Selective inhibitors of bromodomain BD1 and BD2 of BET proteins modulate radiation-induced profibrotic fibroblast responses

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Abstract

Radiotherapy can induce various adverse effects including fibrosis in cancer patients. Radiation-induced aberrant expression of profibrotic genes has been associated with dysregulated epigenetic mechanisms. Pan-BET (bromodomain and extraterminal domain) inhibitors, such as JQ1 and I-BET151, have been reported to attenuate the profibrotic response after irradiation. Despite their profound preclinical efficacy, the clinical utility of pan-inhibitors is limited due to observed cytotoxicities. Recently, inhibitors were developed that selectively target the first (BD1) and second (BD2) bromodomain of the BET proteins (iBET-BD1 [GSK778] and iBET-BD2 [GSK046]). Here, their potential to attenuate radiation-induced fibroblast activation with low-toxicity was investigated. Our results indicated that cell proliferation and cell cycle progression in fibroblasts from BJ cells and six donors were reduced when treated with I-BET151 and iBET-BD1, but not with iBET-BD2. After irradiation, induction of DGKA and profibrotic markers, especially COL1A1 and ACTA2, was attenuated with all BET inhibitors. H3K27ac enrichment was similar at the DGKA enhancer region after I-BET151 treatment and irradiation, but was reduced at the COL1A1 transcription start site and the ACTA2 enhancer site. iBET-BD2 did not change H3K27ac levels in these regions. BRD4 occupancy at these regions was not altered by any of the compounds. Cell migration activity was measured as a characteristic independent of extracellular matrix production and was unchanged in fibroblasts after irradiation and

Abbreviations: ACT, antibody-guided chromatin tagmentation; ACTA2, smooth muscle α-actin; BD, bromodomain; BET, bromodomain- and extraterminal domain; ChIP-qPCR, chromatin immunoprecipitation and real-time polymerase chain reaction; COL1A1, collagen 1a1; COL3A1, collagen 3a1; DGKA, diacylglycerol kinase alpha; DMR, differentially methylated region; ECM, extracellular matrix; EGR1, early growth response 1; ELISA, enzyme-linked Immunosorbent assay; H3K27ac, histone H3 lysine 27 acetylation; IR, ionizing radiation; NF-κB, nuclear factor κB; NHDFs, normal human dermal fibroblasts; Promo, promoter; RTK, receptor tyrosine kinase; TSS, transcription start site.
BET inhibitor-treatment. In conclusion, iBET-BD2 efficiently suppressed radiation-induced expression of DGKA and profibrotic markers without showing cytotoxicity. Thus BD2-selective targeting is a promising new therapeutic avenue for further investigations to prevent or attenuate radiotherapy-induced fibrosis.

KEYWORDS
BET, fibroblast activation, radiation, selective bromodomain inhibitors

What’s new?
Radiation therapy for cancer can cause fibrosis by disrupting epigenetic control mechanisms that activate pro-fibrotic genes. Inhibitors that broadly target the bromodomain and extra-terminal (BET) domain family can combat this activity, but aren’t clinically useful due to high toxicity. Here, the authors tested bromodomain-selective inhibitors and found that iBET-BD2, which targets the second bromodomain of BET proteins, lessens the activation of pro-fibrotic genes with only minor cytotoxicity. This inhibitor could be a promising option for reducing fibrosis in cancer survivors.

INTRODUCTION
Radiation-induced fibrosis is a common late side-effect of radiotherapy in cancer patients. Around 20% of breast cancer patients and 68% of head and neck cancer survivors suffer from subcutaneous fibrosis after radiotherapy. Radiation-induced cell loss and tissue damage initiates regeneration and wound healing processes which, if not deactivated in time, may cause fibrosis. Transdifferentiation of fibroblasts to myofibroblasts and perpetuated expression of extracellular matrix (ECM) proteins including collagens and smooth muscle α-actin (ACTA2) are involved in wound healing and may cause inductions and scars. Fibrotic scars can reduce tissue elasticity and lead to organ failure, causing deterioration in the quality of life of cancer survivors. Despite the fact that molecular mechanisms in fibrosis are rather well studied, therapeutic options are still very limited.

Differential gene expression and epigenetic predisposition have been observed in radiation-induced fibrosis of lung and skin. A recent study showed that differential DNA methylation at an enhancer of diacylglycerol kinase alpha (DGKA) was associated with fibrosis risk and modulated profibrotic gene expression after irradiation through an EGR1-DGKA-COL1A1/3A1 axis in human fibroblasts. The profibrotic response was suppressed by targeting the histone acetyltransferase CBP/p300 or acetylation-sensitive bromodomain- and extraterminal domain (BET) proteins. BET-family proteins such as bromodomain containing (BRD) 2, BRD3 and BRD4 contain two tandem bromodomains (BD1 and BD2) that allow binding to acetylated histones and act as epigenetic “readers” to facilitate transcription. Alteration of BET protein activity results in transcription reprogramming in cancer, in autoimmune, cardiovascular and metabolic diseases, and in embryonic development. First-generation BET inhibitors such as I-BET151 target both bromodomains and are found to be anti-inflammatory and anti-cancer agents in preclinical models and clinical trials. Their clinical usage is, however, hampered due to cytotoxicity, probably due to interference with basic cellular functions such as cell growth and cell cycle progression occurring in all dividing cells. Bromodomain-selective BET inhibitors have been recently developed and experimental data suggest that individual bromodomain sites have different biological functions. Here, we investigated whether the pan-BET inhibitor I-BET151 and the bromodomain-selective inhibitors IBET-BD1 and iBET-BD2 can target the EGR1-DGKA-COL1A1/3A1 axis and alleviate the radiation-induced production of profibrotic proteins in fibroblasts. Our findings may provide insights for the development of novel therapeutic opportunities to prevent or attenuate radiotherapy-induced fibrosis.

MATERIALS AND METHODS

2.1 Cell lines and culture

Human foreskin-derived BJ fibroblasts (CRL-2522, RRID:CVCL_3653) obtained from American Type Culture Collection and normal human dermal fibroblasts (NHDFs, L1, L3, L4 and H1-3) were cultivated as described. NHDFs were established from female donors aged 47 to 84 years (median age, 66 years) at the Universitätsmedizin Mannheim, Germany, as part of the EURATOM/ESTRO GENEPI project. Fibroblasts were outgrown from skin biopsies taken from the un-irradiated inner upper arm of donors. The methylation status of NHDFs was as described. All cell lines were recently authenticated using short tandem repeat or SNP profiling by Multiplexion (Heidelberg, Germany) (December 2020), and all experiments were performed with mycoplasma-free cells.

2.2 Radiation and drug treatment

Cells were irradiated using the Cs Gammacell 40 Exactor (Best Theratronics) at 1 Gy/min for 6 Gy. I-BET151 (GSK1210151A), iBET-BD1 (GSK778) and iBET-BD2 (GSK046) were provided by GlaxoSmithKline plc (GSK, London, UK). If not otherwise indicated,
cells were pretreated with I-BET151, iBET-BD1, iBET-BD2 or vehicle (DMSO) for 48 hours, irradiated with 0 or 6 Gy, and incubated for additional time intervals with concurrent drug or vehicle treatment. Maximal DMSO concentration in assays was 0.1%.

2.3 Flow cytometry analysis of cell cycle

Cells were collected and fixed in ice-cold 75% ethanol, permeabilized using 1% Triton X-100, treated with RNase A (#19101, Qiagen, Hilden, Germany) and finally incubated with 25 μg/mL propidium iodide, (#P4864, SigmaAldrich). Samples were run on a BD FACSCanto II flow cytometer (BD Bioscience, San Jose, CA), and at least 10,000 events/sample were acquired. The cell cycle distribution was evaluated using FlowJo software version 10 (BD Bioscience).

2.4 Cell proliferation and viability assay

CellTiter Blue reagent (#G8081, Promega, Madison, WI) was used to measure cell viability and reflected proliferation. Cells were cultivated in 96-well plates, treated as described, and further processed according to the manufacturer’s protocol.

2.5 RNA isolation and real-time quantitative reverse transcription PCR (RT-qPCR)

RNA isolation and quantitative real-time PCR (RT-qPCR) were performed as previously described using universal probe library hydrolysis probes (Roche, Basel, Switzerland) on a LightCycler 480 system (Roche). Primer sequences are listed in Table S1. Gene expression was normalized to the average of two housekeeping genes, HPRT1 and GAPDH.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The secreted collagen 1a1 was quantified using the COL1A1 ELISA kit (#ab210966, Abcam, Cambridge, UK). In short, conditioned media were collected from BJ cells or NHDFs treated with indicated conditions, diluted (1:200), and further processed according to the manufacturer’s protocol.

**FIGURE 1** Cell proliferation and cell cycle in BJ fibroblasts treated with BET inhibitors and ionizing irradiation. (A) Proliferation of BJ fibroblasts after treatment with increasing concentrations of the BET inhibitors I-BET151, iBET-BD1 and iBET-BD2 for 4 days. (B) Proliferation of inhibitor-treated BJ fibroblasts with and without irradiation. Cells were incubated with increasing concentrations of inhibitors for 48 hours, irradiated, and incubated for another 48 hours with fresh inhibitor-containing media before analysis. (C) Cell cycle analysis and (D) G2-M arrest in BJ fibroblasts. Cells were treated as described above before harvest. Results are presented as means ± SD from four (A and B) or two (C and D) biological replicates [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 2  Legend on next page.
2.7 Antibody-guided chromatin tagmentation and real-time PCR (ACT-qPCR)

The experiment was performed as described. Briefly, the pA-Tn5 transposome (pA-Tn5ome) was mixed with indicated antibodies (H3K27ac, ab4729, Abcam or rabbit IgG, #pp64, Millipore, Burlington, MA). Approximately 24,000 cells were used for pA-Tn5ome-Ab complex binding and tagmentation. DNA libraries were generated under real-time conditions with a LightCycler 480 (Roche), and cycling was stopped when the increase of fluorescence units (FUs) was 5 or higher. Libraries were purified with AMPure XP beads (#A63880, Beckman, Brea, CA) with a bead:DNA ratio of 1:4:1. Specific regions were quantified for enrichment using a primoQUANT CYBR green kit (#SL-9902-10mL, Steinbrenner Laborsysteme GmbH, Wiesbaden, Germany) with the indicated primers (Table S2) on a LightCycler 480. Signals reflect the relative H3K27ac enrichment of treated samples vs nonirradiated, DMSO-treated control samples.

2.8 Chromatin immunoprecipitation and real-time PCR (ChIP-qPCR)

The experiment was performed as described with some modifications. Briefly, nuclei were isolated, lysed, and sheared by using a Covaris M220 sonicator (Covaris, Brighton, UK). Antibody against BRD4 (#13440, Cell Signaling, Danvers, MA) and rabbit IgG (#pp64, Millipore) were used. Specific regions were quantified for enrichment as described for ACT-qPCR. Enrichment was expressed relative to the sample without IP (% of input).

2.9 Cell migration assay

100,000 BJ cells in 2 mL medium per well were cultivated in 6-well plates and treated with 1 μM BET inhibitors or DMSO for 48 hours. The cell monolayer was scratched using a 200 μL plastic pipette tip, irradiated with 0 or 6 Gy, and incubated for another 20 hours with fresh inhibitor-containing media. Cells were fixed using 70% ethanol and stained using 1% crystal violet. The wound area was photographed using an inverted microscope (Leica DM IL LED) and images were analyzed using ImageJ software (version 1.53c, Rayne Rasband, National Institutes of Health; https://imagej.net/software/imagej/).

2.10 Protein extraction and Western blotting

Protein extraction and Western blotting were performed as previously described. The primary antibodies used in our study were: DGKA (1:1000, 11 5471-1-AP, Proteintech, Manchester, UK), ACTA2 (1:1000, NBP2-22120SS, Novus Biologicals, CO) and beta-actin (1:3000, sc-47778HRP, Santa Cruz, CA). Quantification was performed using ImageJ software, and normalization was done using beta-actin as loading control.

2.11 Statistics

Statistical significances were determined by two-tailed Student’s t-test, and results with P-values <.05 were considered statistically significant. Data processing and statistical analyses were performed in R 4.0.5, and visualized using ggplot2 (3.3.4). The graphical abstract was created with BioRender.com.

3 RESULTS

3.1 Cell proliferation and cell cycle in BJ fibroblasts treated with BET inhibitors and ionizing irradiation

To investigate the effects of BET inhibitors in normal human fibroblasts, cell proliferation of BJ fibroblasts was measured after treatment with the inhibitors I-BET151 (GSK1210151A), iBET-BD1 (GSK778) and iBET-BD2 (GSK046) or vehicle control (DMSO) for 4 days. Cell proliferation was reduced in cells incubated with both I-BET151 and iBET-BD1 but remained unaffected in iBET-BD2-treated cells (Figure 1A). Similar drug effects were obtained in irradiated BET inhibitor-treated BJ fibroblasts (Figure 1B).

Since iBET-BD1 phenocopied the effects of I-BET151, investigations of exploring the effects of I-BET151 and iBET-BD2 were focused on cell cycle progression in BJ fibroblasts with and without irradiation. None of the inhibitors changed the distribution of cell cycle phases in unirradiated cells after 48 hours (Figure 1C,D); however, after irradiation, the G2-M phase was significantly increased in fibroblasts treated with I-BET151. This was not observed in cells treated with iBET-BD2 (Figure 1D). Taken together, under our experimental conditions, iBET-BD2 shows no...
**FIGURE 3** Legend on next page.
3.2 Radiation-induced fibroblast activation is reduced in BET inhibitor-treated BJ fibroblasts

As the expression of profibrotic extracellular matrix proteins can be indicative of fibroblast transactivation, the radiation-induced increase of DGKA expression, which in turn controls collagen (COL1A1 and COL3A1) and alpha smooth muscle actin (ACTA2) mRNA expression in fibroblasts, was measured. Endogenous DGKA expression was not significantly changed by increasing concentrations of I-BET151, iBET-BD1 and iBET-BD2 whereas the radiation-induced increase of DGKA expression was suppressed by all three inhibitors (Figure 2A).

Similarly, the radiation-induced mRNA expression of COL1A1, COL3A1 and ACTA2 was reduced by all inhibitors (Figure 2B). When measuring protein expression, radiation-induced secretion of the Collagen 1a1 pro-peptide into the culture medium was reduced after treatment with I-BET151, iBET-BD1 and iBET-BD2 (Figure 2D). In addition, radiation-induced DGKA and ACTA2 protein levels were attenuated when treated with I-BET151 and iBET-BD2 (Figure 2E). Taken together, these results show that inhibiting either both bromodomains, using the pan-BET inhibitor tool, or targeting selectively each of the bromodomains of the BET proteins can impede radiation-induced expression of DGKA and profibrotic markers.

3.3 Effects of BET inhibitors in normal human dermal fibroblasts with high and low DNA methylation at the DGKA enhancer

The epigenetic pattern at the DGKA enhancer in NHDFs of breast cancer patients can be modulated by epigenetic drugs, which results in altered expression of profibrotic markers. To verify whether the effects of the novel bromodomain selective BET inhibitors depend on the DNA methylation status at the DGKA enhancer region, primary NHDFs from donors with high and low DNA methylation at this site were used. Cell viability was significantly reduced in both hypo- and hyper-methylated NHDFs when treated with I-BET151 and iBET-BD1, but not with iBET-BD2 (Figure 3A). DGKA induction by irradiation was not affected by the BET inhibitors neither in hyper- nor in hypo-methylated NHDFs (Figure 3B). Remarkably, radiation-induced DGKA expression was rather heterogeneous in fibroblasts with low DNA methylation and treatment with inhibitors compared to fibroblasts with high DNA methylation. Endogenous COL1A1 expression was reduced in hypo-methylated fibroblasts by I-BET151 and iBET-BD1, whereas the radiation-induced increase in COL1A1 expression was suppressed by all three inhibitors (Figure 3C). In fibroblasts with high DNA methylation, COL1A1 expression was not changed by irradiation or drug treatment. Expression of other profibrotic markers (COL3A1 and ACTA2) was suppressed after I-BET151, iBET-BD1 and iBET-BD2 treatment in both hypo- and hyper-methylated NHDFs with and without irradiation (Figure 3D). Baseline collagen 1a1 secretion was significantly suppressed by I-BET151 in fibroblasts with low DNA methylation (Figure 3E). Radiation-induced collagen 1a1 secretion was significantly reduced only in hypo-methylated NHDFs when treated with I-BET151 or iBET-BD2. Taken together, the results in NHDFs support that iBET-BD2, which was shown to not affect cell viability, can attenuate fibroblast activation by preventing profibrotic marker induction. This depends on the DNA methylation status at the DGKA enhancer region.

3.4 Histone modifications after inhibitor treatment in irradiated BJ fibroblasts

BRD4 is the most abundant BET isoform expressed in BJ fibroblasts (Figure 4A) and activates transcription by reading the active histone H3 lysine 27 acetylation chromatin mark (histone H3 lysine 27 acetylation, H3K27ac). Therefore, it was investigated whether iBET-BD2 induced changes in H3K27ac levels and BRD4 occupancy at specific genomic regions using antibody-guided chromatin tagmentation (ACT)-qPCR and chromatin immunoprecipitation (ChIP)-qPCR, respectively. For comparison, BJ fibroblasts were also treated with the pan-BET inhibitor I-BET151. Regions of interest were the DGKA promoter (DGKA_Promo), its enhancer including the two EGR1 binding sites (DGKA_EGR1-1 and DGKA_EGR1-2) and a downstream control region (DGKA Ctrl) (Figure 4B). In unirradiated cells, H3K27ac enrichment at the DGKA_Promo site was reduced when treated with I-BET151, increased at the DGKA_EGR1-1 when treated with iBET-BD2, and it remained unchanged at the DGKA_EGR1-2 and downstream control regions with both inhibitors. Neither I-BET151 nor iBET-BD2 altered H3K27ac enrichment after irradiation at the measured DGKA regions (Figure 4C). In contrast, BRD4 enrichment at these DGKA regions was identical after treatment with I-BET151 or
FIGURE 4

Legend on next page.
iBET-BD2, with or without irradiation (Figure 4D). We further observed that H3K27ac enrichment at the COL1A1 transcription start site (COL1A1_TSS) was reduced in I-BET151 treated BJ fibroblasts without irradiation, and this reduction was also detectable after irradiation (Figure 4E). At the ACTA2 enhancer site (ACTA2_enh), enriched H3K27ac was only suppressed in I-BET151-treated cells after irradiation. BRD4 binding was unaltered with or without irradiation neither at the COL1A1_TSS or the ACTA2_enh regions (Figure 4F). Taken together, I-BET151 showed stronger effects than iBET-BD2 on the H3K27ac pattern of our regions of interest. Of note, none of the drugs interfered with the BRD4 enrichment at these regions.

3.5 | Cell migration after BET inhibitor treatment and ionizing irradiation of BJ fibroblasts

An early step in wound healing requires that fibroblasts migrate into the wounded area to stabilize the tissue by excreting extracellular matrix proteins. It was therefore investigated whether the selective BET inhibitors affect cell migration after irradiation using the cell scratching assay. DMSO-treated cells with and without irradiation efficiently migrated into the wounded area. This migration activity was unaltered when treated with I-BET151, iBET-BD1 or iBET-BD2 (Figure 5). This result shows that the selective BET inhibitors did not specifically affect the migration of BJ fibroblasts into the wounded area, although they attenuated the secretion of profibrotic proteins.

4 | DISCUSSION

Our study revealed that the pan-BET inhibitor I-BET151 and the BD1-selective BET inhibitor iBET-BD1 reduced cellular proliferation and attenuated the expression of radiation-induced profibrotic markers efficiently. This is in line with other reports that pan-BET inhibitors can prevent fibroblast activation and fibrosis in hepatic stellate cells, in lung fibroblasts derived from idiopathic pulmonary fibrosis.
fibrosis, in radiation-induced lung fibrosis, in cardiomyocytes and in renal interstitial fibroblasts. BRD4 silencing or treatment with the pan-BET inhibitor JQ1 also impeded fibroblast activation in human dermal fibroblasts with low or high DNA methylation at the DGKA enhancer after bleomycin treatment or irradiation. In our studies, the BD2-selective inhibitor iBET-BD2 showed potent inhibition of profibrotic markers induced by irradiation with no detectable effects on cell proliferation and cell cycle progression in the investigated fibroblasts, pointing at a low cytotoxicity of this compound which might be beneficial for patient treatment.

Important hints for toxic effects of a drug can be derived from its ability to change the cellular transcriptome. More than 700 genes were significantly altered in a pan BET inhibitor JQ1-treated hepatocellular carcinoma HepG2 cells and in l-BET151-treated acute myeloid leukemia MV-4-11 cells. Also, more than 800 genes were changed by the selective BD1 inhibitor iBET-BD1 (GSK778), but only less than 50 genes by the selective BD2 inhibitors apabetalone (RVX-208) or iBET-BD2 (GSK046). The broad transcriptional changes by the pan-BET and BD1 inhibitors interfered with the inflammatory response by nuclear factor xB (NF-xB) and facilitated proliferation and survival by Myc and receptor tyrosine kinase (RTK) signaling cascades. Thus, it was suggested to be responsible for the considerable cytotoxicity observed in clinical trials. Clinical data on BD2-selective BET inhibitors are not yet available, but based on the more selective impact on overall gene expression, adverse effects are expected to be lower than for pan-BET inhibitors. In our fibroblast experiments, iBET-BD2 had no detectable cytotoxic effects on important basic cellular functions such as cell proliferation, cell cycle progression, and migration. Nevertheless, our results indicated a selective iBET-BD2 inhibition of the inducible profibrotic radiation response. These observations are in line with the low impact on baseline expression patterns coupled with efficient effects on an inducible inflammatory response reported by Gilan et al. They also observed low toxicity of the drug in an animal model for liver fibrosis.

Human BET-family proteins contain two bromodomains, each formed by four α helices linked by hydrophobic ZA and BC loops, which surround a conserved acetyl-lysine (KAc)-binding pocket. Although both BD1 and BD2 of the BET proteins share highly conserved sequences (about 95%) and prefer to engage to a motif with two acetylated lysines bridged by two amino acids (KAc-XX-KAc), different sequence variants at the ZA and BC loops still modulate their binding preferences. In general, BD1 selectively binds to a motif diacetylated at K5 and K8 on histone H4, while BD2 can bind to multiple proteins with diacetylated peptides. Accordingly, BD1 is mainly responsible for chromatin binding at promoter and enhancer regions, whereas BD2 stabilizes transcription complexes, in this way facilitating transcription elongation. The novel BD-selective BET inhibitors used in our study were designed to address specific differences in the BC loop of the two binding sites which explains their diversity in biological function and toxicity. Using these tools, it has been reported that steady-state gene expression primarily requires BD1 whereas the rapid increase of gene expression induced by inflammatory stimuli requires both BD1 and BD2 of all BET proteins. The pan-BET and BD1-selective BET inhibitors show broad anti-cancer and anti-inflammatory activities, whereas BD2 inhibitors only retain the anti-inflammatory phenotype.

When we analyzed H3K27ac enrichment at our genomic loci of interest, we observed a loss of H3K27ac enrichment only at the COL1A1 transcription start site (COL1A1_TSS) in l-BET151-treated cells, and no changes at any other region that we measured in iBET-BD2 treated cells. Similar observations were made at the IFNG locus where the H3K27ac pattern remained constant when treated with JQ1 in TH1 polarized PBM C cultures. A genome-wide analysis further revealed that the H3K27ac enrichment was almost not changed during macrophage differentiation in the presence of iBET-BD1, iBET-BD2 or l-BET151. These results indicate that both pan-BET and selective-bromodomain BET inhibitor-mediated transcriptional changes are independent of H3K27ac enrichment. In addition, we did not find changes in BRD4 occupancy after drug exposure. However, we did not investigate whether BRD2 and BRD3 occupancy could be altered by irradiation or inhibitor treatment, as only BRD4 shows a strong expression in our fibroblast system studied. Thus, we might have missed effects of these enzymes propagated by the treatments as observed by Gilan et al.

We investigated the drug effects in fibroblasts in vitro, as these cells are considered to be among the main players in the cellular response of wound-healing and fibrosis evoked by irradiation. Fibroblasts participate in specific steps such as migration to the wound and production of ECM, which can be readily investigated in cultivated fibroblasts from skin or tissues. We focused our analysis on DGKA and ECM proteins as DGKA can control multiple critical steps including the immune response, lipid signaling, cell migration and cell proliferation. DGKA was also reported to control profibrotic gene expression after irradiation through an EGR1-DGKA-COL1A1/3A1 axis in human fibroblasts. To further translate our promising data from fibroblasts to clinical applications in fibrosis prevention and treatment, further investigations in preclinical models and clinical trials are, however, required.

Strikingly, we observed considerable variation in the radiation response among fibroblasts with low DNA methylation. We suggest that this observation might reflect the high plasticity and heterogeneity, which fibroblasts require for their multiple physiologic functions in healthy and damaged tissues. Fibroblasts can be trans-activated by various stimuli to increase ECM secretion and remodeling, to foster secretion of signaling factors to surrounding cells, to generate mechanical force, and regulate tissue metabolism and metabolite secretion. During the pathogenesis of fibrosis, this plasticity might be harmful and could even drive the disease. Thus, variation was strongest in the fibroblasts with hypo-methylation at the DGKA enhancer, which was reported to be associated with fibrosis risk. In addition to measuring ECM production, we determined the migration activity was not significantly reduced when treated with BD-selective BET inhibitors at concentrations where the transcription
of ECM proteins is already decreased. In pancreatic ductal adenocarcinoma cells; however, the pan-BET inhibitor i-BET762 suppressed migration and invasion. This difference might be caused by the different compounds and cell types tested, but it might also suggest that tumor cells are more sensitive to BET inhibition than normal fibroblasts. However, cell migration is an important early step which is required in wound healing and tissue regeneration after irradiation. Inhibiting this step would strongly increase side effects after radiotherapy.

Overall, our studies indicate that selective inhibition of the second bromodomain (BD2) of the BET proteins could offer a novel pharmacological approach to prevent the profibrotic response after irradiation. Future and more detailed investigations in different types of fibroblasts are needed to elucidate in more detail how iBET-BD2 affects the radiation-induced profibrotic response. Moreover, extensive preclinical studies are required to verify the efficacy and toxicity of these drugs and whether they can be used to prevent fibrosis development after radiotherapy.

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CONFLICT OF INTEREST
Rab K. Prinjha and Inmaculada Rioja are employees and shareholders of GSK. Elena Sperk received travel support and honorary for lectures/webinars from Carl Zeiss Meditec AG. All other authors declare that they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT
All data analyzed during this work are available from the corresponding author upon reasonable request.

ETHICS STATEMENT
The study protocol was approved by the Ethics Committee of the University Hospital of Mannheim. All patients provided informed written consent.

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\textbf{SUPPORTING INFORMATION}

Additional supporting information may be found in the online version of the article at the publisher’s website.

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