The pleiotropic profile of the indirubin derivative 6BIO overcomes TRAIL resistance in cancer

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TRAIL (TNF-related apoptosis-inducing factor) has been promoted as a promising anti-cancer agent. Unfortunately, many tumor cells develop resistance towards TRAIL due to numerous defects in apoptotic signaling. To handle this problem, combination therapy with compounds affecting as many different anti-apoptotic targets as possible might be a feasible approach. The bromo-substituted indirubin derivative 6BIO overcomes this challenge: Treatment of breast cancer and bladder carcinoma cell lines with micromolar concentrations of 6BIO abrogates cellular growth and induces apoptosis. Combination of subtoxic amounts of 6BIO with ineffective doses of TRAIL completely abolishes proliferation and long-term survival of cancer cells. As shown in two-dimensional as well as three-dimensional cell culture models, 6BIO potently augments TRAIL-induced apoptosis in cancer cell lines. The potent chemosensitizing effect of 6BIO to TRAIL-mediated cell death is due to the pleiotropic inhibitory profile of 6BIO. As shown previously, 6BIO abrogates STAT3, PDK1 as well as GSK3 signaling and moreover, inhibits the expression of the anti-apoptotic Bcl-2 family members Bcl-xL and Mcl-1 on mRNA as well as on protein level, as demonstrated in this study. Moreover, the expression of cFLIP and cIAP1 is significantly downregulated in 6BIO treated cancer cell lines.

In sum, the multi-kinase inhibitor 6BIO serves as a potent chemosensitizing agent fighting TRAIL-resistant cancer cells.

1. Introduction

A major cause in development and progression of cancer are defects in apoptosis pathways. Tumor cells develop modulated apoptotic mechanisms, thereby evading cell death and encompassing aberrant survival. Therefore, overcoming apoptosis resistance mechanism is one of the main goals in the therapy of cancer.

Tumor necrosis factor (TNF) α-related apoptosis inducing ligand (TRAIL) is a member of the TNF family that initiates apoptosis through binding to its receptors TRAIL-R1 and TRAIL-R2, thus leading to the formation of the death-inducing signaling complex (DISC), which consists of the receptors, the adaptor protein FAS-associated death domain (FADD) and caspase 8 or 10. Autoactivation of these caspases induces proteolytic cleavage of the effector caspase 3 and Bcl2 homology domain 3-interacting domain death agonist (BID). Activated caspase 3 in turn cleaves numerous intracellular targets, hence triggering apoptosis. Translocation of the truncated form of BID to the mitochondria induces cytochrome C release and activates the intrinsic apoptotic pathway [1]. Since recombinant TRAIL (also named Apo-2L) induces cell death in a wide variety of different cancer cells without showing any significant cytotoxicity in normal cells, it is a highly promising anti-cancer drug [2]. Unfortunately, many tumors, including breast and bladder cancer cells, are resistant to TRAIL-induced cytotoxic effects [3–5]. The insensitivity of the cells towards TRAIL is mediated by multiple defects in the TRAIL pathway, including inactive or decreased expression of TRAIL receptors, overexpression of anti-apoptotic proteins like the inhibitor of apoptosis proteins (XIAP, cIAP), cellular FLICE-like protein (cFLIP) and Bcl-2 family members Bcl-2, Bcl-xL, and Mcl-1 [6–10]. Moreover, it was reported that also activation of survival pathways like Akt, GSK3 and NFκB signaling cascades contributes to TRAIL resistance in numerous cancer cell types [11–13]. Thus, in order to potentiate TRAIL-induced cytotoxicity in cancer cells and to increase the
efficiency of cancer therapies, TRAIL sensitizers targeting the resistance mechanisms are urgently needed.

The indirubin derivative 6-bromo-indirubin-3'-oxime (6BIO) was originally synthesized and characterized as a very potent inhibitor of GSK-3β by the group of L. Meijer and L. Skalskounis in 2003 [14]. In a previous study of our group, we were able to show that 6BIO not only targets GSK-3β, but also PDK1/AKT and Jak/STAT3 signaling pathways in cancer cells [15]. Since it is known that these signaling cascades are implicated in apoptosis mechanisms by regulating the expression of multiple anti-apoptotic proteins, we aimed to elucidate the chemosensitizing effect of 6BIO to TRAIL-induced apoptosis in TRAIL-resistant breast and bladder cancer cells.

2. Materials and methods

2.1. Compounds

6-Bromo-indirubin-3'-oxime (6BIO) was partly synthesized by the authors (L. Meijer and L. Skalskounis) as well as purchased from Enzo Life Sciences (Farmingdale, NY, USA). SuperKiller/TRAII was obtained from Enzo Life Sciences, Paaclaxelf was from Sigma-Aldrich (St. Louis, MO, USA), Q-VD-Oph was from R&D Systems (Minneapolis, MN, USA). Recombinant human Apo2L/TRAII was a gift from Genentech (South San Francisco, CA, USA). MG-132 was purchased from Selleck Chemicals (Houston, TX, USA).

2.2. Cell culture

The breast cancer cell line MDA-MB-231 was obtained from CLS Cell Lines Service (Eppelheim, Germany) and cultured in DMEM containing 10% FCS and 0.1% penicillin/streptomycin at 37 °C and 5% CO₂. The urinary bladder carcinoma cell T24 was kindly provided by Dr. B. Mayer (University of Munich, Germany) and cultured in McCoy5A containing 10% FKS and glutamine.

2.3. Cell viability assay

1500 cells/well were seeded into 96-well-plates and treated as indicated. After a proliferation period of 72 h at 20 μl CellTiter-Blue™ reagent (CTB) from Promega (Madison, WI, USA) was added per well and incubated for 4 h at 37 °C. Using a Tecan SpectraFluorPlus™ microplate reader (Tecan, Männedorf, Switzerland) the reduction of the CTB dye resazurin into fluorescent resorufin, which correlates with the metabolic activity of the cells, was measured.

2.4. Clonogenic assay

Clonogenic survival of MDA-MB-231 and T24 cells treated with 6BIO and TRAIL, respectively, was analyzed as described previously [16].

2.5. Apoptosis assay

Subdiploid DNA content was determined according to Nicoletti et al. using a FACScalibur (Becton Dickinson, Heidelberg, Germany) [17]. Nuclei to the left of the G1-peak containing hypodiploid DNA were considered apoptotic.

2.6. Hoechst staining

20,000 cells/well were seeded into 8-well IBIDI µ-slides (IBIDI, Martinsried, Germany). Cells were treated as indicated for 48 h and stained with 125 ng/ml of the DNA intercalating dye Hoechst 33258® (Sigma-Aldrich) for 20 min. Fluorescent cells were analyzed using a Zeiss Axioskop 25 microscope (Zeiss, Oberkochen, Germany).

2.7. Western blotting

Proteins of 40 μg cell lysates were separated by SDS-PAGE and blotted onto a PVDF membrane (Immobilon-P, Millipore, Eschborn, Germany). The membranes were then blocked and incubated with BID (# 2002; Cell Signaling Technology, Danvers, MA, USA), Bcl-xL (#2762; Cell Signaling), cIAP1 (AF8181; R&D Systems) Caspase-3 (#610323; BD Biosciences, San Jose, CA, USA), Caspase-8 (#9746, Cell Signaling), FLIP (#8510: Cell Signaling), Mcl-1 (#4572; Cell Signaling), PARP (#9542; Cell Signaling) and actin antibody (#MAB1501R, Merck Millipore, Billerica, MA, USA) according to manufacturer’s instructions. Staining was performed using the ECL Prime Western Blotting Detection reagent (GE Healthcare, Buckinghamshire, UK). Band intensities were quantified using NIH ImageJ software (Bethesda, Maryland, USA).

2.8. Expression of TRAIL receptor 2 (DR5)

Cells were treated with indicated concentrations of 6BIO for 24 h, detached, washed, blocked with 1%BSA/PBS and incubated for 30 min with Alexa Fluor488 conjugated anti-TRAILR2 (R&D Systems, Minneapolis, MN, USA) or mouse IgG2b isotype control antibody, respectively. After further washing steps, surface expression of TRAILR2 was analyzed by flow cytometry using a FACScalibur (Becton Dickinson).

2.9. RNA isolation and reverse transcription

RNA was isolated and transcribed into cDNA using the RNEasy Mini Kit (Qiagen, Hilden, Germany) and the First-Strand cDNA Kit (Bio-Rad, Munich, Germany), respectively, according to manufacturer’s instructions.

2.10. Analysis of gene expression by quantitative real-time PCR

Quantitative real-time PCR was performed and analyzed on an ABI 7300 RealTime PCR system with the TaqMan Gene expression Assays using probe and primers for Mcl-1, Bcl-xL, cIAP1, FLIP and GAPDH (housekeeping gene) supplied as a mix by Life Technologies (Darmstadt, Germany). Calculation of relative mRNA levels in treated vs. nontreated cells was performed according to Pfaffl [18].

2.11. Luciferase reporter gene assay

MDA-MB-231 breast cancer cells were transfected with 1 μg of Stat3 reporter constructs (kindly provided by J. Bromberg, NY, USA) using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). For normalization, 0.1 μg of a pGL4.74 plasmid (Promega) was co-transfected. Twenty-four hours after transfection, the cells were treated with indicated concentrations of 6BIO or DMSO, respectively. Cellular lysis was performed after 24 h using Passive Lysis Buffer (Promega and luciferase activity was determined by using the Dual-Luciferase Reporter Assay (Promega) according to manufacturer’s instructions.

2.12. Intra-spheroidal cell death assay

Spheroids were generated according to the hanging drop protocol as described previously [19] and embedded in collagen [15]. To determine cell death, spheroids were stained on day 0 and day 4 after treatment with 125 ng/ml Hoechst 33258 for 4 h and 50 μg/ml PI per well for 1 h prior to confocal laser scanning microscopy using a Zeiss LSM 510 Meta microscope. A series of images in z-stacks was recorded, each image with a distance of 20.3 μm.
2.13. Chorioallantoic membrane (CAM) assay

CAM assay was done as described previously [20]. Briefly, 1 \times 10^6 T24 cells were resuspended in 10 μl NaCl 0.9% (B. Braun, Melsungen AG, Germany) and 10 μl Matrigel matrix (BD Biosciences, Heidelberg, Germany), implanted on the CAM of fertilized chicken eggs on day 8 of incubation and allowed to form tumors. The next day, tumors were treated with 10 μM 6BIO and/or 100 ng/ml TRAIL or DMSO daily for three days. Four days after inoculation, tumors were excised with the surrounding CAM, fixed in 4% paraformaldehyde, embedded in paraffin, cut in 3 μm sections and stained with 1:1 hematoxylin and 0.5% eosin solution for histological analysis.

2.14. Statistical analysis

Results are expressed as the mean ± SD (range) or as percentage value. Comparisons between groups were made using the One-way

![Fig. 1. Influence of 6BIO on cancer cell growth and survival.](image)

(A) Treatment of MDA-MB-231 and T24 cells with increasing concentrations of 6BIO for 72 h reduced the proliferation rate up to 50%. (B) Clonogenic growth of cancer cells is diminished by high concentrations of 6BIO (10 μM). Exemplary pictures of MDA-MB-231 and T24 cells treated with increasing concentrations of 6BIO are shown on the lower part. (C) Incubation of MDA-MB-231 and T24 cells with 3 μM 6BIO revealed no induction of cell death, whereas treatment with 10 μM 6BIO for 48 h resulted in a significant increase of cell death. 50 ng/ml paclitaxel (pac) was used as positive control. Bars always show the mean ± SD of 3 independent experiments with n = 3. ns: not significant; *p < 0.5; **p < 0.01; ***p < 0.001 (D) Hoechst staining showed enhanced fragmentation of nuclei in cells treated with 10 μM 6BIO compared to control cells after 48 h. (E) T24 spheroids were treated with 3 μM 6BIO, embedded in collagen and monitored over 3 days. Staining with propidium iodide (red) and Hoechst dye (blue) revealed enhanced cytotoxicity in spheroids incubated with 6BIO compared to DMSO control after 3 days (lower part), whereas at day 0 no induction of cell death is visible (upper part); (F) T24 cells were seeded on the CAM of chicken embryos and treated with 10 μM 6BIO and/or 100 ng/ml TRAIL or DMSO for 3 days. Tumor growth was analyzed using hematoxylin/eosin-stained paraffin sections of the CAM as described in Materials and Methods. Tumor area as percentage of the untreated control group is shown with mean ± S.E.M. of two independent experiments with 6 samples per group, *p < 0.05, (left panel); representative pictures of hematoxylin/eosin-stained sections are shown in the right panel, scale bars: 500 μM.
ANOVA/Dunnett and Student’s unpaired t-test. A p-value < 0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism software package (GraphPad Software Inc, San Diego, USA). Synergism was calculated according to Bliss [21].

3. Results

3.1. Proliferation and survival of cancer cells is inhibited by 6BIO

The breast cancer cell line MDA-MB-231 and urinary bladder carcinoma cells T24 were incubated with increasing concentrations of 6-Bromo-Indirubin-3’-oxime (6BIO) for 72 h and proliferation rates were determined. 6BIO dose-dependently diminishes growth of both cell lines up to 50% compared to control cells (Fig. 1A). Next, cells were treated with 6BIO for 4 h, freshly seeded and incubated for further seven days. As shown in Fig. 1B, long-term survival is significantly inhibited after treatment with high doses of 6BIO. Exemplary pictures of the wells are depicted in Fig. 1B, lower part. Analysis of cell death, either by determining the amount of cells containing hypodiploid DNA content or Hoechst staining, respectively, revealed that 10 μM 6BIO has cytotoxic effects on MDA-MB-231 and T24 cells (Fig. 1C and D). Incubation of the cells with 50 ng Paclitaxel (Pac) for 48 h was used as positive control for cell death induction (Fig. 1C).
In the following set of experiments, the impact of 6BIO on urinary bladder carcinoma cells grown as three-dimensional spheroids was analyzed. Therefore, spheroids were generated, treated with 6BIO, embedded in a collagen matrix and stained with propidium iodide (PI) and Hoechst. In order to assess intraspheroidal cell death, a series of images in z-stacks was recorded.
over 3 days, each image with a distance of about 20 μM. As shown in Fig. 1E, upper part, 6BIO as well as DMSO treated spheroids featured a similarly low number of PI positive cells on day 0, indicating that 6BIO does not affect cell death by an immediate action. After 3 days, the amount of PI positive cells increased dose-dependently in 6BIO treated spheroids (Fig. 1E, lower part). Cell death could be observed at various spheroid layers throughout the cross-sectional area, thus indicating that 6BIO efficiently diffuse even to the core zone of the tumor model. In addition, CAM (chick chorioallantoic membrane) assay confirmed the cytotoxic effects of 6BIO in a three-dimensional tumor model (Fig. 1F).

3.2. 6BIO synergizes with TRAIL to abrogate cell proliferation and induce apoptosis

Subsequently, a potential chemosensitizing effect of the multi-kinase inhibitor 6BIO in combination with the well-known anti-cancer drug TRAIL was examined. Therefore, MDA-MB-231

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

Fig. 3. 6BIO in combination with TRAIL induces caspase-dependent cell death. (A) Apoptosis of cells treated with a high concentration of 6BIO (10 μM) was partly blocked by simultaneous treatment with the pan-Caspase inhibitor QVD for 48 h (i). QVD completely abrogated apoptosis in cells treated with a combination of 3 μM 6BIO and 25 ng/ml TRAIL (ii). (B) Western blot analysis showed reduced expression of the mature caspase-3 and enhanced PARP cleavage in cells incubated with increasing concentrations of 6BIO for 48 h. (C) Combined treatment with 3 μM 6BIO and 25 ng/ml TRAIL resulted in cleavage of caspase-8, BID, caspase-3 and PARP after 48 h. Band densities were quantified and normalized to actin. Bars show the mean ± SD of 3 independent experiments. Measurements were performed in triplicate. *p < 0.5.
and T24 cells were treated with small doses of 6BIO in combination with 25 ng/ml TRAIL. As shown in Fig. 2A, 3 μM 6BIO led to an about 40% reduction of the proliferative capacity, whereas in combination with TRAIL cellular growth was completely abrogated. Further investigations revealed that 3 μM 6BIO in combination with 25 ng/ml TRAIL synergistically reduced the long-term survival of the cancer cells up to 60% compared to each drug alone (Fig. 2B, exemplary pictures of MDA-MB-231 cells are displayed in the lower part of the figure).

To determine whether the growth inhibitory effect of 6BIO and TRAIL was due to induction of apoptosis, MDA-MB-231 as well as T24 cells were incubated with 6BIO in combination with or without TRAIL. As shown in Fig. 2C, whereas 3 μM 6BIO alone had virtually no effect on cell death, combined treatment with 25 ng/ml TRAIL resulted in a more than 3-fold induction of apoptosis compared to each drug alone. In addition, Hoechst staining showing compact and fragmentized nuclear chromatin confirmed enhanced cell death rates in cells treated with low concentrations of 6BIO (3 μM) in combination with TRAIL (25 ng/ml) (Fig. 2D). Next, spheroids were treated with 3 μM 6BIO in the presence of 10 ng/ml TRAIL and intra-spheroidal cell death was monitored after 3 days. Compared to single agent treatment, combination therapy severely promotes the cytotoxic effects in three-dimensional spheroids (Fig. 2E). By comparison, the addition of TRAIL did not further reduce tumor growth as compared to treatment with 6BIO alone in the CAM model (Fig. 1F).

To further unravel the apoptosis induction mediated via 6BIO and TRAIL, cells were treated with the pan-Caspase inhibitor QVD. Indeed, additional treatment with QVD inhibited apoptosis induced by 10 μM 6BIO up to 50% in MDA-MB-231 and T24 cells (Fig. 3A i). Moreover, in the presence of TRAIL, QVD completely abrogates cell death in both cancer cell lines, thus indicating that 6BIO in combination with TRAIL augments caspase-dependent apoptosis (Fig. 3A ii). Western Blot experiments showed decreased expression of the mature form of caspase-3 and enhanced cleavage of its main substrate PARP in cells incubated with increasing concentrations of 6BIO for 48 h (Fig. 3B). 6BIO in combination with TRAIL led to strong activation of the extrinsic caspase cascade, as shown by cleavage of the initiator caspase-8, its downstream target BID and the main effector caspase-3. Subsequently, PARP was processed in cells incubated with 6BIO and TRAIL for 48 h (Fig. 3C).

3.3. Underlying mechanism of 6BIO mediated chemosensitization

In order to elucidate the molecular mechanism being associated with the chemosensitizing impact of 6BIO to TRAIL, key players known to be involved in TRAIL resistance were investigated. As shown in Fig. 4A, incubation of the cancer cell lines with increasing concentrations of 6BIO for 24 h results in an elevation of TRAILR2 (DR5) expression on the cell surface as measured via FACS analysis. Furthermore, 6BIO treatment for 48 h led to a strongly diminished expression of the anti-apoptotic proteins Mcl-1, Bcl-xL, FLIP and cIAP1 in MDA-MB-231 and T24 cells, indicating that 6BIO targets both the extrinsic apoptotic pathway by abrogating FLIP and cIAP1 expression, as well as the intrinsic pathway via downregulation of Mcl-1 and Bcl-xL levels (Fig. 4B).

Subsequently, we aimed to gain further insights into the 6BIO-dependent regulation of these anti-apoptotic proteins. Treatment of MDA-MB-231 cells with increasing concentrations of 6BIO dose-dependently decreased the mRNA expression of Mcl-1, Bcl-xL, FLIP and cIAP1 after 6 h (Fig. 4C).
Former studies identified the transcription factor STAT3 as an important positive regulator of the expression of the anti-apoptotic proteins cIAP1, FLIP and the Bcl-2 family members Mcl-1 and Bcl-xL [22–24]. Moreover, it is known, that STAT3 signaling pathways are constitutively active in breast and bladder cancer cells [25]. Therefore, in line with our previous study [15], the influence of 6BIO on the activity of STAT3 was analyzed. As shown by luciferase reporter gene assays, treatment of MDA-MB-231 breast cancer cells with increasing concentrations of 6BIO clearly abrogated STAT3 promoter activity (Fig. 4D), thus indicating that the observed downregulation of Mcl-1, Bcl-xL, cIAP1 and FLIP mRNA levels in 6BIO treated cells is mediated at least partly via inhibition of STAT3.

Next, to reveal whether 6BIO is also capable to regulate the protein levels of Mcl-1, Bcl-xL, FLIP and cIAP1 via proteasome-mediated degradation, cells were treated with 6BIO in combination with MG132, a well-known proteasome inhibitor. As shown in Fig. 4E, the 6BIO mediated downregulation of Mcl-1 is inhibited by MG132, whereas protein levels of Bcl-xL, FLIP and cIAP1 are not rescued by additional treatment with the proteasome inhibitor. These data demonstrate that 6BIO regulates the expression of...
numerous anti-apoptotic proteins both on mRNA and on protein level.

Combined treatment of MDA-MB-231 and T24 with low doses of 6BIO in combination with TRAIL led to a dramatic decline of the pro-survival proteins Mcl-1, Bcl-xL, FLIP and cIAP1 compared to each drug alone (Fig. 5), thus further illustrating the mode of action how 6BIO is able to overcome the resistance mechanism of TRAIL induced apoptosis in breast and bladder cancer cells.

4. Discussion

Since apoptosis is aberrantly regulated in many cancer types, which subsequently leads to uncontrolled growth of tumors and development of resistance to chemotherapeutic agents, compounds that restore the normal apoptotic pathway serve as promising drugs in treatment of cancer [2]. In the present study, we demonstrated that treatment of breast and bladder carcinoma cell lines with subtoxic concentrations of the indirubin derivative 6BIO results in diminished expression of the anti-apoptotic proteins Mcl-1, Bcl-xL, FLIP and cIAP1, hence strongly sensitizing to TRAIL-induced apoptosis.

It was previously shown that members of the indirubin family and their derivatives induce apoptosis in several cancer types, e.g. breast cancer, melanoma, colon and pancreatic cell lines [26–29]. This is in line with our study showing inhibition of proliferation, diminished long-term survival and increased apoptosis rate in breast and bladder carcinoma cells treated with micromolar concentrations of the bromo-substituted indirubin derivative 6BIO. Even more interestingly, subtoxic amounts of 6BIO in combination with TRAIL completely abrogate the proliferative capacity of the cancer cells. Whereas TRAIL alone has virtually no effect on cytotoxicity, combined treatment of these cancer cells with 6BIO led to profound synergistic effect on apoptosis induction as shown in two- as well as three-dimensional cell culture experiments. In a CAM based tumor model TRAIL alone has also no cytotoxic effect but rather induces tumor growth. Consequently, combination treatment was not as efficient in inhibiting cellular growth as 6BIO alone. It might well be that TRAIL due to the CAM specific tumor microenvironment activates different signal transduction pathways in the CAM model compared to two or three-dimensional cell culture conditions which may account for the lack of cooperativity of 6BIO and TRAIL that we observed in our present study.

Indeed, although the usage of TRAIL (tumor necrosis factor (TNF) – related apoptosis – inducing ligand) is a promising therapeutical approach to treat various cancer types effectively, multiple different mechanisms of TRAIL resistance have been identified so far. Next to diminished expression and/or activity of the TRAIL receptors DR4/DR5 on the surface of the cancer cells and upregulation of several anti-apoptotic proteins during cancer
progression, insensitivity to TRAIL induced cell death is frequently based on deregulation of multiple signaling cascades [1,30]: For instance GSK3 signaling affects TRAIL induced cytotoxicity in hepatocellular as well as pancreatic cancer cells [12,31]. Moreover, enhanced activity of the PDK1 downstream target Akt accounts for the insensitivity of the cancer cells to TRAIL mediated apoptosis [11]. Besides, the STAT signaling pathways have a fundamental impact in regulation of apoptosis: Members of the Bcl-2 family, namely Bcl-xL and Mcl-1, that act as inhibitor of the intrinsic apoptosis pathway by preventing the release of cytochrome C from mitochondria to cytosol, are important downstream targets of STAT3 and known to be involved in TRAIL resistance [32–35]. In sum it is quite evident that fighting TRAIL resistance strongly demands the application of a multifunctional inhibitor for combination therapy. 6BIO perfectly meets this challenge as demonstrated by our work.

It inhibits the activity of the transcription factor STAT3, which subsequently diminishes Bcl-xL mRNA and protein expression. This is in line with the study of Kim et al., demonstrating that cancer cells harboring increased expression of Bcl-xL are strongly resistant to TRAIL [9]. In addition, treatment of cells with 6BIO results in reduced mRNA and protein expression of Mcl-1 and triggers the proteasomal degradation of Mcl-1. Quite recently, several studies illustrated that TRAIL resistance is conferred to enhanced protein expression of Mcl-1 [10]. A variety of cancer cell lines show enhanced sensitivity towards TRAIL when Mcl-1 is downregulated by the anti-cancer drug sorafenib [36–38]. Thus, by inhibiting several important antiapoptotic members of the Bcl-2 family, namely the STAT3 downstream targets Bcl-xL and Mcl-1, the chemosensitizing effect of 6BIO to TRAIL induced apoptosis is well founded.

Next to the proteins acting on the intrinsic apoptosis pathway also components of the extrinsic apoptosis cascade are implicated in TRAIL resistance [39]. FLIP (cellular FLICE-like inhibitory protein), which is regulated by PI3K/Akt signaling cascades, is a non-redundant antagonist of the initiator caspases 8 and 10, preventing their binding to the DISC (death inducing signaling complex) and subsequently inhibiting activation of effector caspases and apoptosis [40,41]. As shown previously, overexpression of FLIP is linked with TRAIL resistance in many tumors and consequently, downregulation of FLIP increases TRAIL induced cytotoxicity [42,43]. This could be substantiated by our study, showing that by abrogating the protein levels of FLIP by using the PDK1 inhibitor 6BIO, the cytotoxic effect of TRAIL in MDA-MB-231 as well as in T24 cells is restored.

Further targets of 6BIO that are implicated in apoptosis regulation are members of the IAP (inhibitor of apoptosis proteins) family. By inhibiting the activation of effector caspases and regulating the ubiquitin-dependent activation of NFκB (nuclear factor-kB) these proteins promote cancer cell survival and proliferation [44]. Different studies already used IAP antagonists in combination with chemotherapeutics and death receptor ligands, especially TRAIL, and showed strong synergistic effects on apoptosis induction in several cancer types [45,46]. As the expression of IAP members is regulated at least partly by STAT3 and PDK1 [47,48], 6BIO is capable to diminish the protein levels of

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**Fig. 5.** Combined treatment with 6BIO and TRAIL abrogate expression of pro-survival proteins.
Expression of anti-apoptotic proteins Mcl-1, Bcl-xL, FLIP and cIAP1 is strongly downregulated in cancer cells treated with 3 µM 6BIO in combination with 25 ng/ml TRAIL for 48 h. Arrows indicate major bands of FLIP and cIAP1. Quantification of protein expression was normalized to actin using NIH Imagej software.
Not available.