from the nucleus and repression of transcriptional activity [56]. Lapatinib treatment progressively increased MYC via suppressing AKT-mediated FOXO phosphorylation [26] This FOXO-mediated MYC overexpression provided HER2+ cells with increased resistance to lapatinib. BET inhibition increased sensitivity to lapatinib in HER2+ cell lines and HER2+ xenograft models by decreasing lapatinib-induced MYC upregulation without affecting FOXO levels [26]. Moreover, we previously described a mechanism of resistance to inhibition of several kinases, like PI3K/AKT, MEK, mTOR and ERBB2/ERBB3 via adaptive upregulation of alternative kinase pathways. Lapatinib treatment gradually increased the expression of many of these kinases conferring resistance to treated cells [28]. This resistance mechanism was blocked by BET inhibition through dissociation of BRD4 and thus RNA PolII from lapatinib-induced kinase genes. Concomitant treatment with lapatinib and BET inhibitors eliminated growth of lapatinib-resistant cells [28].

3.10. AKT inhibitors

As previously described, FOXO phosphorylation by AKT kinase induces its inactivation and migration to cytoplasm [56]. On the other hand, inhibition of AKT suppresses FOXO3a phosphorylation, allowing its nuclear translocation and its interaction with BD2

domain of BRD4. FOXO3a and BRD4 complex catalyze CDK6 expression, an oncogenic kinase that regulates cell cycle [71]. Synchronous AKT and BET inhibition proved to be more efficient than either monotherapy by concomitant blocking of both PI3K/AKT and BRD4/FOXO3a/CDK6 pathways [71]. In vivo experiments in mice confirmed synergistic activity of BET and AKT inhibitors [71]. Furthermore, BET proteins control AKT3 expression, which is upregulated in AKTi-resistant cells [72]. BET inhibitors could potentially restore sensitivity to AKT inhibitors by suppressing AKT3 [72].

3.11. Immune checkpoint inhibitors

BET inhibitors impaired BRD4 localization at the Cd274 promoter, the gene that encodes the immunoregulatory transmembrane protein PD-L1, suppressing its surface expression in both tumor and immune cells [73,74]. JQ1 offered an additive in vitro and in vivo effect to anti-PD1 antibody in lymphoma-bearing mice, whereas either monotherapy was only partially effective [73]. The efficacy of combination treatment of BET and immune checkpoint inhibitors was studied in a mathematical model based on anti-CTLA4 (ipilimumab) that can also be expanded to anti-PD1 and anti-PDL1 molecules [75].

3.12. Antimitotic estradiol Analogue (ESE-15-ol)

ESE-15-ol is a microtubule-targeting agent, which interferes with normal formation of mitotic spindle and thus leads to mitotic arrest [76]. ITH-47 BRD4-selective inhibitor showed synergistic activity with ESE-15-ol in TNBC cells [77].

3.13. Baz2/Brd9 bromodomain inhibitor (GSK2801)

BAZ2A/B bromodomain proteins play a major role in chromatin remodeling and regulation of non-coding RNAs, while BRD9 is a component of SWI/SNF chromatin remodeling complex that alters chromatin structure via changing DNA-histone contacts [78]. Combination treatment with GSK2801 and JQ1 exhibited increased efficacy in a series of TNBC cell lines, by causing BRD2 dissociation from promoter and enhancer regions additionally to JQ-1-induced BRD4 loss [45].

BETi resistance is not mediated by MDR1 transporter or increased drug efflux, as verapamil MDR1 inhibitors did not resensitize resistant cells to BETis. However, alternative mechanisms of resistance to BET inhibitors have been recognized in breast cancer. PIK3CA mutation is associated with resistance to BET inhibition [27]. A66, a PIK3CA inhibitor increased susceptibility to JQ1 treatment [27]. Moreover, sustained expression of BCL-xL anti-apoptotic protein may provide both TNBC and ILC cells with resistance to BET inhibitors [38,48]. ABT737, an anti-BCL-xL molecule sensitized cells to JQ1 and enhanced its activity [48]. ABT-263, a BH3 mimetic which inhibits BCL-2, BCL-xL and BCL-w, rendered ILC BETi resistant cells sensitive to JQ1 and demonstrated a synergistic activity with JQ1 [38]. Another mechanism of BETi resistance is the inactivation of the PP2A phosphatase tumor suppressor gene, which results in hyperphosphorylation of BRD4 and increased binding of BRD4 to MED1 [42]. Phenothiazine (PTZ) functions as an activator of PP2A enzymatic activity, causing dephosphorylation of BRD4. Combination treatment with JQ1 and PTZ reversed resistance to BET inhibition [42]. FGFR1,2,3 and 4 (**fibroblast** growth factor receptors) were significantly upregulated by JQ1 treatment in ILC resistant cell lines, indicating another possible mechanism of resistance [38]. FGFR1 is amplified in both ILC and **invasive ductal carcinoma (IDC)** cell lines intrinsically resistant to BET inhibitors. Indeed, treatment with FGFR1 inhibitor PD173074 and JQ1 enhanced cytotoxic effect in ILC and IDC resistant cell lines [38]. Voltage-dependent anion channels (VDAC) are located in the mitochondrial outer membrane and function as gatekeepers for the entry and exit of metabolites, ions, reactive oxygen species (ROS) between mitochondria and cytosol [79]. VDAC is also a key player in mitochondria-mediated apoptosis. VDAC1, a VDAC family member, is overexpressed in breast cancer compared to normal breast tissue and is downregulated by JQ1 in a BRD4-mediated manner [80]. Silence of VDAC1 in breast cancer cell lines increased sensitivity to JQ1 treatment [80]. Other previously reported mechanisms of BETi resistance include suppression of PCR2 complex [81], the activation of 5' AMP-activated protein kinase (AMPK)/Unc-51 like autophagy activating kinase 1 (ULK1) pathway [82,83], the upregulation of Wnt/β-catenin pathway [84], all described in leukemia cells as well as the increase of GLI2 of the Sonic hedgehog pathway in pancreatic cell lines [85]. However, these mechanisms of BETi resistance are not yet described in breast cancer.

4. Clinical data

There are several ongoing studies assessing the clinical profile of BET inhibitors in solid and hematological malignancies. In this paper, we are focusing on the trials of BET inhibitors in breast cancer or in solid tumors including breast cancer patients (Table 1).

OTX015/MK-8628 (Birabresib) is a triazolothienodiazepine selective inhibitor of BRD2, BRD3, BRD4 which has exhibited efficacy in hematological malignancies [86,87] and solid tumors [88,89] Moreover, OTX015 exhibited antitumor activity in different triple negative breast cancer cell lines as a single agent or in combination with everolimus [44]. Concomitant treatment with OTX015 and everolimus was effective in TNBC xenograft models and also more potent than either monotherapy or paclitaxel. A Dose Finding Study of MK-8628 was designed to determine the recommended dose in participants with NUT midline carcinoma (NMC), triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC) or castration-resistant prostate cancer (CRPC) (NCT02698176) [90]. The study was prematurely terminated due to limited efficacy and not because of safety reasons. Thirteen patients were enrolled, including nine CRPC patients, three NMC patients and one triple negative breast cancer patient. Participants did not complete the study because of progressive disease ($n = 8$), clinical progression ($n = 3$) or adverse event ($n = 2$). Stable disease was achieved in six patients, but none of the participants achieved an objective response. Another Phase 1, multicenter, dose-finding study of MK-8628 was conducted to determine the recommended dose and administration schedule for Phase II studies and the clinical efficacy in different solid tumors (NCT02259114) [91]. The recommended phase II dose selected was 80 mg once daily, however there were no breast cancer patients enrolled.

Mivebresib (ABBV-075) is a selective BET bromodomain inhibitor that causes G1 cycle arrest and apoptosis in several cancer cell lines by affecting the intrinsic apoptotic pathway [92]. ABBV-075 exhibited a higher affinity to BRD2, BRD4 and BRDT bromodomains comparing to BRD3 bromodomains and efficiently downregulated MYC expression. Furthermore, ABBV-075 showed a broad antitumor activity with a greater efficacy in hematologic cell lines [92]. A Phase I study was initiated to evaluate the pharmacokinetic and safety of ABBV-075 in advanced solid and hematologic malignancies, including breast cancer (NCT02391480) [93]. Focusing on solid tumor patients, a total of seventy-two patients (14% uveal melanoma; 11% colorectal; 11% breast; 8% pancreatic; 7% head/neck; 49% others) were initially enrolled in the dose-escalation and another twelve patients with prostate cancer in the dose-expansion cohort [94]. Adverse events related to ABBV-075 were reported in the majority of participants (88%) with dysgeusia (49%),

Table 1
Clinical trials of BET inhibitors in breast cancer or solid tumors including breast cancer subjects.

Study	Phase	Drug	Design	Status	Results
NCT02698176	I	MK-8628 (Birabresib)	NUT midline carcinoma, TNBC, NSCLC, CRPC	Terminated (due to limited efficacy)	0% (0/13) objective response 46% (6/13) stable disease [90]
NCT02391480	I	ABBV-075 (Mivebresib)	Advanced solid tumors and hematological malignancies (NSCLC, BC, SCLC, Prostate cancer, AML, MM, Non Hodgkin lymphoma)	Completed	26/61 (43%) stable disease 35/61 (57%) progressive disease [93,94]
NCT02711137	I/II	INCB057643 Gemcitabine Paclitaxel Rucaparib Abiraterone Ruxolitinib Azacitidine	Advanced solid tumors and hematologic malignancies. (CRPC, BC, HGSC, CRC, Glioblastoma multiforme, Ewing sarcoma, Pancreatic adenocarcinoma, AML, MDS)	Terminated (due to safety issues)	6/134 (4%) objective response (2 CR/ 4 PR) 27/134 (20%) stable disease 41/134 (30%) progressive disease [97,98]
NCT02431260	I/II	INCB054329	Advanced solid tumors and hematologic malignancies. (CRPC, BC, HGSC, CRC, Ewing sarcoma, Pancreatic adenocarcinoma, AML, MDS, MF, MM)	Terminated (due to PK variability)	0/69 (0%) objective response 21/69 (30%) stable disease 30/69 (43%) progressive disease [98,100]
NCT02964507	I/II	GSK525762 (Molibresib) + Fulvestrant	HR(+)/HER2(-) advanced breast cancer	Ongoing (not recruiting)	No results [102]
NCT01587703	I/II	GSK525762 (Molibresib)	NUT midline carcinoma, SCLC, CRPC, TNBC, ER(+) BC, GIST	Completed	5/196 (2%) objective response (CR/PR) [103]
NCT02630251	I	GSK2820151	Advanced solid tumors	Terminated (due to development of GSK525762)	No results [104]
NCT03292172	I	RO6870810 Atezolizumab	Advanced Ovarian Cancer, TNBC	Terminated (due to portfolio priorization)	No results [105]
NCT01987362	I	RO6870810	Advanced Solid Tumors	Completed	No results [106]
NCT02392611	I	GS-5829 (Alobresib) Exemestane Fulvestrant	Advanced solid tumors and lymphomas HR (+) Breast cancer	Completed	No results [108]
NCT02983604	I/II	GS-5829 (Alobresib) Exemestane Fulvestrant	HR (+)/HER2(-) Advanced Breast cancer	Terminated	Has results [109] (No patients were enrolled in Phase II study)
NCT02683395	I	PLX51107	Advanced solid tumors and hematological malignancies	Terminated (business decision)	9/36 (25%) stable disease [111,112]
NCT02419417	I/II	BMS-986158 Nivolumab	Advanced solid tumors (TNBC, SCLC, serous ovarian cancer BRCA1/2 wt) and hematological malignancies	Recruiting	DLT: gr3/4 thrombocytopenia [114,115]
NCT03901469	II	ZEN003694 Talazoparib	Triple Negative Breast Cancer (TNBC)	Recruiting	No results [116]
NCT03035591	I/II	ODM-207	Advanced Solid tumors	Completed	No results [118]
NCT02369029	I	BAY1238097	Advanced solid tumors and hematological malignancies	Terminated	DLTs: gr3 vomiting, gr3 headache, gr2/3 back pain 8/8 (100%) TEAEs 2/8 (25%) stable disease 2/8 (25%) progressive disease [124]

thrombocytopenia (48%), **gastrointestinal [GI]** disorders (nausea [25%], diarrhea [21%]), fatigue (26%), decreased appetite (24%) and anemia (18%) ranking as the most frequent ones. Serious treatment-related adverse were reported in 10% of patients, with thrombocytopenia being the most common. Participants were initially administered Mivebresib on a daily schedule, but the DLT of reversible thrombocytopenia imposed a switch to intermittent schedules. Regarding clinical efficacy, 26 of 61 evaluable patients exhibited stable disease (43%) regardless of the dosing schedule, whereas 35 exhibited disease progression (57%). Median progression-free survival was 1.8 months. Finally, pharmacokinetics of mivebresib include a half-life of 16.1–19.9 h and a clearance of 4.94 L/h, which is quite equal to birabresib but lower than molibresib [94].

INCB057643 is a BET inhibitor effective in AML, MM, DLBCL and CRPC xenograft models [95,96]. A Phase I/II dose escalation-expansion study was designed to explore the safety and maximum tolerated dose of INCB057643 in advanced malignancies (Part 1,2) and the combination of INCB057643 and standard-of-care (SOC) agents (gemcitabine, paclitaxel, rucaparib e.g.) in selected advanced solid tumors and hematological malignancies, including breast cancer (Part 3,4) (NCT02711137) [97]. However, the study was terminated due to safety reasons. As of September 2018, 134 patients were enrolled and administered INCB057643 at doses from 8 to 16 mg once daily continuously every three or four weeks [98]. Thirteen of 134 patients received the combination treatment. Median duration of treatment was 50.5 days and the main reason for treatment discontinuation was disease progression in 57% of patients. Of 134 patients receiving INCB057643, 6 patients achieved objective response, with two patients exhibiting complete response (follicular lymphoma and relapsed AML), 4 patients exhibiting partial response (three patients with follicular lymphoma and one breast cancer patient in combination treatment with paclitaxel) and 27 patients achieved stable disease, 6 of whom remained on SD for more than six months [98]. On the other hand, 41 patients had disease progression. Overall, treatment related AEs of grade 3 and 4 were reported in 36% of patients and serious TRAEs in 13% of patients. The majority of patients experienced TRAE of any grade (115/134; 86%). Overall, most frequent grade 3 or 4 TRAEs were thrombocytopenia (18%), anemia (10%), hyperglycemia (3%), dehydration (2%), diarrhea, vomiting and nausea (2% each), increased INR (2%) hyponatremia (2%) and syncope (2%). The most frequent serious TRAE was thrombocytopenia. Pharmacokinetic assessment revealed a linear relationship between AUC and thrombocytopenia induced. In addition to this, fed state reduced the time to maximum plasma concentration (Tmax) from 6 to 2 h, creating the need of administration in a fasted state.

INCB054329 has shown similar antitumor activity to INCB057643 in hematologic malignancies and CRPC [95,96]. INCB054329 has also been studied in triple negative breast cancer cell lines [99]. A Phase I/II dose-escalation and safety study of INCB054329 was initiated in subjects with advanced malignancies including breast cancer, but it was then terminated due to pharmacokinetic variability (NCT02431260) [100]. As of April 2018, 69 patients were treated with INCB054329 at daily doses of 15–30 mg given once daily or 15–25 mg given twice daily in continuous or intermittent dosing schedules. Twentyone of 69 patients (30%) had stable disease, of whom four remained on SD for more than 6 months [98]. Specifically, one patient with breast cancer remained on SD for two years. Thirty patients (43%) reported progressive disease. INCB054329 exhibited a faster absorption and clearance rate and a shorter half-life than INCB057643. However, treatment with INCB054329 was characterized by high interpatient variability in drug clearance of the same drug doses [98]. 54 patients were enrolled in the dose-escalation part, divided in eleven cohorts of different dosing schedules. Two dose-limiting toxicities were reported, both grade 4 thrombocytopenia at 30 mg QD and 22.5 mg BID, while no DLT or grade 4 AE was reported at 20 mg BID. Pharmacologically active dose was the 20 mg twice daily, however the recommended phase II dose could not be determined because of the high interpatient variability mentioned before. In order to overcome this limitation, a dose titration cohort in part 2 was developed to individualize the recommended dose in each patient starting from 20 mg twice daily. However, the dose of 20 mg twice daily was not tolerated as eight patients experienced TRAEs (thrombocytopenia [n = 4], anemia [n = 3], bilirubin increase [n = 1], epistaxis [n = 1], fatigue [n = 1], AST increase [n = 1]). In terms of safety, 54 patients experienced TRAEs of any grade (78%) and seven patients experienced a serious TRAE, including thrombocytopenia (n = 4) and anemia (n = 20).

GSK525762 (Molibresib) is a pan-BET inhibitor that has exhibited potent antitumor activity both in vitro and in preclinical breast mouse models [101]. Molibresib is currently being tested in combination with fulvestrant in hormone receptor positive HR (+)/HER2-negative advanced or metastatic breast cancer. (NCT02964507) [102]. This is a Phase I/II dose escalation and expansion study, still recruiting, expected to be completed by February 2021. However, no results are published yet. NCT01587703 is a Phase I study evaluating the safety and pharmacokinetics of GSK525762 in subjects with NUT Midline Carcinoma and other solid tumors, including breast cancer [103]. This phase I/II multicenter study enrolled 196 participants in total, including 40 breast cancer patients. In the dose expansion cohort, patients were administered **with** GSK525762 at 75 mg once daily dose. Severe adverse events were reported in 54% of the patients (106/196 patients), most commonly thrombocytopenia (n = 44), anemia (n = 13) and gastrointestinal disorders [nausea (n = 10), vomiting (n = 10), abdominal pain (n = 3), diarrhea (n = 2)]. Other frequent severe AEs included asthenia (n = 5) and fatigue (n = 4) and a decrease in coagulation factor VII (n = 6) [103]. However, despite the high incidence of decreased platelet and factor VII reported, events of GI hemorrhage, hemoptysis or other bleeding conditions were quite low. Non-serious adverse events (AEs) were reported in the majority of patients (98%), including anemia (41%), thrombocytopenia (50%), hyperbilirubinemia (29%) and gastrointestinal disorders. GSK2820151, is a structurally differentiated selective BET inhibitor that exerted efficacy in preclinical models. A dose escalation Phase I trial was conducted to investigate the safety and clinical activity of GSK2820151 in solid tumors (NCT02630251) [104]. The study was then terminated due to the development of GSK525762, which offered a better insight into safety and efficacy profile.

Another Phase I Study was designed to assess the combination of R06870810 BET inhibitor and Atezolizumab anti-PD-L1 antibody in advanced ovarian and triple negative breast cancer (NCT03292172) [105]. The trial started in November 2017 but was then terminated due to portfolio priorization. The same BET inhibitor R06870810 is explored in the two-part, Phase I trial with a dose escalation cohort in solid tumors and a dose expansion cohort in selected malignancies (NCT01987362) [106]. Study results are still pending.

Alobresib (GS-5829) is a BET inhibitor with preclinical antiproliferative activity in hematological malignancies as well as uterine serous carcinoma cell lines overexpressing c-Myc [107]. A Phase I Study was designed to evaluate GS-5829 as monotherapy in advanced solid tumors or in combination with Exemestane or Fulvestrant in ER (+) breast cancer (NCT02392611) [108]. The trial consists of three arms, a dose escalation group A, a treatment group B with breast cancer patients receiving combination treatment with GS-5829 and either exemestane or fulvestrant and a

lymphoma expansion group C in non-hodgkin lymphoma patients. The study has been completed, however results are not yet published. Another Phase I/II exploring GS-5829 combination with fulvestrant or exemestane in advanced ER (+), HER2(-) breast cancer was initiated (NCT02983604) [109]. The study was terminated prior to the dose expansion part enrollment, so no data was collected for this endpoint. The Phase 1 dose escalation part enrolled 14 participants in four arms, arm A receiving alobresib in combination with exemestane and arms B, C, D receiving fulvestrant and alobresib at different doses. From 13 participants administered at least one drug dose, only one in twelve evaluable patients experienced a drug limiting toxicity. Serious AEs were reported in three of 13 participants (23%), including asthenia and dehydration in one patient and dysphagia and hypercalcemia in the other two ones. All of the participants experienced a non-serious adverse event, mainly consisting of GI disorders (diarrhea [n = 7], nausea [n = 8]), fatigue (n = 6) and dysgeusia (n = 3) [109].

PLX 51107 is a novel selective BRD4 BET inhibitor which demonstrated preclinical antileukemic activity. There are two ongoing studies of PLX 51107, one in hematologic malignancies (NCT04022785) and another one in hematologic malignancies and solid tumors, including breast cancer (NCT02683395) [110,111]. This is a Phase Ib/IIa, two-part dose escalation and expansion study of PLX 51107, composed of two arms in solid tumor patients (arm A) and hematologic malignancies (arm B). The study was terminated due to business decision, however preliminary results were published. Thirtysix patients with advanced solid tumors (uveal melanoma [n = 11], sarcoma [n = 6], breast cancer [n = 2], NSCLC [n = 2], CRPC [n = 2]) received PLX 51107 at 20, 120 and 160 daily doses. Eight patients exhibited confirmed stable disease for 4–14 months and one uveal melanoma patient remained on stable disease for 14 months [112]. Three treatment related serious AEs were reported (gr3 nausea, gr2 vomiting and gr2 kidney injury). Drug limiting toxicities consisted of grade 3 nausea, grade 3 thrombocytopenia and grade 2 kidney injury. Other treatment related AEs were mostly of lower grade and included fatigue, GI disorders, bilirubin and INR increase.

BMS-986158 is another potent BET inhibitor which caused >70% tumor inhibition in patient derived xenograft models (triple negative breast cancer, lung and colorectal) [113]. A Phase I/IIa trial is ongoing to assess BMS-986158 as monotherapy or in combination with nivolumab in selected advanced solid tumors (TNBC, SCLC, serous ovarian cancer BRCA1/2 wild type) and hematological malignancies (NCT02419417) [114]. The trial is still recruiting, however preliminary results have been published. As of March 2018, 68 patients received BMS-986158 at doses ranging from 0.75 to 4.5 mg once daily in three dosing schedules: five consecutive days every week (arm A), 14 consecutive days every three weeks (arm B) and seven consecutive days every three weeks (arm C). 43% of patients were heavily pretreated with four or more previous treatments [115]. Pharmacokinetic assessment demonstrated a fast absorption (Tmax: 2–4 h), a dose–dependent AUC and a prolonged half-life of 33–82 h. Grade 3/4 thrombocytopenia was the dose limiting toxicity reported. Treatment related AEs occurred in 63% of patients but grade 3/4 ones only in 22% of them. Most frequent AEs were mild diarrhea, thrombocytopenia and fatigue, all expected [115]. More results are anticipated.

There are several other BET inhibitors currently under clinical investigation in solid tumor and breast cancer subjects. ZEN003694 BET inhibitor has shown efficacy in castration-resistant prostate cancer and triple negative breast cancer xenograft models. A Phase II study of ZEN003694 and a PARP inhibitor, Talazoparib in triple negative breast cancer is currently ongoing (NCT03901469) [116]. ODM-207 is a novel BET inhibitor with significant activity against HR-positive breast cancer. ODM-207 inhibited the growth of ER + breast cancer cell lines and patient derived xenografts by altering MYC, estrogen, CDK4 and Cyclin D1 gene expression as a single agent but also in combination with palbocilib [117]. These results provide strong evidence of its potential role in breast cancer treatment. ODM-207 is currently under investigation in a Phase I/II multicenter study in advanced solid tumors (NCT03035591) [118]. BI 894999 is a novel potent, selective BET inhibitor with a greater affinity to BRD4-BD2 bromodomain and an established efficacy in AML cell lines [119]. BI 894999 is currently evaluated in a Phase Ia/b dose-escalation and expansion study in advanced malignancies (NCT02516553) [120]. However, the trial does not include breast cancer patients therefore it is no further analyzed. BAY 1238097 is a BET inhibitor with greater affinity to BRD4 than BRD2 or BRD3, which has shown a potent anti-lymphoma activity though down-regulation of MYC and E2F1 genes and impairment of NFKB and JAK/STAT pathways [121]. Furthermore, there is evidence of a synergistic activity between mTOR inhibitors and BAY 1238097, a property that could be exploited in breast cancer [121]. BAY 1238097 demonstrated preclinical activity in other solid tumors, including KRAS-mutated pancreatic ductal adenocarcinoma, KRAS-mutated NSCLC as well as melanoma [122,123]. A Phase I Dose Escalation Study was conducted to determine the maximum tolerated dose and safety profile of BAY 1238097 in advanced malignancies, other than breast cancer (NCT02369029) [124]. The study was prematurely terminated due to unexpected toxicity. All patients treated with BAY 1238097 discontinued from treatment due to either disease progression or adverse events [124]. One patient died from bilateral ischemic stroke not drug-related while two patients experienced DLTs including grade 3 headache and vomiting and Grade 2 nausea and back pain in doses below therapeutic threshold.

5. Conclusion & future perspectives

BET inhibitors initiate a new era of epigenetic drugs in breast cancer treatment. Clinical data from Phase I/II studies of BET inhibitors are gradually becoming available. Although BET inhibitors exhibited a potent antitumor activity in breast cancer cell lines and murine xenograft models, their toxicity profile should be carefully managed. Thrombocytopenia was the main dose limiting toxicity reported in clinical trials. Gastrointestinal disorders, anemia and fatigue constitute treatment-related adverse events most commonly found [94,103,115,125,126] These toxicities indicate a need for development of well tolerated BET inhibitors that will maximize clinical efficacy without exceeding the toxicity threshold.

Apart from BET inhibitors already described, current technological advances led to the development of alternative BET inhibitor molecules. Nanoparticles containing JQ1 inhibitor and a polymeric compound as a carrier achieved higher plasma stability, overcoming limitations due to JQ1 short half-life [127]. These JQ1-loaded nanoparticles exerted activity in TNBC both in vitro and in vivo. Novel technologies led to the development of BET-PROTACS, proteolysis targeting chimeric molecules that bind BRD4 and cause its proteasome-mediated degradation [128]. ARV-825 and MZ1 are PROTACs that recruit BRD4 to E3 ubiquitin ligase, promoting its rapid degredation. ARV-825 is based on OTX-015 compound, whereas MZ1 on JQ1. Both PROTACS attenuated cell growth in JQ1-sensitive and resistant TNBC cell lines and murine models by inducing G2/M cycle arrest [128]. Another BET degrader, BETd-246 effectively decreased BRD2, BRD3 and BRD4 protein levels and induced apoptosis in TNBC cell lines [129]. Collectively, extensive interest in BET inhibition has led to the development of unconventional agents that yield BET protein suppression via indirect pathways.

BET inhibitors displayed a cytostatic rather than a cytotoxic

activity by inducing G1 cell cycle arrest [43,46]. This property could potentially be related to lower response rates comparing to existing cytotoxic drugs. However, BET inhibition augmented cytotoxic activity of a variety of agents currently in use. BET inhibitors efficiently sensitized breast cancer cell lines and murine models to PI3K, MEK and MTOR inhibitors and enhanced tumor growth inhibition. This synergism is reported in other solid tumors as well. BET inhibitors provided an additive effect to MEK inhibitor Trametinib in ovarian and colorectal cancer cell lines via synchronously blocking MAPK pathway [130–132] There is strong evidence that concomitant anti-androgen treatment with enzalutamide and BET inhibition increases efficacy in castration resistant prostate cancer [133]. In-human studies were designed to explore this notion. Apart from studies already described in breast cancer, a Phase I study is evaluating synergism of Molibresib (GSK525762) with an HDAC inhibitor, Entinostant in solid tumor or lymphoma patients (NCT03925428) [134]. Another study was designed to explore Molibresib and Trametinib combination in RAS-mutated solid tumor patients, including SCLC, colorectal cancer, NSCLC and pancreatic adenocarcinoma (NCT03266159) [135] Therefore, BET inhibitors should be exploited to enhance response to already existing chemotherapy and molecular therapies.

Overall, BET inhibitors constitute a promising field of clinical search in hematologic malignancies and solid tumors. Breast cancer heterogeneity creates a therapeutic challenge that needs to be addressed by new therapeutic options. Results from ongoing studies remain to prove if BET inhibition can serve this purpose.

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Declaration of competing interest

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