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Indirubin Derivative 6BIO Suppresses Metastasis

Simone Braig[1], Christine A. Kressirer[1], Johanna Liebl[1], Fabian Bischoff[1], Stefan Zahler[1], Laurent Meijer[2], and Angelika M. Vollmar[1]

Abstract

While metastasis is the chief cause of cancer mortality, there nonetheless remains a lack of antimetastatic therapies that are clinically available. In this study, we present the indirubin derivative 6-bromo-indirubin-3′-oxime (6BIO) as a promising antimetastatic agent. 6BIO strongly reduced formation of lung metastasis in the well-established 4T1 mouse model of aggressive breast cancer. Several major hallmarks of the metastatic process were affected by subtoxic concentrations of 6BIO, which inhibited adhesion, migration, and invasion of a variety of metastatic cell types *in vitro*. Mechanistic analyses focused on known targets of 6BIO, which were silenced by this compound. Unexpectedly, RNAi-mediated silencing of glycogen synthase kinase 3β (GSK3β) and phosphoinositide-dependent protein kinase 1 (PDK1), both modulators of cellular metastasis targeted by 6BIO, were not found to affect invasive migration in this study. Instead, the Jak/STAT3 signaling pathway appeared to play a major role through modulation of its downstream migration regulators C-terminal tensin-like protein and matrix metalloproteinase 2. However, PDK1 and GSK3β contributed to the overall response to 6BIO, as silencing of all three pathways resulted in almost complete inhibition of migration, phenocopying the 6BIO response. Taken together, our findings illustrate the antimetastatic activity of 6BIO on the basis of its ability to simultaneously inhibit several kinase cascades involved in metastasis of cancer cells, supporting the concept of "polypharmacology" in developing drugs to attack metastasis, the most deadly aspect of cancer. *Cancer Res; 73(19); 6004–12. ©2013 AACR.*

Introduction

The high mortality rates in metastatic cancer are primarily due to invasion and progression of tumor cells into distant organs rather than to the primary tumors themselves (1, 2). Metastasis is composed of several discrete steps: loss of cellular adhesion, increased motility and invasiveness, intravasation of the circulatory system, transport and survival in circulation, adherence to vessel walls, extravasation, and finally colonization of a distant site (3). Despite huge advances in understanding the molecular mechanisms underlying these metastatic processes during the last decades, metastasis is still responsible for as much as 90% of cancer-associated mortality because specific therapies are lacking (4). By now it is well known that development, survival, and metastasis of cancer cells depend on a crosstalk of numerous signal transduction pathways (1, 5). Therefore, a change of paradigms has occurred in drug design during recent years: Instead of monospecific agents, those hitting rather a range of metastatic signaling targets are urgently needed (6).

Throughout history, natural products have played an essential role in the treatment of diseases. Along this line, more than 3,000 species of plants have been used and analyzed for the treatment of cancer since the mid 1970s (7, 8).

Numerous natural sources (mostly plant extracts) constitute the basis for multiple prescriptions in traditional Chinese medicine, being widely used as complementary approach in addition to conventional treatment of cancer (9). One is Danggui Longhui Wan, which consists of eleven medicinal ingredients and was shown to be clinically active in chronic myeloid leukemia (CML; ref. 10). The active component of this recipe is *Indigo naturalis*, itself composed of high levels of the well-known dye indigo and, to a minor extent, its 3,2′-isomer indirubin (11, 12). Further investigations in animal models and clinical studies revealed that not indigo, but indirubin exhibited pronounced effects in the treatment of CML (10). Nowadays, because of its poor water solubility and its adverse gastrointestinal effects, indirubin is no longer in clinical use (13).

However, a variety of indirubin derivatives isolated from indigo dye–producing plants and gastropod mollusks have been discovered and identified as protein kinase inhibitors (14). In 2003, Meijer and colleagues characterized several bromo-indigos and synthetic derivatives of various bromo-substituted indirubins, and among these the cell permeable product 6-bromo-indirubin-3′-oxime (6BIO) has been described as a potent inhibitor of glycogen synthase kinase 3β (GSK3β; ref. 15).

Authors' Affiliations: [1]Pharmaceutical Biology, Department of Pharmacy, University of Munich, Munich, Germany; and [2]Centre Nationale de la Recherche Scientifique, Station Biologique, Roscoff, France

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Corresponding Author: Simone Braig, Department of Pharmacy, Center for Drug Research, Pharmaceutical Biology, University of Munich, Butenandtstr, Butenandtstr. 5-13 81377 Munich, Germany. Phone: 49-8921-8077-189; Fax: 49-8921-8077-170; E-mail: simone.braig@cup.uni-muenchen.de

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In this study, we investigated the effect of 6BIO on cancer cell attachment, migration, and invasion both *in vitro* and *in vivo*. Furthermore, the molecular targets of the indirubin derivative mediating the antimetastatic effect of 6BIO in cancer cells were identified.

Materials and Methods

Compounds

6BIO, 5BIO, 7BIO, and Methyl-6-bromo-indirubin-3′-oxime (Me-6BIO) were partly synthesized by the authors (L. Meijer) as well as purchased from Enzo Life Sciences. Further compounds are listed in Supplementary Methods.

Cell culture

Acquisition and cultivation of the human cancer cell lines T24, HuH-7, MDA-MB-231, and the murine mammary carcinoma cell line 4T1-luc are described in Supplementary Methods.

Cell adhesion to fibronectin

Adhesion assays were carried out as described previously (16). For detailed information, see Supplementary Methods.

Attachment assay using the xCELLigence system

Attachment and spreading of the cells was determined using the xCELLigence System from Roche Diagnostics. T24 cells were treated with 6BIO for 24 hours and seeded at a density of 5,000 cells per 100 μL in equilibrated E-plates.

Scratch assay

Scratch assays on confluent cell monolayers were conducted as described previously (17). Details are described in Supplementary Methods.

Migration and invasion assay using Boyden chambers

Assays were conducted using Transwell plates containing polycarbonate filters (Corning). For further details, see Supplementary Methods.

Chemotaxis

Chemotaxis of 6BIO-treated T24 cells was investigated by μ-Slide Chemotaxis assay (IBIDI) according to manufacturer's instructions. Chemotaxis analysis was conducted as described previously (18).

Apoptosis assay

Quantification of apoptosis was conducted according to the procedure described in the work of Nicoletti and colleagues using a FACSCalibur (Becton Dickinson; ref. 19). Nuclei to the left of the G_1 peak containing hypodiploid DNA were considered apoptotic.

Transfection experiments

Cells were transfected for 24 to 48 hours using DharmaFECT Transfection reagent (Thermo Scientific) and Dharmacon ON-TARGETplus SMARTpool siRNAs phosphoinositide-dependent protein kinase 1 (PDK1), GSK3β, and JAK1, and nontargeting siRNA (Thermo Scientific) according to manufacturer's instructions.

Western blotting

Protein expression was analyzed by using commercially available primary and HRP-conjugated secondary antibodies. Further details are described in Supplementary Methods.

Confocal laser scanning microscopy

6BIO-treated T24 cells were fixed, permeabilized, and stained with the respective antibodies. Images were taken by confocal microscopy (LSM 510 Meta, Zeiss). Details are described in Supplementary Methods.

Spheroid migration assay

Spheroids were generated according to the hanging drop protocol as described previously (20). For further details, see Supplementary Methods.

In vivo experiments

Six-week-old female Balb/cByJ mice obtained from Janvier (Centre d'Elevage R. Janvier, Le Genest Saint-Isle, France) were pretreated with 1 mg/kg 6BIO [dissolved in dimethyl sulfoxide (DMSO), 10% Cremophor EL, and PBS] or vehicle 2 hours before intravenous injection of 0.1×10^6 4T1-luc2 breast cancer cells. The number of animals analyzed was 11 in the control group and 13 in the treated group. Eight days after injection, the lungs of the mice were isolated and the number of metastasis was determined visually. The luciferase activity of the lungs was measured by using Dual-Luciferase Reporter Assay (Promega). All animal experiments were conducted according to the guidelines of the German law for protection of animal life and approved by the local ethics committee.

Statistical analysis

Results are expressed as the mean ± SD (range) or as percentage value. Comparisons between groups were made using the one-way ANOVA/Dunnett and Student unpaired *t* test. A *P* value less than 0.05 was considered statistically significant. All calculations were carried out using the GraphPad Prism software package (GraphPad Software Inc).

Results

The indirubin derivative 6BIO prevents migration and invasion of metastatic cancer cells

6BIO treatment decreases the ability of T24 urinary bladder carcinoma cells to bind to fibronectin by approximately 20% upon treatment with 3 μmol/L 6BIO. A reduction of adhesion up to 35% was observed when cells were treated with 10 μmol/L 6BIO compared with control cells (Fig. 1A). In addition, the attachment capacity of 6BIO-treated T24 cells to uncoated wells was reduced in a similar dose-dependent manner, as determined by xCELLigence experiments (Supplementary Fig. S1).

To exclude cytotoxicity being involved and to find appropriate subtoxic concentrations of 6BIO, apoptotic cell death as well as loss of cell membrane integrity was analyzed after

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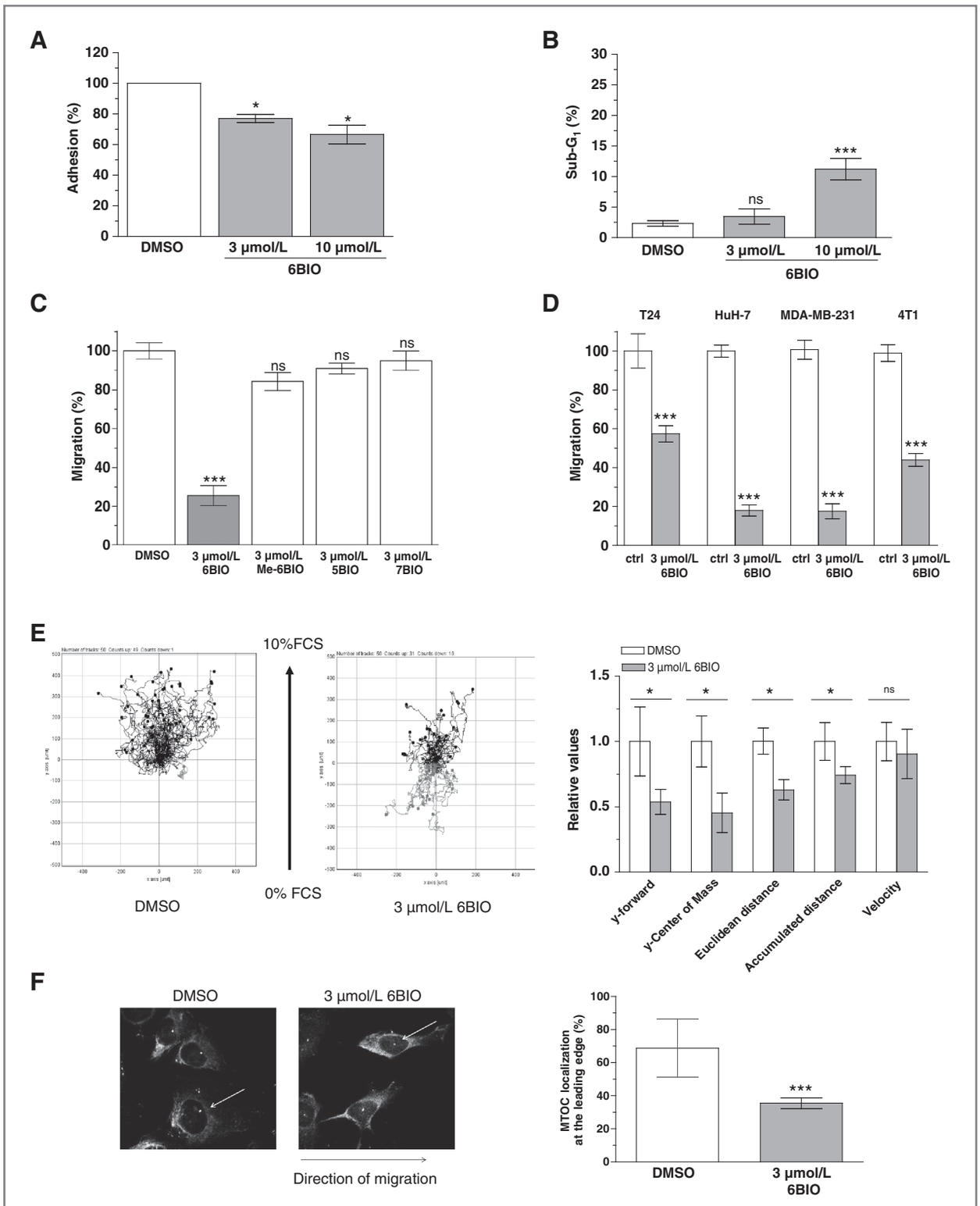

Figure 1. 6BIO inhibits adhesion and migration of various invasive cancer cells. A, treatment of T24 urinary cancer cells with 3 µmol/L and 10 µmol/L 6BIO resulted in a reduced ability to bind to fibronectin-coated plates compared with control cells. B, apoptosis measurement revealed no induction of cell death upon incubation with 3 µmol/L 6BIO for 72 hours, whereas 10 µmol/L 6BIO treatment resulted in a slight increase of apoptotic cells. (*Continued on the following page*.)

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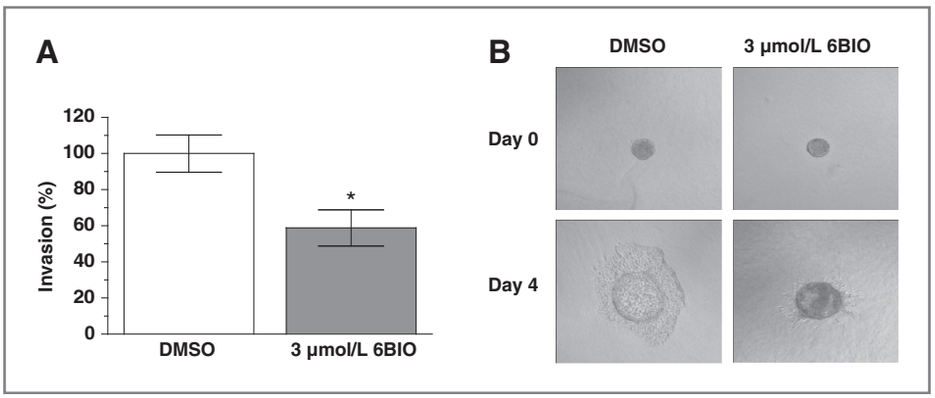

Figure 2. 6BIO diminishes the invasive potential of cancer cells. A, modified Boyden assays revealed a significant inhibition of the invasive capacity of T24 cells upon treatment with 6BIO. Bars show the mean ± SD of 3 independent experiments. Measurements were carried out in triplicate. *, $P < 0.05$. B, T24 cell spheroids treated with 3 μmol/L 6BIO and embedded in collagen showed a diminished invasion capacity compared with control treated cells.

treatment with increasing concentrations of 6BIO for 72 hours. While incubation with 3 μmol/L 6BIO had clearly no effect on cell survival, a slight, but significant induction of apoptosis in T24 cells treated with 10 μmol/L 6BIO was observed (Fig. 1B). On the basis of these results, all subsequent experiments on migration and invasion of cancer cells were conducted using a subtoxic dose of 6BIO (3 μmol/L).

6BIO strongly inhibited the migration of T24 cells to 13% of untreated cells as shown in a wound-healing assay. Noteworthy, neither the kinase-inactive form Methyl-6BIO (Me-6BIO), nor the structurally related bromo-substituted indirubin derivatives 5BIO and 7BIO had an effect on the migratory potential of the cells (Fig. 1C). Exemplary pictures of the Scratch assay, as well as the chemical structures of 5BIO, 6BIO, Me-6BIO, and 7BIO are shown in Supplementary Figs. S2 and S3.

To further study the effect of 6BIO on migration in different types of invasive cancer cell lines, next to the highly invasive urinary bladder carcinoma cell line T24, the moderate invasive HuH-7 hepatocarcinoma cells, and the triple-negative breast cancer cell line MDA-MB-231 as well as the murine mammary carcinoma cell line 4T1 were used. As shown by a wound-healing assay, the migratory potential of 6BIO-treated T24, HuH-7, MDA-MB-231, and 4T1 cells was strongly diminished compared with control cells (Supplementary Fig S4). Apoptosis assays confirmed that treatment of the cell lines with 3 μmol/L 6BIO had no cytotoxic effects (Supplementary Table S1).

Furthermore, directed migration of the T24, HuH-7, MDA-MB-231, and 4T1 cells towards a chemoattractant was also significantly reduced after treatment with 6BIO for 24 hours, as determined by Boyden chamber assays (Fig. 1D). Representative images of migrated T24 cells are shown in Supplementary Fig. S5. In addition, analysis of movements of single cells along a chemoattractant gradient (chemotaxis assay) revealed that 6BIO not only affected directed migration (indicated by y-forward index, y-center of mass, and euclidean distance) but also the motility of cells (accumulated distance) in comparison with control cells. In contrast, the overall velocity of the cells was not disturbed by 6BIO treatment (Fig. 1E).

As a further indicator for a loss of directionality, 6BIO-treated cells exhibited altered localization of the microtubule-organizing center (MTOC), as shown by confocal laser scanning microscopy. While in control cells, the MTOC is localized in front of the nucleus at the leading edge of the migrating cell, the MTOC of 6BIO-treated cells was randomly distributed throughout the cell, thereby impeding directed migration of the cells (Fig. 1F).

Two sets of experiments addressed the effect of 6BIO on the invasive potential of cancer cells. First, T24 cells pretreated with 6BIO for 20 hours and seeded into Transwell inserts on top of a defined layer of Matrigel showed significantly reduced invasive activity as compared with control cells (Fig. 2A). Second, a three-dimensional model was used: T24 cells were grown as spheroids, treated with 6BIO or DMSO and embedded in collagen. 6BIO clearly abrogated the invasion of the cells into the surrounding collagen matrix (Fig. 2B).

6BIO inhibits GSK3β, PDK1, and STAT signaling pathways

Next, we aimed to identify the molecular targets of 6BIO responsible for the antiinvasive action of 6BIO. The known fact that 6BIO inhibits glycogen synthase kinase 3β (GSK3β; ref. 14) was confirmed also for our experimental setting by analyzing the amount of β-catenin. β-catenin is phosphorylated by GSK3β, thereby leading to its degradation. T24 cells treated for 24 hours with 6BIO displayed increased levels of β-catenin compared with control cells. Lithium chloride, another reported inhibitor of GSK3β, was used as positive control (Fig. 3A, i).

To analyze the functional link of inhibition of GSK3β by 6BIO and migration, we treated T24 cells with 10 mmol/L LiCl and specific siRNAs against GSK3β for 48 hours. Successful silencing of GSK3β protein levels was confirmed by Western blot

(*Continued*.) C, scratch assays showed a significant reduction in the migratory potential of T24 cells after treatment with 3 μmol/L 6BIO, whereas neither the kinase-inactive form of 6BIO (Me-6BIO), nor 5BIO, nor 7BIO had an effect on migration. D, treatment of T24, HuH-7, MDA-MB-231, and 4T1 breast cancer cells with 3 μmol/L 6BIO resulted in a diminished migration capacity as shown by Boyden chamber experiments. E, directed migration towards an FCS gradient was analyzed by live cell imaging (left). 6BIO significantly abolished directed migration and migration distance of T24 cells (right). F, localization of the MTOC in migrating cells is altered by 6BIO. Representative images of T24 cells are shown on the left. The fluorescent signals of MTOC (stained by anti-γ-tubulin antibody, arrows) are shown. Bars on the right indicate percent of cells with MTOCs oriented towards the leading edge. Bars always show the mean ± SD of 3 independent experiments with $n = 3$. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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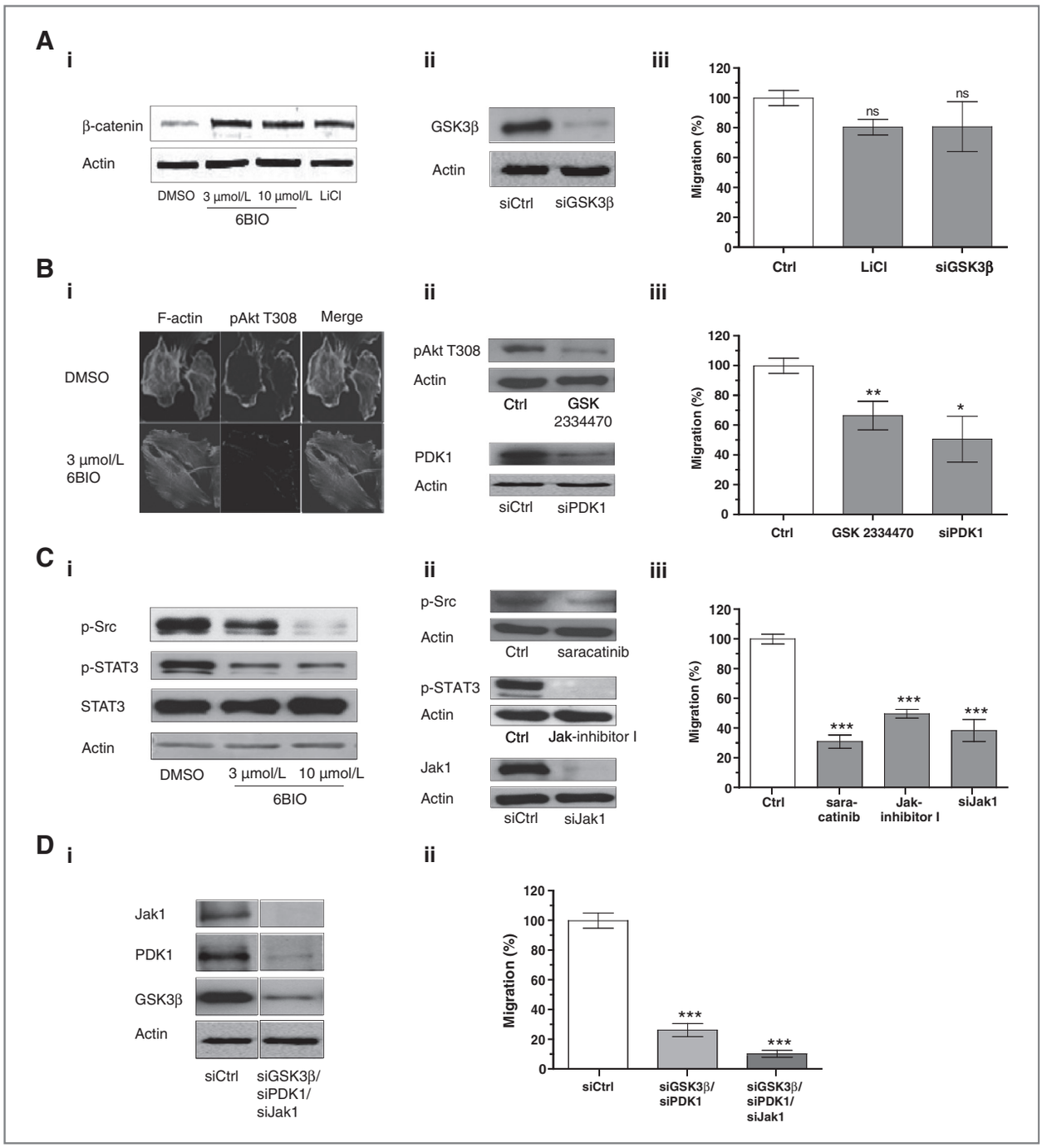

Figure 3. Impact of 6BIO targets on cancer cell migration. A, 6BIO inhibits GSK3β in T24 cells, but GSK3β does not affect migration. β-catenin is stabilized in 6BIO-treated cells. This effect is comparable to incubation with the GSK3β inhibitor lithium chloride (LiCl; i). Transfection of T24 cells with siRNAs against GSK3β resulted in a clear reduction of GSK3β protein expression after 48 hours. Transfection with a non-targeting siRNA served as a control (ii). Migration is neither diminished in siGSK3β-transfected nor in LiCl-treated T24 cancer cells as determined by Boyden chamber assays (iii). B, impact of inhibition of PDK1 by 6BIO. Confocal microscopy experiments showed reduced phosphorylation of Akt (T308) after treatment with 6BIO for 24 hours (i). Migration of T24 cells (iii) was significantly affected after knockdown of PDK1 using the PDK1 inhibitor GSK 2334470 or specific siRNAs (ii). C, inhibition of JAK/STAT3 by 6BIO. Phosphorylation of Src and STAT3 is strongly diminished in 6BIO treated urinary cancer cells after 24 hours as shown by Western blot (i). Incubation with the Src kinase inhibitor saracatinib and the pan Jak-Inhibitor I, as well as transfection with specific siRNAs, revealed diminished expression of pSrc, pSTAT3, and Jak1, respectively (ii). Migratory potential of the treated cells is strongly reduced compared with control cells, as determined by Boyden chamber experiments (iii). D, impact of combined knockdown of GSK3β, PDK1, and Jak1 on bladder cancer cell migration. T24 cells were transfected with specific siRNAs against GSK3β, PDK1, and Jak1 (i). Migratory potential of the cells is reduced upon combined transfection with GSK3β and PDK1, whereas triple knockout of GSK3β, PDK1, and Jak1 strongly impeded the migration of the cells compared with control cells. Bars always show the mean ± SD of 3 independent experiments. Measurements were carried out in triplicate. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$.

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analysis (Fig. 3A, ii). Interestingly, inhibiting GSK3β had no significant effect on the migratory potential of T24 cells (Fig 3A, iii). Because it is known that 6BIO targets cyclin-dependent kinases (CDK; ref. 15), cells were treated with 10 μmol/L roscovitine, a known inhibitor of CDKs. However, Boyden chamber assays revealed no diminished migratory potential of roscovitine-treated cells (Supplementary Fig. S6), indicating that the effect of 6BIO on migration impairment is not mediated via CDK inhibition.

PDK1 was recently identified by our group as a target of 6BIO, yet unknown (21). PDK1 phosphorylates the downstream kinase Akt on Threonine 308, which results in activation of Akt. As shown by immunofluorescent staining, Threonine 308-phosphorylation of Akt indeed was strongly diminished after 6BIO treatment of urinary bladder carcinoma cells (Fig. 3B, i). Next, the expression of PDK1 was abrogated by treatment with GSK2334470, a specific PDK1 inhibitor, or siRNA transfection (Fig. 3B, ii). Boyden chamber assays revealed a significant decrease in the migratory capacity of cells incubated with 10 μmol/L GSK2334470 or with siRNAs targeting PDK1 compared with related controls (Fig. 3B, iii).

Figure 3C, i shows a further target of 6BIO, i.e., the Jak/STAT pathway in our setting. 6BIO-treated cells showed reduced phosphorylation and activation of Src and STAT3. To investigate the functional relevance of Src/Jak/STAT3 signaling in the migration inhibition mediated by 6BIO, we treated T24 with 1 μmol/L saracatinib, a known inhibitor of Src kinase, 10 μmol/L of the pan Jak-Inhibitor I and siRNAs against Jak1, the upstream regulator of STAT3 activity. Reduction of Src, STAT3, and Jak1 expression was confirmed by Western blot experiments (Fig. 3C, ii). Boyden chamber assays revealed a reduction of the migratory potential of saracatinib and Jak-Inhibitor I–treated cells up to 3-fold and 2-fold, respectively, whereas transfection with siRNA against Jak1 resulted in more than 60% diminished migration compared with controls (Fig. 3C, iii). Notably, treatment with the aforementioned small-molecule inhibitors (LiCl, roscovitine, GSK2334470, saracatinib and Jak-Inhibitor I) had no cytotoxic effect on the cancer cells (Supplementary Fig. S7).

Having shown that 6BIO inhibits multiple signaling pathways in metastatic cancer cells, we carried out a double- and triple knockdown of GSK3β, PDK1, and Jak1 (Fig. 3D, i) and analyzed their impact on cell migration. Boyden chamber assays revealed a significant migration-inhibition of siGSK3β and siPDK1 double transfected cells compared with control cells. Moreover, triple knockdown of GSK3β, PDK1, and Jak1 almost completely abrogated the migration of T24 cells (Fig. 3D, ii). Noteworthy, silencing the expression of these proteins had no impact on survival or proliferation of the cells, indicating that the effect of GSK3β, PDK1, and Jak/STAT signaling pathways on migration inhibition is not due to an induction of apoptosis (Supplementary Fig S8).

JAK/STAT-3 pathway regulated genes affected by 6BIO

Because inhibiting the Jak/STAT signaling pathway has the most evident effect on migration inhibition, we investigated potential target genes of Jak/STAT that might be involved in the regulation of migration and invasion. Thereby, we identified C-terminal tensin-like protein (CTEN, Tensin-4) and matrix metalloproteinase 2 (MMP-2) as being strongly diminished in siJak1-transfected cells (Fig. 4A). To confirm that these STAT3 target genes were also downregulated by treatment with 6BIO, we conducted Western blot experiments. While a reduction of MMP-2 expression is only visible in cells incubated with high concentrations of 6BIO, the protein levels of CTEN were in fact dramatically reduced upon treatment of T24 cancer cells with low concentrations of 6BIO (Fig. 4B). Hence, 6BIO impedes the expression of CTEN and MMP-2 by blocking the Jak/STAT signaling cascade, thus most likely leading to inhibition of migration and invasion of cancer cells.

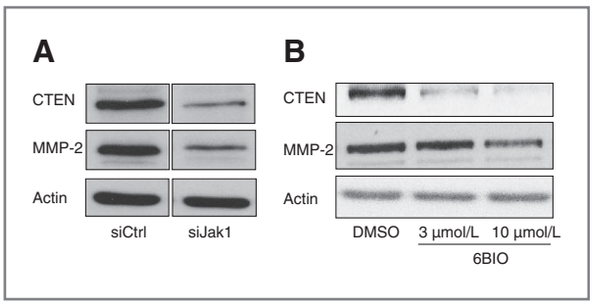

Figure 4. Expression of STAT target genes CTEN and MMP-2 is diminished in 6BIO-treated cells. A, silencing of Jak1 resulted in strongly diminished expression of CTEN and MMP-2 in T24 cells. B, protein levels of CTEN and MMP-2 are reduced in T24 cells upon treatment with increasing concentrations of 6BIO for 24 hours.

6BIO inhibits cancer metastasis *in vivo*

Finally, to transfer our *in vitro* results to an *in vivo* system, we made use of the 4T1-luc2 metastatic mouse breast cancer model (22). 4T1 cells rapidly disseminate to distant organs, predominantly to the lungs after intravenous application. Balb/cByJ mice were treated once with 1 mg/kg 6BIO 2 hours before intravenous injection of 4T1-luc2 cells. Mice were sacrificed 8 days after injection, lungs were isolated and the number of lung metastasis was determined visually. As shown in Fig. 5A, treatment with 6BIO strongly diminished the number of lung-metastasis compared with vehicle-treated mice. In addition, the luciferase activity of the isolated lungs of 6BIO-treated mice was reduced up to 80% in comparison with the lungs of control-treated animals (Fig. 5B).

Discussion

This study designates the synthetic indirubin derivative 6BIO as a potent antimetastatic agent for treatment of invasive cancer. 6BIO at a subtoxic concentration potently inhibits migration and invasion of various metastatic cells by interfering with distinct pathways implicated in the regulation of cell migration, namely GSK3β, PDK1, and Src/Jak/STAT signaling cascades.

Initially, 6BIO was identified as a very potent inhibitor of GSK3β. Cocrystal structures showed a direct interaction of 6BIO within the ATP-binding pocket of GSK3β (15). Along this line, 6BIO has been used to maintain pluripotency of human

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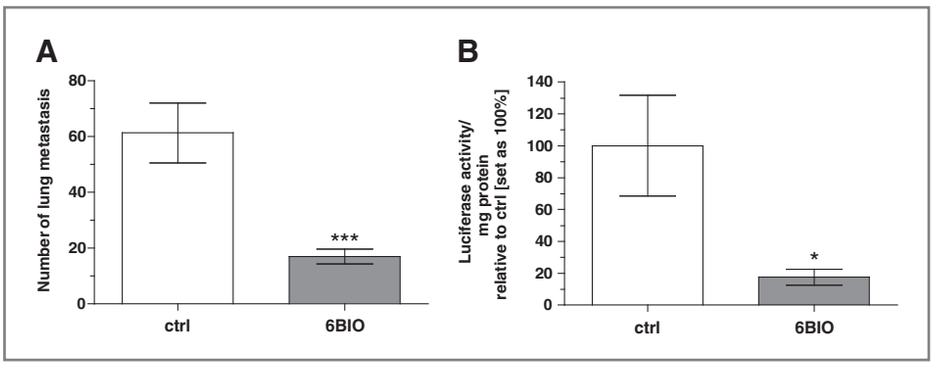

Figure 5. 6BIO inhibits breast cancer metastasis *in vivo*. 1 mg/kg 6BIO or DMSO vehicle was injected intravenously into Balb/c mice 2 hours before injection of 4T1luc2 cells. After 8 days, lungs were isolated and number of lung metastasis was determined visually (A) and by measuring the luciferase activity of the lung lysates (B). Eleven control mice and 13 6BIO-treated mice were analyzed. *, $P < 0.05$; ***, $P < 0.001$.

embryonic stem cells (23), to prevent GSK3β-induced glucocorticoid bone loss (24), and to counteract tau protein aggregation in tauopathies such as Alzheimer disease (25).

Recently, to extend the therapeutic application of 6BIO beyond its usage as a GSK3β inhibitor, a panel of various isolated kinases has been tested for inhibition by 6BIO (21, 26). However, until now, most of the data obtained have not been validated in cellular systems in detail.

It is known that 6BIO is able to induce apoptosis in melanoma and breast cancer cells (27, 28). Liu and colleagues showed that 6BIO exerts its proapoptotic effects on human melanoma cells by inhibiting Jak/STAT3 signaling pathways (27). Interestingly, 6BIO also suppressed tumor growth in a human melanoma xenograft model. The fact that the compound was administrated orally makes it a very promising therapeutic agent for treatment of cancer in general. However, the authors had to use very high concentrations of 6BIO (50 mg/kg) to observe inhibition of tumor growth *in vivo* (27).

Up till now, there is only one study illustrating the effect of 6BIO on migration and invasion of cancer cells *in vitro* and *in vivo*. Williams and colleagues showed that 6BIO reduces the invasive potential of glioma cells through the specific suppression of GSK3β signaling pathways (29). They state that activation of GSK3β promotes glioma migration through phosphorylation and degradation of β-catenin. Therefore, inhibition of this signaling pathway by 6BIO stabilizes β-catenin and subsequently reduces cell migration and invasion. However, the influence of GSK3β on cellular migration is controversial. Conflicting reports show decreased cell migration after inhibition of GSK3β (30), whereas other reports provide strong evidence that GSK3β inhibition most likely promotes cancer cell invasion and metastasis (31). According to this, silencing of β-catenin did not result in complete rescue of 6BIO's antimigratory effects in glioma cells (29), thus indicating that GSK3β is not the only regulator of migration inhibition mediated by 6BIO.

These data were reinforced and extended by our own work showing a strong inhibition of migration and invasion in 6BIO-treated cancer cells. Although we were able to confirm GSK3β as a target of 6BIO, inhibiting its expression by LiCl and siRNAs surprisingly had no effect on migration. This implies that, at least in the case of our metastatic cancer cells, 6BIO exerts its effects on metastasis inhibition by suppressing further molecular targets besides GSK3β.

Recently, by inverse *in silico* screening, PDK1 was identified as a potential target of 6BIO (21). This could be verified by our experiments showing reduced phosphorylation of Akt, the main substrate of PDK1 after treatment with 6BIO. It has been reported that inhibition of PDK1 in MDA-MB-231 breast-cancer cells strongly reduced adhesion, migration, and motility of the cells by impairing EGF-induced activation of Akt and PKCζ (32). The fact that depending on cell type and cellular conditions, one isoform of the PDK1 downstream target Akt (namely Akt2) enhances the migratory and invasive phenotype of cancer cells, whereas the other isoform Akt1 inhibits migration (33, 34), might be the reason why abrogating the expression of PDK1 by siRNA as well as by using a specific PDK1 inhibitor in our T24 urinary carcinoma cells resulted in a visible, but not robust reduction of migration.

STAT3 is one of the main regulators of tumorigenesis (35). Besides the well-known effect of STAT3 signaling on cellular growth and apoptosis, growing evidence arises showing that STAT3 target genes are implicated in migration, invasion, and metastasis of different kinds of cancer (36). STAT3 signaling pathways are activated by Jak-family tyrosine kinases, which cooperate with Src kinases to activate STAT proteins (37, 38). Furthermore, because STAT3 is constitutively active in the majority of highly invasive cancer types, inhibition of tyrosine kinase pathways mediating STAT3 activation might be a promising therapeutic option for treatment of metastatic cancers (39). Indeed, blocking of STAT3 signaling cascades using small-molecule inhibitors suppresses migration and invasion of breast cancer cells (40). Liu and colleagues showed that 6BIO is a pan-JAK inhibitor and thus induces apoptosis in melanoma cells (27). We were able to identify the Src/Jak/STAT3 signaling as a target of 6BIO in cancer cell migration, even at a subtoxic concentration. Treatment of T24 cells with 6BIO strongly diminished the phosphorylation of Src and STAT3. This is in accordance to the study of Ribas and colleagues, showing by isolated kinase screens that 6BIO

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targets Src kinase (26). Importantly, inhibition of this pathway by using specific inhibitors of Src kinase (saracatinib) and Jak (Jak- Inhibitor I) as well as siRNAs against Jak1, resulted in a strong reduction of migratory behavior of the cells. Thus, by impeding STAT3 signaling pathways, 6BIO exerts both cytotoxic effects on melanoma cells (27), as well as antimetastatic, but virtually no cytotoxic impact on urinary, hepatocarcinoma, and breast cancer cells, as shown in this study. However, although the STAT signaling pathway is the main downstream target of Src kinases, we cannot rule out that by inhibiting Src activity, 6BIO also modulates other signaling cascades activated by Src, like mitogen-activated protein kinase pathways (41).

STAT3 signaling pathways are known to promote the expression of MMPs, which are involved in dissemination of cancer cells. Senft and colleagues reported that inhibition of STAT3 signaling diminishes the expression and activity of MMP-2, thus impeding the migration and invasion of glioblastoma cells (42). Moreover, invasion of colon carcinoma cells is enhanced by STAT3-mediated MMP induction (43). These reports are in accordance with our own data showing that inhibiting the Jak/STAT3 by 6BIO results in significant downregulation of MMP-2 in T24 urinary cancer cells.

Next to MMPs, several other metastasis-related genes are affected by STAT3 signaling cascades. A largely unknown target of STAT3 is a member of the Tensin family, namely CTEN. Besides our study, which shows that 6BIO-mediated inhibition of STAT3 signaling resulted in strongly reduced expression of CTEN, there is just one further publication that links STAT3 with CTEN. Barbieri and colleagues illustrated in 2010 that constitutively-active STAT3 triggers the migration and invasion of mammary tumors via overexpression of CTEN (44). Tensin family members are localized to focal adhesion sites, where they link the cytoskeleton to integrin-based adhesion sites. CTEN is the only tensin that lacks the actin-binding domain necessary for the interaction of integrins and actin fibers (45). Katz and colleagues showed that CTEN displaces tensin-3 from integrins, thus promoting actin disassembly and stimulating mammary tumor invasion (45). Therefore, next to MMP-2, CTEN as a yet unknown target gene of STAT3 in T24 cancer cells might be, in part, responsible for the effect of 6BIO on migration inhibition.

Nevertheless, besides STAT3, 6BIO also targets GSK3β and PDK1 signaling, both of which are implicated in migration regulation. Because metastasis clearly depends on the crosstalk of numerous signal transduction pathways, the combined inhibition of these signaling cascades by 6BIO might have a synergistic impact on migration inhibition. Indeed, silencing GSK3β, PDK1, and STAT3 pathways almost completely abrogates the migration of metastatic cancer cells. Hence, by using multi-targeted agents like 6BIO, that combine the advantage of synergistic effects together with the circumvention of potential counter regulation mechanisms, greater efficiency in the treatment of cancer will be achieved (46, 47).

The feasibility of this approach is shown by the fact that the effect of 6BIO on migration and invasion inhibition in cellular systems could also be shown in a mouse model of tumor metastasis. There, treatment with low concentrations of 6BIO (1 mg/kg) almost completely inhibited metastatic spreading of breast cancer cells into the lungs. We propose that 6BIO diminished metastasis mainly due to its antimigratory potential. Certainly, we cannot rule out that next to the direct effects of 6BIO on cancer cells, 6BIO influences the vascular or immune system and thus contributes to the inhibition of cancer metastasis. However, because the micrometastases are quite small, it is very unlikely that either inhibition of proliferation or angiogenesis play pivotal roles in the observed effect of 6BIO on lung metastases. Furthermore, although treatment of cancer cells with high concentrations of 6BIO led to about 10% apoptosis induction *in vitro*, dissemination of tumor cells is inhibited more than 80% after treatment with 6BIO *in vivo*. This implies that apoptosis does not account substantially to 6BIO's antimigratory effects.

By using 6BIO as a chemical tool to characterize the impact of various signaling pathways on cancer cell migration, we identified this compound as a promising therapeutic agent in the treatment of metastatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were declared.

Authors' Contributions

Conception and design: S. Braig, C.A. Kressirer, L. Meijer, A.M. Vollmar
Development of methodology: S. Braig, C.A. Kressirer, F. Bischoff, S. Zahler, A.M. Vollmar
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Braig, C.A. Kressirer, J. Liebl, S. Zahler
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Braig, C.A. Kressirer, J. Liebl, F. Bischoff, S. Zahler
Writing, review, and/or revision of the manuscript: S. Braig, C.A. Kressirer, F. Bischoff, L. Meijer, A.M. Vollmar
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.A. Kressirer, A.M. Vollmar
Study supervision: A.M. Vollmar

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