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Induction of discrete apoptotic pathways by bromo-substituted indirubin derivatives in invasive breast cancer cells

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ABSTRACT

Indirubin derivatives gained interest in recent years for their anticancer and antimetastatic properties. The objective of the present study was to evaluate and compare the anticancer properties of the two novel bromo-substituted derivatives 6-bromoindirubin-3′-oxime (6BIO) and 7-bromoindirubin-3′-oxime (7BIO) in five different breast cancer cell lines. Cell viability assays identified that 6BIO and 7BIO are most effective in preventing the proliferation of the MDA-MB-231-TXSA breast cancer cell line from a total of five breast cancer cell lined examined. In addition it was found that the two compounds induce apoptosis via different mechanisms. 6BIO induces caspase-dependent programmed cell death through the intrinsic (mitochondrial) caspase-9 pathway. 7BIO up-regulates p21 and promotes G_2/M cell cycle arrest which is subsequently followed by the activation of two different apoptotic pathways: (a) a pathway that involves the upregulation of DR4/DR5 and activation of caspase-8 and (b) a caspase independent pathway. In conclusion, this study provides important insights regarding the molecular pathways leading to cell cycle arrest and apoptosis by two indirubin derivatives that can find clinical applications in targeted cancer therapeutics.

1. Introduction

Indirubin is a bisindole alkaloid of red/purple color and is an isomer of the blue color indigo. Indirubins can be extracted from about 200 indigo dye-producing species. They are also present in about 15 species of Muricidae mollusks [1] and in some bacteria [2,3]. In addition, these compounds have been detected in human urine [4]. Indirubin is considered to be the active ingredient of the Danggui Longhui Wan, an extract that was used for the treatment of chronic myelocytic leukemia in traditional Chinese medicine [5]. Indirubins' low solubility in water and their poor bioavailability [6] lead to the synthesis of a series of derivatives with improved qualities, some of which turned out to be potent glycogen synthase kinase-3b (GSK-3b) inhibitors [1,7]. Furthermore, several of these synthetic derivatives have been shown to act as potent CDK1 and CDK5 inhibitors by competing with ATP for binding to the catalytic site of the kinase [8].

Much of the recent literature on indirubins has been concentrating on 6-bromoindirubin-3′-oxime (6BIO) and 7-bromoindirubin-3′-oxime (7BIO), two indirubin derivatives that differ only in the position of the bromine on their aromatic ring (Fig. 1). Although both 6BIO and 7BIO had been reported to induce rapid cell death in different tumor cell lines, the death mechanisms are not fully understood and there are conflicting reports in the literature as to the exact pathways activated by each agent. 6BIO, a strong inhibitor of GSK-3b (IC_{50} = 0.005 μM), CDK1 (IC_{50} = 0.320 μM), and CDK5 (IC_{50} = 0.083 μM), has been shown to induce apoptosis in various cancer cell lines including colon (HT-29, HCT116), breast (MDA-MB-231), lung (A549), prostate (PC3), hepatoma (5L, BP8, F1, Huh7) and neuroblastoma (SH-SY5Y) [1,9,10]. On the other hand 7BIO, having only marginal inhibitory activity

Abbreviations: 6-BIO, 6-bromoindirubin-3′-oxime; 7-BIO, 7-bromoindirubin-3′-oxime; Cas-9, caspase-9; Cas-8, caspase-8; Cas-3, caspase-3; Z-VAD, Z-VAD (OMe)-FMK; PARP, poly-(ADP-ribose) polymerase; DR4, death receptor TRAIL-R1; DR5, death receptor TRAIL-R2; FADD, fas associated death domain protein; CDK, cyclin-dependent kinase protein; GSK-3β, glycogen synthase kinase-3-beta.

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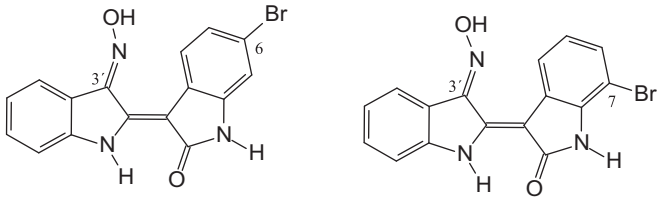

Fig. 1. Chemical structure of 6-bromoindirubin-3′-oxime (6BIO) and 7-bromoindirubin-3′-oxime (7BIO).

against GSK-3 (IC_{50} = 32 μM), CDK1 (IC_{50} = 22 μM) and CDK5 (IC_{50} = 33 μM) [8], has been reported to cause non apoptotic or a special type of necrotic cell death in neuroblastoma (SH-SY5Y), leukemia (Jurkat) and breast (MDA-MB-231) cancer cell lines [8,11]. From these results it is becoming apparent that the ability of the two derivatives to kill cancer cells is independent of their ability to inhibit GSK-3b and CDKs and the mode of death may be also dependent on cell content.

The objectives of the present study were to: (a) identify breast cancer cells that are especially susceptible to 6BIO and 7BIO, and (b) to thoroughly elucidate the programmed cell death pathways engaged by each compound in the selected cell line. Our results show that although both compounds effectively retard the growth of breast cancer cell lines, 6BIO clearly induces a caspase-9 dependent apoptotic pathway, whereas 7BIO produces the induction of a G_2/M phase cell cycle arrest and the activation of both a caspase-8 dependent and a caspase independent pathway of apoptosis.

2. Materials and methods

2.1. Synthesis of 6BIO and 7BIO

The derivatives 6BIO and 7BIO were prepared by Dr. Skaltsounis laboratory as previously described [9,10].

2.2. Cell lines

The MDA-MB-231 derivative cell line MDA-MB-231-TXSA was kindly provided by Dr. Andreas Evdokiou (Royal Adelaide Hospital, University of Adelaide, Australia). These cells have been selected for their increased propensity to metastasize to the bone [12]. Cells were cultured in DMEM purchased from Invitrogen (Carlsbad, CA), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 160 μg/ml gentamicin and 10% fetal bovine serum from Invitrogen (Carlsbad, CA) and maintained in a 5% CO_2 atmosphere.

2.3. Measurement of cell viability

Cells (1×10^5) were seeded in 96-well microtiter plates and 24 h latter treated with 6BIO or 7BIO as indicated. Cell viability was assessed after 24 h or 48 h using the CellTiter-Blue assay from Promega (Madison, MI) which measures the indicator dye resazurin at 570 nm wavelength to evaluate the metabolic capacity of cells. The Crystal Violet staining assay was also used by measurement of absorbance at 570 nm wavelength [13]. Unless otherwise indicated, in all subsequent experiments 6BIO and 7BIO were used at a final concentration of 15 μM.

2.4. Measurement of DEVD-caspase activity

A total of 1×10^5 cells per well were seeded in 6-well microtiter plates and allowed to adhere for 24 h. Cells were then treated with 6BIO or 7BIO as indicated for several time points (0–48 h). Measurement of caspase-3 activity was done as described before [13,14].

2.5. Western blot analysis

Cells were treated as indicated for 0–48 h, lysed and analyzed as previously described [13]. Anti-caspase-8, anti-caspase-9 and Bax antibodies were purchased from Cell Signaling Technology (Danvers, MA), anti-caspase-3 and anti-FADD antibodies from Transduction Laboratories and anti-poly ADP ribose polymerase (PARP) antibodies from Roche Diagnostics (Indianapolis, IN). Anti-p21 antibody was purchased from Santa-Cruz biotechnology (Heidelberg, Germany) and anti-p53 antibody from ZYMED laboratories (San Francisco, CA). TRAIL receptors (DR4, DR5) were purchased from R&D systems (Minneapolis, MN). Anti-actin monoclonal antibody from Sigma (St. Louis, MO) was used as a loading control.

2.6. DAPI staining

Circular TC-treated cover slips were placed at the bottom of each well of a 24-well plate. A total of 1×10^5 of MDA-MB-231-TXSA cells were seeded in each well and incubated for 24 h. Growth medium was removed, replaced with fresh DMEM or DMEM supplemented with 15 μM of 6BIO or 7BIO. After 24 h, the medium was removed and the cells were incubated for 10 min with 1 μg/ml DAPI stain purchased from Roche Diagnostics (Indianapolis, IN). The morphology of the cell nuclei was observed using a fluorescence microscope (Olympus BH Series) at excitation wavelength 350 nm.

2.7. Cell death detection ELISA

A total of 1×10^5 MDA-MB-231-TXSA cells per well were seeded in 96-well microtiter plates and allowed to adhere for 24 h. Cells were treated with the indirubin derivatives as indicated, and lysed after 24 and 48 h respectively. Lysates (for apoptosis) and supernatant (for necrosis) were analyzed for the presence of nucleosomes using the Cell Death Detection ELISA Plus kit purchased from Roche, Diagnostics (Indianapolis, IN). Absorbance was measured at 405 nm.

2.8. Tali™ Apoptosis Kit

Cells were plated in 60-mm plates and treated with 6BIO and 7BIO. After 24 h and 48 h of treatment, cells were harvested and stained using annexin-V Alexa Fluor® 488/PI staining, as described by the Tali™ apoptosis kit from Invitrogen, (Carlsbad, CA). As a positive control we used etoposide (100 μg/ml). Cell viability, death and apoptosis were evaluated using the Tali™ Image-based Cytometer.

2.9. Cell cycle analysis

MDA-MB-231-TXSA cells (3×10^6) were incubated in complete growth medium in the presence or absence of 15 μM 7BIO or 6BIO for several time points. The cells were collected by centrifugation and fixed with 70% cold ethanol for 2 h on ice. Cells were then incubated with the Propidium Iodide (PI) staining solution (containing 10 μg/ml PI and 0.2 mg/ml RNase A) for 45 min at 37 °C. Cells were then analyzed by a Becton Dickinson FACS Calibur flow cytometer.

The percentage of cells in each cell cycle phase (Sub-G_1, G_0/G_1, S, G_2/M) were calculated using CellQuest 3.3 software.

2.10. Statistical analysis

Experiments were done in triplicates and repeated at least two times. Values are presented as the mean ± SEM. Statistical significance was evaluated using Student's two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Anti-proliferative effects of 6-BIO and 7-BIO

To compare the two indirubin derivatives (6BIO and 7BIO) for their ability to retard growth and induce cell death in human breast cancer cell lines, we determined cell viability at 24 and 48 h after treatment with selected doses (0–50 μM) of the two derivates. The response of five human breast cancer cell lines (MCF-7,

Table 1
IC_{50} values of breast cancer cell lines treated with 6BIO or 7BIO. Values are expressed in μM and were calculated from corresponding data presented in Fig. 2. The data are expressed as the mean (±SE) of the results from three separate experiments.

Cell line	Treatment	
	6BIO	7BIO
MCF-7	21.2 (±5.7)	20.0 (±3.1)
ZR75	22.3 (±2.5)	16.7 (±2.9)
MDA-MB-231	17.0 (±3.6)	17.4 (±4.2)
MDA-MB-468	17.4 (±3.3)	6.6 (±1.0)
MDA-MB-231-TXSA	9.2 (±3.5)	2.3 (±4.6)

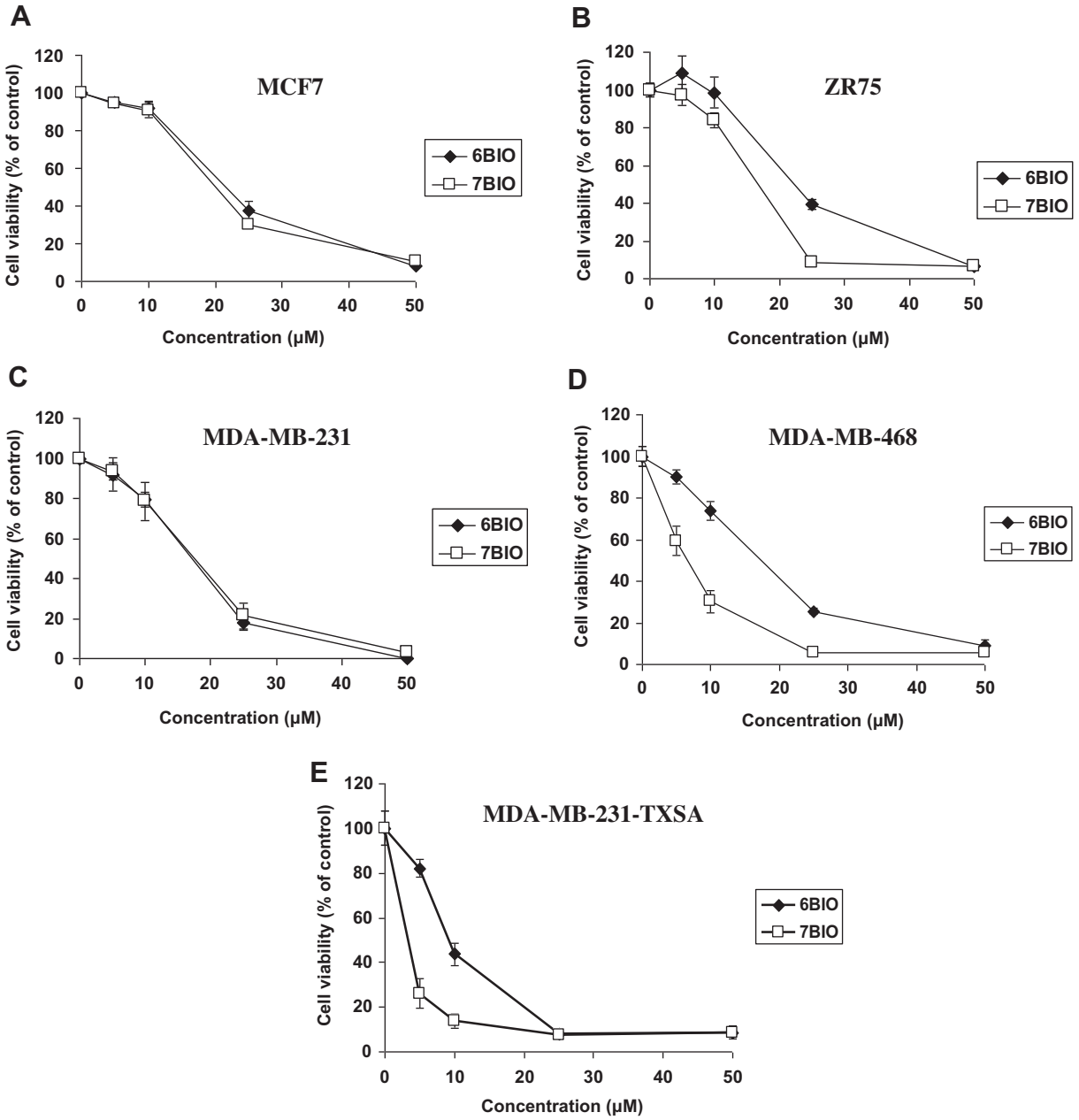

Fig. 2. Effect of 6-BIO and 7-BIO on the survival of (A) MCF-7, (B) ZR75, (C) MDA-MB-231, (D) MDA-MB-468 and (E) MDA-MB-231-TXSA cells. Cells were exposed for 48 h to increasing concentrations of 6BIO and 7BIO. Cell survival was estimated with the CellTiter-Blue cell viability assay and is expressed in percentage of survival in untreated cells. Average ± S.E. of at least three independent experiments with three independent measurements per experiment.

ZR75, MDA-MB-231, MDA-MB-468 and MDA-MB-231-TXSA) to the two derivatives is shown in Fig. 2. 6BIO and 7BIO reduced cell viability in a dose-dependent manner (Fig. 2A–E). The effect of 7BIO was almost identical to the effect of 6BIO in MCF-7 and MDA-MB-231 cells (Fig. 2A and C) whereas for the other three cell lines 7BIO was a stronger inhibitor (Fig. 2B, D and E). This is also reflected in the IC_{50} values presented in Table 1. MDA-MB-231-TXSA was the most sensitive of all cell lines examined with IC_{50} values of 9.2 μM (±3.56) for 6BIO and 2.3 μM (±4.63) for 7BIO (Table 1). Since the highly metastatic MDA-MB-231-TXSA breast cancer cells were the most sensitive to both agents the mechanism of apoptosis was thoroughly investigated in these cells.

3.2. Induction of apoptosis by 6-BIO and 7-BIO

The effects of the two indirubin derivatives on cell death were initially evaluated by a cell death ELISA assay which detects cytoplasmic histone-associated DNA-fragments produced after cell death. Both 6BIO and 7BIO increased apoptosis significantly at 24 h of treatment while no further increase was evident after 48 h of treatment (Fig. 3A). Apoptotic induction was also analyzed using the Tali™ Apoptosis Kit, where Annexin V and Propidium Iodide (PI) are used for determination of apoptotic cell death. Treatment with 6BIO and 7BIO for 24 h resulted in about 38% and 42% of the cells, respectively, staining positive for Annexin V (early

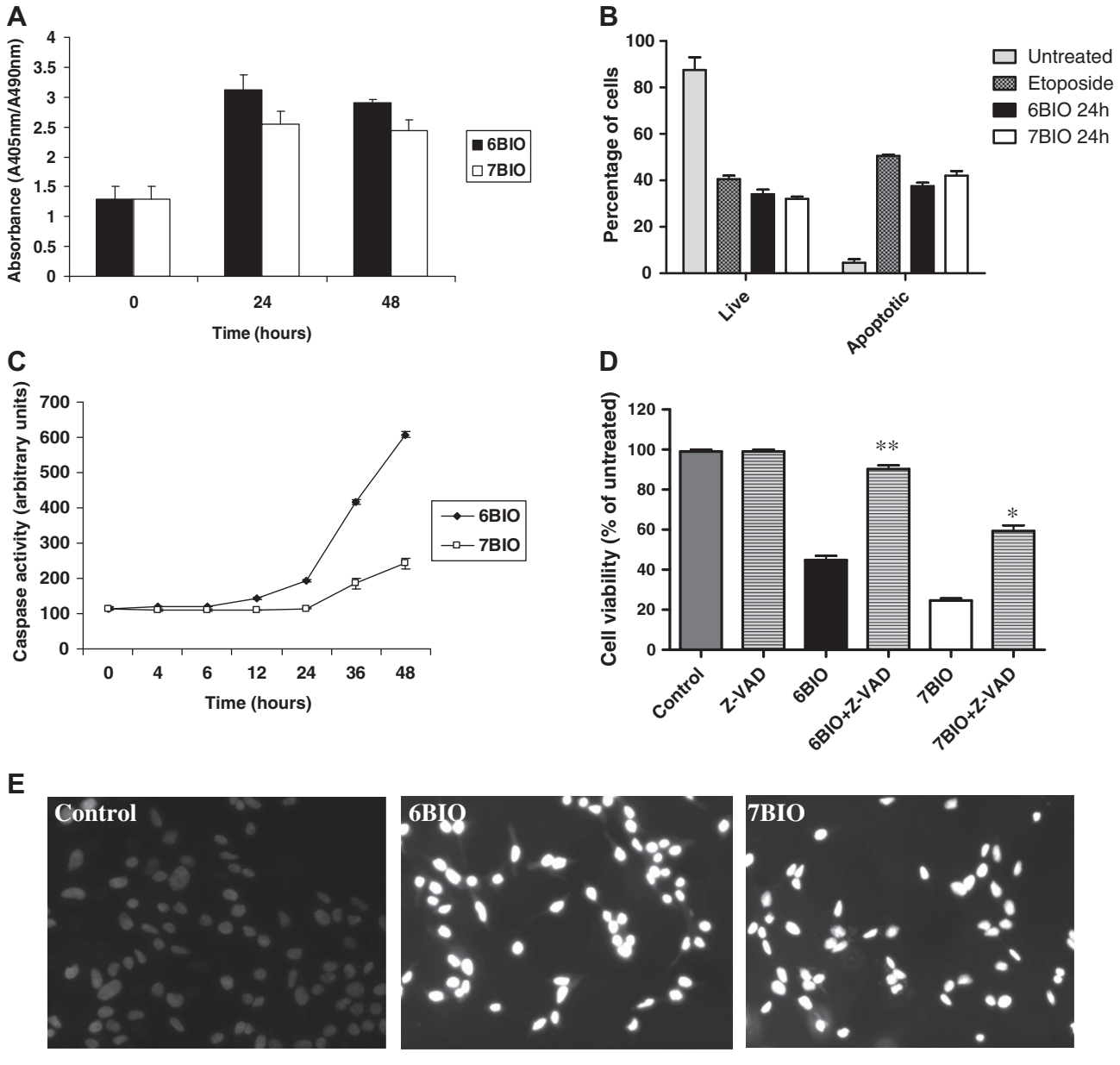

Fig. 3. Effect of 6-BIO (15 μM) and 7-BIO (15 μM) on apoptosis and caspase-3 activity in the MDA-231-TXSA tumor cells. (A) Tumor cells were treated with 6BIO or 7BIO for 24 h and 48 h as shown and analyzed for the presence of nucleosomes using the Cell Death Detection ELISA. (B) Effect of 6BIO and 7BIO on tumor cell apoptosis using Annexin-V Alexa FluorR 488/PI staining. Cells were plated in 60-mm plates and treated with either one of the test agents for 24 h. Cell viability and apoptosis were evaluated with the Tali™ Image-based Cytometer using the Tali™ apoptosis kit. As a positive control we used cells treated with 100 μg/ml etoposide. (C) Cells were left untreated or were treated for (0–48 h) with 6BIO or 7BIO and caspase-3 activity was evaluated using the caspase-3 specific fluorogenic substrate, zDEVD-AFC, as described in Material and Methods. (D) Tumor cells were treated with several concentrations of 6BIO or 7BIO for 48 h and cell viability was evaluated with CellTiter-Blue cell viability assay, in the presence or absence of 50 μM Z-VAD (OMe)-FMK, (p values: 6BIO vs. 6BIO + Z-VAD < 0.01**, 7BIO vs. 7BIO + Z-VAD < 0.05*). (E) Tumor cells were treated with 6BIO or 7BIO for 24 h and morphology of the cell nuclei were observed after DAPI staining using a fluorescence microscope.

apoptotic) (Fig. 3B). Treatment with etoposide (100 μg/ml), which was used as a positive control, resulted in 51% Annexin V positive cells.

To determine if apoptosis was associated with caspase-3 activation we treated MDA-MB-231-TXSA cells with various concentrations of the two indirubin derivatives for 24 h and measured its activity (data not shown). In a time response curve a small increase in caspase-3 activity was evident after 48 h of treatment with 7BIO (Fig. 3C). However, the effect of 6BIO, which became evident at earlier times, was much stronger at 48 h compared to that of 7BIO. In particular, 6BIO induced a 6-fold increase in caspase-3 activity (Fig. 3C). Consistent with this finding, the general caspase inhibitor Z-VAD (OMe)-FMK, increased the viability of the cells that had been treated with 6BIO to almost the control value while a smaller effect was evident in 7BIO/Z-VAD (OMe)-FMK -treated cells (Fig. 3D). These findings show that although both compounds induce apoptosis and caspase activity, 6BIO is a much stronger inducer of the caspase mediated pathway of apoptosis than 7BIO. The apoptotic nature of cell death was also confirmed morphologically with the DAPI staining method which revealed that both 6BIO and 7BIO -treated cells exhibit extensive condensed nuclear morphology (Fig 3E).

3.3. Effects of 6-BIO and 7-BIO on key proteins involved in apoptosis and cell cycle regulation

To elucidate the downstream signaling pathways activated during 6BIO and 7BIO -induced cell death, we evaluated the levels of key proteins known to participate in the apoptotic pathways. Immunoblotting analysis was performed at 0 (control), 6, 12, 24 36 and 48 h post-treatment with the antibodies indicated in Fig. 4. Treatment with 6BIO resulted in an increase in the cleavage of executioner caspase-3 that was associated with an enhanced proteolytic processing of caspase-9 and consistent cleavage of Poly (ADP-ribose) polymerase (PARP) (Fig. 4A and C). Interestingly, no

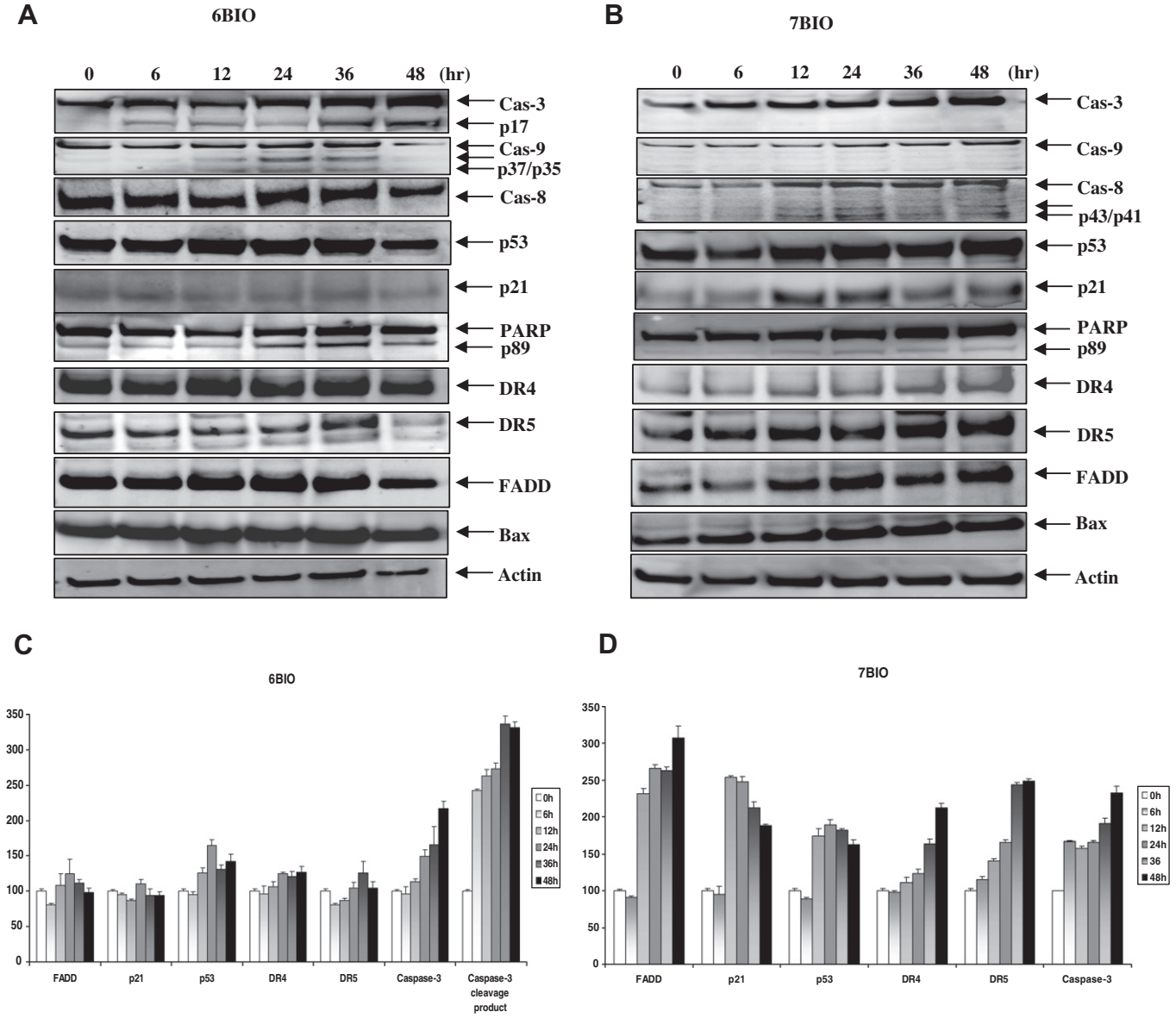

Fig. 4. Effects of 6-BIO and 7-BIO on key proteins participating in apoptosis and cell cycle regulation. MDA-MB-231-TXSA cells were treated with (A) 15 μM of 6BIO or (B) 15 μM 7BIO for the times indicated. Cells were then harvested and protein extracts were used in Western blotting as described in the see Section 2. Quantification of immunoblotting results of (C) 6BIO treatment and (D) 7BIO treatment was performed using the image analysis software Image Quant 5.2. Every point is the mean ± SE of two independent experiments with three independent measurements per experiment.

Table 2
Effect of indirubin derivatives on cell cycle distribution.MDA-MB-231-TXSA cells were treated with 15 μM 6BIO (A) or 15 μM 7BIO (B) for several time points. Distribution of cell cycle was monitored by flow cytometry. The data are expressed as the mean (±SE) of the results from three separate experiments.

	Hour			
Phase	0	6	12	24
(*A*)				
DNA Content (%)				
Sub-G1	3.7 (±0.7)	5.7 (±7.7)	21.1 (±1.7)	25.0 (±1.0)
G0/G1	41.0 (±1.3)	43.0 (±2.1)	48.8 (±0.2)	53.6 (±0.6)
S	22.2 (±0.9)	21.0 (±0.3)	8.8 (±1.2)	9.0 (±1.9)
G2/M	31.8 (±0.7)	29.2 (±0.4)	19.2 (±1.8)	11.1 (±0.6)
(*B*)				
DNA Content (%)				
Sub-G1	1.9 (±1.4)	2.5 (±0.4)	5.5 (±0.7)	39.4 (±2.4)
G0/G1	46.8 (±2.0)	38.3 (±1.7)	28.6 (±1.3)	34.6 (±1.4)
S	12.0 (±0.1)	9.6 (±1.3)	10.1 (±0.8)	7.5 (±1.9)
G2/M	38.6 (±0.7)	47.9 (±0.3)	54.4 (±0.9)	16.0 (±2.7)

cleavage of caspase-8 was observed following treatment with 6BIO (Fig. 4A). On the other hand, treatment with 7BIO resulted in a time-dependent increase in cleaved caspase-8 and an increase in the uncleaved levels of caspase-3 and caspase-9, with no associated cleavage. Furthermore, 7BIO increased the levels of FADD and Bax, PARP, p53 and p21 and the total protein levels of the death receptors DR4 and DR5 were elevated in a time dependent manner (Fig. 4B and D).The death receptors DR4 and DR5 associate through their functional cytoplasmic death domain motifs with FAS-associated death domain protein (FADD) upon activation by apoptotic signals (extrinsic death receptor pathway). Since FADD is known to be involved in the activation of caspase-8 [15], the increased surface expression of DR4 and DR5 observed in 7BIO-treated MDA-MB-231-TXSA cells is likely to contribute to the observed caspase-8 activation. DR4 and DR5 expression have been reported to be transcriptionally regulated by p53 [16,17]. Since the MDA-MB-231-TXSA cell line (derived from MDA-MB-231cells) has a mutant p53 [18], we can presume that the 7BIO-induced up-regulation of p21 is through a p53 independent mechanism. Several studies have reported increased p21 expression [19–21] or increased p21 protein stability [22,23] via p53 independent signaling pathways.

Finally, we determined the effects of 6BIO and 7BIO on cell cycle distribution (Table 2). 6BIO produced a time dependent increase in G_0/G_1 DNA content. This was accompanied by a decrease in the G_2/M phase. A time dependent increase of sub-G_1 phase was observed which is indicative of apoptosis. Interestingly, the accumulation of MDA-MB-231-TXSA cells in G_0/G_1 phase of the cell cycle after 6BIO treatment seems to be independent of p21 (Fig. 4A). In contrast, 7BIO produced a time dependent decrease in G_0/G_1 DNA content. This was accompanied by an initial increase in the G_2/M phase (0–12 h). An accumulation in G_2/M phase of the cell cycle and reduction of G_0/G_1 phase after 7BIO treatment is consistent with the observed increase in p21 levels. p21 inhibits the activity of the cyclin B1/Cdc2 complex [24], while GADD45 binds Cdc2 and disrupts its ability to complex with cyclin B. Therefore, the increased p21 protein levels in MDA-MB-231-TXSA cells treated with 7BIO strongly suggests that 7BIO induces cell cycle arrest at G_2/M phase. G2/M block is initiated as early as 6 h post treatment whereas apoptosis is observed at 24 h post treatment (Table 2B).

In previous studies 7BIO has been characterized as an effective cell killer acting in an apoptosis independent manner inducing necrotic cell death in the human neuroblastoma cell line SH-SY5Y and the T cell leukemia cell line Jurkat [8,11]. The death in these cell lines by has been reported to be independent of p53 [8]. Furthermore, IMR-5, IMR-32, Jurkat and HL-60 cells treated with 7BIO did not activate caspases and no apoptotic features were evident [8,9]. We investigated this possibility in our cell line using the "necrotic assay" of Cell Death Detection ELISA Plus kit. We found no evidence of necrotic cell death after treatment for up to 48 h with 15 μM 7BIO (data not shown).

The results presented here show conclusively that in MDA-MB-231-TXSA cells 7BIO induces clearly apoptotic cell death. This was shown with five different methods (shown in Fig. 3 and Table 2): DAPI staining (showing the characteristic morphology of apoptotic nuclear fragmentation), the ELISA assay (detecting nucleosomes), Annexin-V staining (detecting early apoptotic cells), caspase-3 activity assay and flow cytometry (detecting the number of cells in present in sub-G_1 phase of the cell cycle). However, the response of different cancer cell lines to 7BIO through apoptotic or non-apoptotic mechanisms seems to be a function of cell content. To this end we have found that the PC3 and DU-145 prostate cancer cells respond through non-apoptotic mechanisms to 7BIO (data not shown).

In summary, this study elucidates for the first time, the distinct molecular mechanisms underlying the apoptotic effects of 6BIO and 7BIO on a highly invasive breast cancer cell line. Based on the presented results it has been clearly established that in MDA-MB-231-TXSA cells 6BIO activates the intrinsic caspase-9 and caspase-3 mediated apoptotic pathway. In contrast, 7BIO activates both the caspase-8 extrinsic pathway (which is associated with the upregulation of DR4/DR5) and a caspase-independent pathway of apoptosis. The employment of different molecular modes of anti-tumor activity by two very closely related indirubin derivatives could serve as a valuable tool in combination with chemotherapy protocols for the treatment of highly metastatic breast cancer.

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