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Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative - enhancement of both extrinsic and intrinsic apoptosis pathways

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Abstract

Until today effective therapies are lacking for metastatic melanoma. The death ligand TRAIL appears as promising in cancer treatment; however, melanoma cells reveal both preexisting and inducible TRAIL resistance. Here, we present evidence that the recently described indirubin derivative 8-Rha-β enhances melanoma cell sensitivity for death ligands and overcomes resistance to TRAIL and CD95 agonists. Indirubin is known from traditional Chinese medicine and is a potent kinase inhibitor. Unraveling of apoptotic signaling pathways revealed that TRAIL resulted in a quick (within 8 h) downregulation of both agonistic TRAIL receptors DR4 and DR5, in a kind of negative feed-back loop. Treatment with indirubin, however, mediated upregulation of both receptors, thus compensating this negative feed-back loop by TRAIL. Furthermore, indirubin activated intrinsic apoptosis pathways, seen in loss of mitochondrial membrane potential and release of cytochrome c. The mitochondrial response appeared as related to upregulation of Bax and Bad and to downregulation of Mcl-1. Remarkably, indirubin in combination with TRAIL was also able to overcome apoptosis resistance due to ectopic Bcl-2 overexpression. The tumor suppressor p53 appeared as master regulator of these propapoptotic changes and is the transactivator of proapoptotic proteins which was upregulated by indirubin. Taking into account the physiological role of death ligands in immune surveillance, sensitization of melanoma cells for death ligands may be supportive for an anti-tumor immune response. Furthermore, combinations with kinase inhibitors, such as indirubin 8-Rha-β may help for a breakthrough of TRAIL-mediated strategies in melanoma.

Running title: Indirubin derivative sensitizes for death ligands

Keywords: apoptosis, melanoma, death ligands, indirubin, p53

1. Introduction

Malignant melanoma revealed increased incidences over the past decades and is characterized by an unbroken high mortality [1]. The high metastatic potential and its resistance to conventional chemotherapy is of major importance, related to defects in proapoptotic signaling [2;3]. Overcoming apoptosis resistance is therefore a promising target.

Two main proapoptotic pathways (extrinsic and intrinsic) have been described. The extrinsic pathway is initiated by the binding of death ligands to cell surface death receptors leading to the formation of a death inducing signaling complex (DISC), where initiator caspases 8 and 10 are activated [4]. On the other hand, the intrinsic apoptosis pathway is triggered by intracellular signals such as cellular and DNA damage, also when caused by chemotherapy. Key events are the depolarization of the mitochondrial membrane potential and the release of mitochondrial factors such as cytochrome c into the cytosol, which results in activation of initiator caspase-9 [5;6]. Both pathways may meet at the mitochondria due to caspase-8-mediated activation of the proapoptotic Bcl-2 protein Bid [7]. Mitochondrial activation is critically controlled by the family of Bcl-2 proteins, which consists of antiapoptotic proteins (e.g. Bcl-2, Bcl-x_L and Mcl-1), proapoptotic multidomain proteins (Bax and Bak) and proapoptotic BH3-only proteins (e.g. Bid, Bad, Puma and Noxa) [8]. According to these pathways, caspase cascades appear as central in apoptosis regulation. Initiator caspases cleave and activate downstream effector caspases as caspase-3, which then target a number of death substrates to set apoptosis into work [5;9].

The death ligand TRAIL (TNF-related apoptosis-inducing ligand) appeared as a promising strategy in cancer therapy due to selective killing of tumor cells, whereas normal cells were largely spared [10]. It binds to two agonistic death receptors, DR4/TRAIL-R1 and DR5/TRAIL-R2 [11]. The downstream signaling cascade includes receptor oligomerization, formation of the DISC, activation of initiator caspases, cleavage of effector caspases and

finally DNA fragmentation [12]. In parallel, TRAIL may also trigger additional pathways such as nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinases [13]. In a previous study, we have shown the significant role of DR4 in mediating TRAIL sensitivity in melanoma cells, and as the majority of melanomas revealed immunoreactivity for DR4, TRAIL and DR4-based strategies appeared as promising, also for melanoma [14]. However, pre-existing TRAIL resistance was also seen and in addition, melanoma cell lines may acquire induced resistance upon TRAIL treatment [15].

Indirubin, the red isomer of indigo, is the active ingredient in a traditional Chinese medicinal recipe [16]. It is a potent kinase inhibitor targeting GSK-3β, CDKs (cyclin dependent kinases), c-Src and FGF-R1 (fibroblast growth factor receptor) [17-19]. Here, we show the potential of a recently described indirubin derivative 8-Rha-β [20] to sensitize human melanoma cells for death ligand-induced apoptosis. This effect is mediated by enhancing both extrinsic and intrinsic apoptosis pathways.

2. Materials and methods

2.1 Cell culture

Four human melanoma cell lines were investigated: death ligand sensitive Mel-HO and A-375 as well death ligand resistant Mel-2a and MeWo [15]. TRAIL-resistant subclones of Mel-HO and A-375 (Mel-HO-TS; A-375-TS) derived from a selection with 5 ng/ml TRAIL [15]. CD95-resistant subclones of A-375 (A-375-CS) were generated by selection with 20 ng/ml CH-11 agonistic CD95 antibody. Selected cells were continuously cultured with the respective death ligand. As previously described, other subclones of A-375 resulted from stable transfection with a pIRES-Bcl-2 construct (A-375-Bcl-2) or the pIRES empty plasmid (A-375-Mock) [21]. The pIRES plasmid originated from Clontech (Palo Alto, CA, USA).

Melanoma cells were cultivated at 37°C, 5% CO_2 in DMEM (4.5 g/l glucose; Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and antibiotics (Biochrom, Berlin, Germany). Experiments were performed in 6-well, 24-well or 96-well plates (200,000, 50,000 or 1,250 seeding density per well, respectively). For induction of apoptosis, soluble human killerTRAIL (Alexis, Gruenberg, Germany ALX-201-073-C020; 20 ng/ml) or the agonistic monoclonal CD95 antibody CH-11 (Beckman Coulter, Marseille Cedex, France PN IM 1504; 50 ng/ml) were applied. As indicated, incubation with death ligands took place for 8-24 h. Treatment with indirubin 8-Rha-β, described previously [20], was set up in parallel with death ligands, whereas control cells received only the solvent DMSO (Applichem, Darmstadt, Germany). For inhibition of caspases, cells were preincubated for 1 h with 10 µM of the pancaspase/panprotease inhibitor zVAD-fmk (R&D Systems, Wiesbaden, Germany), which binds the active sites of caspase-like proteases.

In further experiments, the chemotherapeutic substances doxorubicin, paclitaxel and vinblastine (Sigma, Taufkirchen, Germany) were applied in concentrations of 1, 10 and 100

nM. Simultaneous treatments were used for combinations of chemotherapeutics and indirubin 8-Rha-β (24 h).

2.2 Real-time cell analysis and cell viability assay

For obtaining growth curves, cell confluence was continuously monitored by real-time cell analysis (RTCA, xCELLigence, Roche diagnostics; Penzberg, Germany). The technique is based on microelectrodes integrated in the bottom of each well of special 96-well E-plates. The electric impedance corresponds to the cell density. 1,250 cells were seeded per microtiter well, and treatment started after 24 h. The impedance was determined up to 120 h after seeding.

For monitoring cell viability and proliferation, a colorimetric assay (WST-1, Roche diagnostics, Mannheim, Germany) was used. A number of 1,250 cells per well was seeded in 96-well plates and treated the next day for 24 h with 20 ng/ml TRAIL, 2.5 µM indirubin or the combination. For 100 µl culture medium per well, 10 µl WST-1 reagent were added, following by incubation of the cells at 37°C between 0.5 to 4 h, until color changes were seen. The optical density of triplicated samples was determined several times at 450 nm in an ELISA reader. For background correction, the values of untreated controls were subtracted.

2.3 Quantification of apoptosis and cytotoxicity

For quantification of apoptosis, cell cycle analyses were carried out after propidium iodide staining, according to Nicoletti et al. [22]. Cells were harvested by trypsinisation, stained with PBS buffer containing triton-X 100, sodium citrate and propidium iodide (Sigma, Taufkirchen, Germany; 200 mg/ml), centrifuged, washed with PBS and analyzed by flow cytometry in a FACS Calibur. Cytotoxicity was determined in parallel by measuring LDH activity in culture fluids applying a cytotoxicity detection assay (Roche Diagnostics).

2.4 Western blot analysis

For protein analysis, cells were lysed in 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2 mM PMSF; 1 mM leupeptin; 1 mM pepstatin; 0.5% SDS and 0.5% Nonidet P-40. For cytochrome c, AIF and VDAC, cytosolic and mitochondrial cell fractions were separated by a mitochondria/cytosol fractionation kit (Alexis, Gruenberg, Germany). Protocols for protein extraction and Western blot analysis were described previously [23].

The following primary antibodies were used: cleaved caspase-3 (Cell Signaling, rabbit, 1:10000), caspase-3 (Cell Signaling, rabbit, 1:1000), caspase-8 (Cell Signaling, mouse, 1:1000), caspase-9 (Cell Signaling, rabbit, 1:1000), PARP (Biomol, mouse, 1:5000), Bid (Cell Signaling, rabbit, 1:1000), Mcl-1 (Santa Cruz, mouse, 1:200), Bcl-2 (Santa Cruz, mouse, 1:200), Bax (Santa Cruz, rabbit, 1:200), Bak (Dako, rabbit, 1:500), Bad (Cell Signaling, rabbit, 1:1000), Puma (Epitomics, rabbit, 1:1000), Noxa (Pro Sci, rabbit, 1:500), cytochrome c (BD Biosciences, mouse, 1:1000), AIF (Santa Cruz, goat, 1:200), anti-Porin 31 HL (VDAC) (Calbiochem, mouse, 1:5000), XIAP (Cell Signaling, rabbit, 1:1000), Survivin (Santa Cruz, mouse, 1:500), DR4 (abcam, rabbit, 1:500), DR5 (abcam, rabbit, 1:500), p53 (Santa Cruz, rabbit, 1:500) and GAPDH (Santa Cruz, mouse, 1:200).

2.5 Mitochondrial membrane potential

For determination of mitochondrial membrane potential, the fluorescent dye TMRM (Tetramethylrhodamine methyl ester perchlorate) was used. Cells were harvested by trypsinisation, stained with TMRM (Sigma-Alderich, Taufkirchen, Germany; 1 nM, 15 min; 37°C), washed once with PBS buffer and analyzed in PBS by flow cytometry.

2.6 Determination of ROS

For measurement of intracellular ROS levels, the fluorescent dye H_2DCFDA (20,70-dichlorodihydrofluoresceindiacetate) was used. Cells were harvested by trypsinisation, stained with H_2DCFDA (Molecular Probes, Invitrogen, Eugene, Oregon, USA; 15 mM, 30 min), washed once with PBS buffer and analyzed in PBS by flow cytometry.

2.7 Surface Expression of DR4 and DR5

Cells were seeded in 6-well plates ($2x10^5$ cells/well) and treated for 8 h with indirubin or TRAIL, alone or in combination. Then, cells were harvested with 500 µM EDTA, stained with the primary mouse monoclonal antibodies for TRAIL-R1/DR4 (Alexis, clone HS101, ALX-804-297-C100, 1:100), or TRAIL-R2/DR5 (Alexis, clone HS 201, ALX-804-298-100, 1:100), or for control with mouse IgG1 (Ancell, # 278-010). After 15 min of incubation at 4°C and 15 min at room temperature, cells were centrifuged, washed twice with PBS/1% BSA and resuspended in 50 µl PBS/BSA containing the secondary antibody for mouse IgG1 (Alexis, ALX 211-201-050, 1:100). After 30 min incubation at room temperature, cells were centrifuged, washed twice, resuspended in 200 µl of PBS and analyzed by flow cytometry.

2.8 Statistical analyses

Assays consisted of duplicate or triplicate values, and two or three independent experiments were performed. Normal distribution of the samples was proven by the Kolmogorov-Smirnov test. In case of normal distribution, which applied to most of our data sets, the Student's t-test was used. In case that the Kolmogorov-Smirnov test did not prove normal distribution of the data, the Wilcoxon test had to be applied for proving statistical significance. Mean values and SDs were calculated by enclosing all individual values of the independent experiments (at least 6 samples), and a p-value of < 0.05 was considered as statistically significant.

3. Results

3.1 Melanoma cell culture models of TRAIL resistance

We have previously described pre-existing and inducible TRAIL resistance in human melanoma cell lines [15]. MeWo and Mel-2a (DR4-, DR5+) revealed resistance, whereas A-375 (DR4+, DR5+) and Mel-HO (DR4-, DR5+) were sensitive. In addition, A-375 and Mel-HO developed an inducible resistance upon continuous cultivation with TRAIL leading to selected resistant cell populations (Mel-HO-TS, A-375-TS). The apoptotic response to TRAIL of these six cell culture models is demonstrated by determination of sub-G1 cell populations at 24 h of TRAIL treatment (20 ng/ml). At this time, A-375 revealed strongly increased number (18%) and Mel-HO revealed moderately increased (4%) number of apoptotic cells, whereas the other four cell lines remained resistant (Fig 1A). Cytotoxicity was unaffected at this time, as determined by LDH in the cell culture supernatants (Fig 1B).

For targeting melanoma cells, we used the indirubin derivate 8-Rha-β. The synthesis as well as induction of apoptosis and decreased cell proliferation in melanoma cell lines SK-Mel-29 and SK-Mel-147 have been recently described by our group [20]. Decreased cell proliferation by indirubin 8-Rha-β treatment (2.5 µM) was also seen in A-375-TS cells (Fig 1C), which was accompanied by a G2 arrest, seen in A-375, Mel-2a and A-375-TS (Fig 1D).

3.2 Indirubin sensitizes melanoma cells for death ligand-induced apoptosis

Aiming at a sensitization of melanoma cells for death ligand induced apoptosis, we applied different combinations of the new indiribin 8-Rha-β and death ligands in our cell culture models. Significant induction of apoptosis by indirubin itself (10 µM, 24 h treatment) was seen in Mel-2a (4%), A-375 (7%) and A-375-TS (4% apoptotic cells; Fig 2A). Most strikingly, however, was the synergistic enhancement of apoptosis by the combination of indirubin and TRAIL, resulting in increased sub-G1 cell populations in Mel-HO (15%), A-

375 (32%), Mel-2a (14%) and A-375-TS (12%). Also, visible effects such as reduced cell numbers, rounded cells and cell detachment were clearly evident. In contrast, MeWo and Mel-HO-TS remained resistant (Fig 2A).

Enhanced apoptosis also exerted a strong impact on cell proliferation, monitored for A-375-TS by real-time cell analysis. In these selected cells, TRAIL treatment did not decrease cell proliferation, cells rather showed an enhanced proliferation in response to TRAIL. The combination of indirubin and TRAIL, however, appeared as highly efficient (Fig 2B).

For investigation of the effects of indirubin on apoptosis sensitivity via the CD95/Fas system, we selected A-375 cells for CD95 resistance by continuous incubation with the agonistic CD95 antibody CH-11. The resultant CH-11-selected A-375-CS cells revealed a significantly reduced apoptotic response to CD95 stimulation (5% vs. 12% in parental cells). Indirubin 8-Rha-β was able to enhance the sensitivity of A-375 parental cells to CH-11 as well as it reduced the resistance of A-375-CS (Fig 2C; 2D).

Both selected A-375 cell populations revealed cross-resistance for the respective other death ligand. Thus A-375-TS was largely resistant to CH-11, and A-375-CS was largely resistant to TRAIL, suggesting parallel pathways to death ligand resistance. These cross-resistances were also overcome by combinations with indirubin (Fig 2E). For understanding how general the apoptosis sensitization by indirubin was, it was combined with the chemotherapeutics vinblastine, paclitaxel and doxorubicin, respectively. Therefore, the cell line A-375 was simultaneously treated with 8-Rha-β (10 μM) and chemotherapeutics in concentrations of 1, 10 and 100 nM (vinblastine, doxorubicin) or 1 and 10 nM (paclitaxel). For vinblastine, some enhancement of apoptosis was seen in the combinations, however less than additive effects (Fig 3A). Paclitaxel at 10 nM revealed a strong proapoptotic effect on A-375, which was however diminished by the combination with 8-Rha-β (seen in two independent experiments, $p < 0.005$). Only the combination with 100 nM doxorubicin showed a significant

enhancement of apoptosis by about two-fold, when compared to 8-Rha-β single treatment ($p < 0.005$). Cytotoxicity was unaffected for all treatments at this time (24 h) (Fig 3B). A complete series of these combination experiments was also performed with A-375-TS. These cells revealed essentially a similar response to chemotherapeutics as the parental cell line, namely enhancement in the combination with 100 nM doxorubicin but antagonistic effects with 10 nM paclitaxel (data not shown).

3.3 Activation of the full caspase cascade by combination with indirubin with TRAIL

For understanding the way of indirubin-mediated apoptosis sensitization, activation of initiator and effector caspases was investigated in A-375-TS cells by Western blot analysis. The extrinsic caspase cascade with typical cleavage products of the initiator caspase-8 and the main effector caspase-3 was seen in A-375 parental cells upon TRAIL treatment. Further loss of the proform of Bid and processing of caspase-9 were indicative for an amplification loop through the mitochondrial/intrinsic pathway. As a result of caspase-3 activity, cleavage of its characteristic substrate PARP (poly-ADP-ribose polymerase) was seen (Fig 4A).

Interestingly, A-375-TS revealed also visible processing of caspase-8 as well as a 20 kDa cleavage product of caspase-3 upon TRAIL treatment. This 20 kDa form, however, appears as not completely active, as PARP was not cleaved (Fig 4A).

In contrast, at combined treatment with indirubin stronger processing of caspase-8 was seen in A-375-TS, as well as the 17 and 15 kDa cleavage products of caspase-3 representing its completely processed large subunit. This coincided with significant processing of PARP. Characteristically, Bid and caspase-9 were now also activated (Fig 4A).

In order to confirm caspase involvement, the pancaspase inhibitor zVAD-fmk was used. Preincubation for 1 h with zVAD completely abolished apoptosis induction in parental A-375 as well as in A-375-TS cells treated with the combination (Fig 4B). Caspase inhibition also

resulted in improved survival at 24 h of treatment, as determined by WST assay (Fig 4C) and improved cell proliferation, as determined by RTCA (Fig 4D). However, the antiproliferative effects of indirubin were not neutralized by zVAD (data not shown), and the inhibition of cell proliferation by the combination with TRAIL was only partly reduced, thus indicating that indirubin may engage also protease-independent effects (Fig 4D).

3.4 Activation of mitochondrial apoptosis pathways

A clear involvement of the mitochondrial apoptosis pathway became evident after measuring the mitochondrial membrane potential $\Delta\psi$, which is characteristically decreased in apoptotic cells. This effect was completely blocked in A-375-TS, whereas TRAIL strongly decreased $\Delta\psi$ in about 40% of parental A-375 cells. Indirubin, however, resulted in some decrease of $\Delta\psi$ in A-375-TS, which was further strongly enhanced by the combination with TRAIL. Thus, indirubin was able to restore also this TRAIL-mediated effect (Fig 5A; 5B).

Pre-incubation with zVAD only partly prevented the $\Delta\psi$ loss induced of indirubin but completely abrogated any further TRAIL effect, both in parental and selected cells. Thus, the mitochondrial response by TRAIL appeared as downstream of the early caspase cascade (Fig 5A; 5B).

A time kinetic analysis revealed that the $\Delta\psi$ effect of indirubin and the combination came into play at 8 h after start of treatment (Fig 5C), which was right in parallel with the induction of apoptosis (Fig 5D). Thus, the decrease of mitochondrial membrane potential was going hand in hand with apoptosis. Increased ROS levels are frequently associated with mitochondrial activation as we have reported for melanoma cells [24]. However, no effects on ROS levels were seen at 8 and 24 h after treatment with indirubin or with the combination (data not shown).

Release of mitochondrial factors into the cytosol is a hallmark in the mitochondrial proapoptotic pathway. Indeed, Western blot analysis of cytosolic extracts of A-375 treated with TRAIL revealed at 8 h significant release of cytochrome c and apoptosis-inducing factor (AIF),not seen in TRAIL treated A-375-TS. This deficiency of A-375-TS was completely restored by the combination with indirubin (Fig 6).

3.5 Indirubin overrides apoptosis resistance by Bcl-2

Bcl-2 is a critical regulator in melanoma cells. For evaluating whether Bcl-2 or indirubin was stronger in the A-375 cells, we applied A-375 cells stably transfected with a Bcl-2 construct (A-375-Bcl-2) and compared to mock-transfected cells (A-375-Mock). The ectopic overexpression of Bcl-2 resulted in complete CD95 and TRAIL resistance as previously shown [14;21] and also seen here (Fig 7A; 7B). In contrast, the proapoptotic effects of indirubin were not decreased in A-375-Bcl-2 cells as compared to A-375-Mock.

In particular, the TRAIL and CH-11 resistance of A-375-Bcl-2 cells was significantly diminished by the combination with indirubin, resulting in 12% of apoptotic cells (TRAIL, 5% indirubin), 22% (TRAIL, 10% indirubin) and 8% (CH-11, 10% indirubin), respectively (Fig 7A; B). Thus Bcl-2 was able to reduce but not to block apoptosis through indirubin and death ligands. In contrast, the decrease of $\Delta\psi$ was completely blocked in A-375-Bcl-2, proving the complete abrogation of the mitochondrial apoptosis pathway (Fig 7C). Thus, the remaining apoptosis appeared as inpendent of mitochondria.

3.6 Effects of indirubin on Bcl-2 proteins and cIAPs

Kinase-dependent survival pathways, which may be blocked by indirubin, are frequently related to the expression of apoptosis regulators. Of the family of Bcl-2 proteins, antiapoptotic

(Bcl-2, Mcl-1), proapoptotic multidomain (Bax, Bak) and proapoptotic BH3-only proteins (Bad, Noxa, Puma) were investigated by Western blot analysis. In addition, inhibitor of apoptosis proteins (XIAP, Survivin) was enclosed. As concerning proapoptotic Bcl-2 proteins, the central apoptosis regulator Bax was significantly upregulated in A-375-TS at 8 h after indirubin treatment, whereas the functionally related Bak was unaffected. Of three BH3-only proteins investigated, there was significant upregulation of Bad by indirubin. In contrast, the combination treatment resulted in a down-modulation of these proteins in A-375-TS, as a kind of counter-regulation (Fig 8).

As concerning antiapoptotic factors, indirubin alone remained without effect on the expression of antiapoptotic Bcl-2 patterns (Bcl-2, Mcl-1) as well as on cIAPs (Survivin, XIAP). However, in combination with TRAIL, in course of strong apoptosis, there was strong downregulation of Mcl-1 and XIAP (Fig 8).

3.7 Effects of indirubin on death receptors and p53

Expression of the agonistic receptors DR4 and DR5 is elementary for TRAIL sensitivity. Characteristically, A-375-TS cells revealed reduced surface expression of DR4 and DR5 than parental cells, as reported before [15]. Importantly, we found here strongly decreased surface expression of both death receptors in parental cells already at 8 h of TRAIL treatment. This negative feed-back loop helps to understand the quick establishment of TRAIL resistance in melanoma cells (Fig 9A).

Indirubin treatment alone restored a high death receptor expression in A-375-TS, seen for the surface expression (Fig 9A) as well as by Western blot analysis for the total protein (Fig 9B). Further addition of TRAIL in the combination treatment again resulted in downregulation of the receptors, thus reflecting a most sensitive balance regulating TRAIL sensitivity.

As master regulator in apoptosis, the transcription factor and tumor suppressor p53 has been described to trigger the expression of death receptors and of proapoptotic Bcl-2 proteins. In clear agreement with upregulation of Bax, Bad, DR4 and DR5, there was strong upregulation of p53 total protein levels that may in part explain the apoptotic response (Fig 9B).

4. Discussion

An efficient therapy for metastatic melanoma is still outstanding. Thus, chemotherapeutic regimens as well as small molecule inhibitors have been tested in numerous clinical trials, but could not significantly increase overall survival rates of metastasized melanoma patients so far [1;3]. New approaches are urgently needed to improve the prognoses of that dismal disease.

New hope may be set in the death ligand TRAIL, due to its induction of apoptosis in a variety of human cancer cells while normal cells were largely spared [10]. In mouse and primate models, suppression of tumor growth was reported, when applying TRAIL as monotherapy or in combination with chemotherapy [25;26]. Recombinant TRAIL derivatives and agonistic monoclonal antibodies have been developed enabling selective activation of DR4 or DR5 [27;28]. Clinical trials were also performed in patients with advanced colorectal carcinoma and breast cancer, which revealed only little side effects, however, the response on TRAIL monotherapy was also rather limited [29;30].

Limited responsiveness appears as related to inducible TRAIL resistance, which has been reported in different tumor models as in breast cancer and leukemia [31;32]. In melanoma cells, we have previously reported both, pre-existing TRAIL resistance and inducible resistance in course of continuous treatment with TRAIL. Resistance was correlated to downregulation of the agonistic TRAIL receptors, the proapoptotic Bcl-2 protein Bid and the

initiator caspases 8 and 10 [14;15]. As shown here, the downregulation of the TRAIL receptors, seen in total protein levels as well as on the cell surface, appeared as a direct and very early effect, already seen at 8 h of treatment. This finding sheds new light on TRAIL resistance in melanoma; which appears as a fast arising program that is induced by TRAIL itself.

As a second hallmark of TRAIL resistance in melanoma we have shown here the inhibition of complete caspase-3 processing. This effector caspase is expressed as a 35 kDa proenzyme, which is processed in a first step by initiator caspases leading to a 20 kDa intermediate form corresponding to the large subunit. Final processing, suspected to be due to autocatalysis, then results in the 17 kDa and the finally processed 15 kDa form [33]. This cascade was clearly visible in parental A-375 melanoma cells after TRAIL treatment. In contrast, TRAIL-selected A-375 cells did not show the 17 and 15 kDa cleavage products but high levels of the 20 kDa intermediate product and efficient caspase-8 processing. Thus, resistant cells were characterized by a block in caspase-3 autocatalytic activity, which may depend on insufficient activation of the caspase cascade.

Aiming at a sensitization of tumor cells for TRAIL, multiple strategies have been tested. Among these, compounds originating from herbal extracts appeared as promising. Thus, sensitization for TRAIL-induced apoptosis has been reported with ingredients of traditional Chinese medicine such as the flavonoid wogonin and triptolide, tested in leukemia and AML cells, respectively [34;35].

Indirubin, the red isomer of indigo, is the active ingredient of a traditional Chinese medicine. Indirubin and its substituted derivatives are potent kinase inhibitors, and inhibition of GSK-3β, cyclin dependent kinases (CDKs), c-Src and FGF-R1 have been reported [17-19]. In contrast, other kinases appear as activated, in particular a long-term activation of p38 MAP

kinase [19]. As a major appears a the cell cycle arrest in G2/M phase, that has been reported by indirubin derivatives [36] and is also seen here in three of 6 human melanoma cell lines.

Another effect is the induction of apoptosis, that has been attributed to indirubins [37;38]. Recently, we have reported proapoptotic effects also in human melanoma cell lines by the new indirubin derivative 8-Rha-β [20]. Here, we present clear evidence that this substance is able to efficiently enhance death ligand sensitivity of melanoma cells, and it overrides preexisting as well as inducible resistance. Combinations of TRAIL and CD95 agonists with indirubin, as used here, have not been reported, however in lung carcinoma cells the combination of the derivative indirubin-3´-monoxime I3M has been shown to sensitize for TNF-α-induced apoptosis. In these cells, the effects were correlated with decreased activity of NF-κB [39].

In melanoma cells, we present clear evidence that the tumor suppressor p53 was activated by indirubin, seen in enhanced levels of p53, which is usually regulated by its stability [40]. Furthermore, Bax, Bad and both agonistic TRAIL receptors were found upregulated upon indirubin 8-Rha-β treatment, which are characteristic targets of p53 [41;42]. Indeed, enhanced activity of p38 could explain the activation of the p53 pathway in melanoma, as p38 activation has been reported as a major indirubin effect [19], and p53 can be activated by p38 [43;44]. Effects on p53 have also been stated for other indirubin derivatives, such as in cervical cancer and lung cancer cells, which had been further related to upregulation of the cell cycle inhibitor p21 [45;46]. Thus, activation of p53 may be a critical issue in indirubin-mediated cell cycle inhibition and induction of apoptosis.

Activation of the p53 pathway may also result from chemotherapy treatment [40]. The combination of chemotherapeutic agents with indirubin however resulted in variable responses. Whereas indirubin 8-Rha-β was able to enhance apoptosis induction by high dose doxorubicin, it revealed antagonistic inhibition of apoptosis in combination with paclitaxel.

These results are indicative for the selectivity of this compound, which appears to particularly affect death ligand sensitivity thus supporting extrinsic apoptosis pathways.

Of note, the role of p53 in melanoma cells is a non-dissolved question so far, namely despite a lack of inactivating mutations, the p53 pathway appears as blocked in melanoma cells, also underlined by the pronounced chemotherapy resistance [3;47]. Thus, indirubin appears to overcome the block of p53 activation in melanoma cells at least to some extend, as concerning death ligand sensitivity.

The upregulation of Bax and Bad by indirubin 8-Rha-β correlated with parallel activation of intrinsic mitochondrial pathways in A-375-TS cells, as demonstrated by decreased mitochondrial membrane potential, release of cytochrome c and AIF as well as by caspase-9 processing. Expression of Bcl-2 is of major importance for apoptosis deficiency of melanoma cells [48]. Thus, we used A-375 cells stably transfected with Bcl-2 [21], which completely blocked apoptosis by TRAIL or CH-11 agonistic CD95 antibody. Indeed, the decrease of the mitochondrial membrane potential upon indirubin treatment was also completely blocked in these cells. However, apoptosis induction by the combination of indirubin and TRAIL appeared as only partly reduced. This indicates that indirubin affects both the intrinsic and the extrinsic apoptosis pathway, of which only the intrinsic part was blocked by Bcl-2. Previously, we had shown that enhancement via the mitochondrial pathway was essential for death ligand-induced apoptosis in melanoma cells [49]. Indirubin, however, was able to override the Bcl-2-mediated block of apoptosis.

In addition, the combination with TRAIL resulted in downregulation of Mcl-1 and XIAP. Simultaneous decrease of these two proteins was also reported for leukemia cells upon induction of apoptosis [50], possibly indicating their parallel regulation. As XIAP is a potent inhibitor of caspase-3 [51], its downregulation may partly explain the full caspase-3 processing observed in A-375-TS after combined treatment.

The present study presents clear evidence of an upregulation of both DR4 and DR5 by indirubin in melanoma cells. The subsequent enhancement of the extrinsic apoptosis pathway was evident by enhanced caspase-8 processing. Upregulation of death receptors has been reported in HeLa cervix carcinoma cells by the indirubin derivative I3M [45]. Upregulation of both death receptors in cancer cell lines was also seen for celastrol, another ingredient of traditional Chinese medicine [52]. As TRAIL resistance in melanoma cells was associated with a quick TRAIL-mediated downregulation of death receptors, their upregulation by indirubin appears as a suitable compensating effect, preventing inducible death ligand resistance.

In summary, these results present an efficient strategy for overcoming and preventing death ligand resistance in melanoma cells. Taking into account that death ligands are physiological signals of T-lymphocytes in anti-tumor immune surveillance [53;54], indirubin may reveal a therapeutic effect also by supporting the immune response against melanoma. Regarding possible proapoptotic therapies, TRAIL monotherapy may not be sufficient in melanoma cells, due to the quickly arising resistance. However, combinations with kinase inhibitors, such as indirubin 8-Rha-β, may help for a breakthrough of TRAIL-mediated strategies.

Indirubin derivatives were largely well tolerated in animal tumor models as shown in rat, mouse and fish [55-57]. Also in clinical trials for treatment of chronic myelogenous leukemia (CML), indirubin and the derivative meisoindigo have already been tested in China [16]. Nevertheless, this derivative is an investigational compound, and further investigations are needed to prove its applicability and its anti-tumor effects in the clinical situation.

Acknowledgment

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Figure legends

Figure 1: Effects of TRAIL and indirubin alone

(A) Sub G1 cell populations (in %) were determined by flow cytometry in six melanoma cell lines, 24 h after starting treatment with 20 ng/ml TRAIL. (B) LDH-release indicative for cytotoxicity (fold change vs. control) was determined parallel. Values of untreated cells were set to 1. (A, B) Bars represent mean values and SDs of a representative of two independent experiments, each consisting of triplicate values. Independent experiments revealed largely comparable results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.005$), when comparing TRAIL-treated with control cells. (C) Real-time cell analysis (RTCA) is shown for A-375-TS cells treated with 1.25 and 2.5 µM indirubin. Treatment started at 24 h. The determined cell index, normalized to the time of treatment, gives a relative measurement of cell numbers. The experiment was performed two times, each time with triple values, which revealed largely comparable results. (D) Cell cycle analysis by flow cytometry after PI staining is shown for Mel-2a, A-375 and A-375-TS treated with 10 µM indirubin for 24 h, as compared to control cells. The numbers of cells in G1 and G2 (%) are given within the histograms. G1/G2 ratios were also determined for Mel-HO, MeWo and Mel-HO-TS, but not reveal a difference with indirubin treatment (data not shown).

Figure 2: Sensitization for death ligand-induced apoptosis

(A) Apoptosis values are given as percentage of sub-G1 cell populations, as determined by flow cytometry after PI staining. Cells were treated with indirubin (10 µM) and/or TRAIL (20 ng/ml), as indicated. (B) Real-time cell analysis (RTCA) of A-375-TS cells treated with 2.5 µM indirubin, 20 ng/ml TRAIL or the combination. (C) RTCA of A-375-CS cells treated with indirubin and/or 50 ng/ml CH-11. (B, C) Seeding density was 1,250 cells per microtiter well.

The cell index was normalized at 24 h, when treatment had started. The experiment was performed twice (each time with triple values), which revealed largely comparable results. (D) Induction of apoptosis by CH-11 (50 ng/ml), indirubin (10 µM) or the combination is shown for A-375 and A-375-CS cell. (E) Cross resistance for the death ligands TRAIL and CH-11. Left, A-375-TS were treated with CH-11 (50 ng/ml), indirubin (10 µM) or the combination; right, A-375-CS cells were treated with TRAIL (20 ng/ml), indirubin (10 µM) or the combination. (A, D, E) Means and SDs of triple values of representative experiments are shown. All experiments were repeated three times, which revealed largely comparable results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.005$), when comparing combined treatment with indirubin alone.

Figure 3: Combination of indirubin with chemotherapeutics

(A) Apoptosis values of A-375 cells are given as percentage of sub-G1 cell populations, as determined by flow cytometry and PI staining 24 h after treatment. Simultaneous treatment was with indirubin 8-Rha-β (10 µM) in combinations with vinblastine (1, 10, 100 nM), paclitaxel (1, 10 nM) and doxorubicin (1, 10, 100 nM), respectively. The experiment was performed twice (each time with triple values), which revealed highly comparable results. Statistical significance ($p < 0.05$) is indicated by asterisks, when comparing combined with single treatments alone. (B) LDH-release indicative for cytotoxicity (fold change vs. control) was determined parallel. Values of non-treated cells were set to 1. Parallel experiments were performed with A-375-TS, which revealed a highly comparable response.

Figure 4: Enhanced caspase activation

(A) Proteins extracted of A-375 and A-375-TS after 8 h of treatment with TRAIL (20 ng/ml), indirubin (10 µM) or the combination were analyzed by Western blotting and compared with

control cells. Equal protein amounts (30 µg per lane) were separated by SDS-PAGE, and consistent blotting was proven by Ponceau staining as well as by evaluation of GAPDH expression. Molecular weights (in kDa) of identified protein bands are indicated. (B) Induction of apoptosis was determined for A-375 and A-375-TS cells without addition of zVAD-fmk (above) or with preincubation of zVAD-fmk (10 µM) for 1 h (below). Further treatments: indirubin (10 µM) and/or TRAIL (20 ng/ml). (C) Cell viability (fold change vs. control) for A-375 and A-375-TS cells was determined by WST-1 assay. Cells were preincubated for 1 h with zVAD-fmk (10 µM, below) before indirubin (2.5 µM) and/or TRAIL (20 ng/ml) treatment started. The comparison was to cells without zVAD (above). (D) RTCA of A-375-TS cells treated with indirubin (2.5 µM) alone, in combination with TRAIL (20 ng/ml) or in triple combination with TRAIL and zVAD-fmk (10 µM). Seeding density was 1,250 cells per microtiter well. The cell index was normalized at 24 h, when treatment had started. The experiment was performed twice (each time with triple values), which revealed largely comparable results.

Figure 5: Response of mitochondria to indirubin

(A) Decreased mitochondrial membrane potential ($\Delta\psi$) was determined by flow cytometry after TMRM staining in A-375 and A-375-TS cells treated with TRAIL (20 ng/ml), indirubin (10 µM) or the combination (above). Histograms of non-treated controls, C, are in grey. A comparison is shown with cells preincubated with zVAD-fmk for 1 h (below). (B) Quantification of A). (C) Loss of $\Delta\psi$ is shown in a time kinetic for A-375-TS cells treated for 2, 4, 8 and 24 h with 10 µM indirubin, 20 ng/ml TRAIL or the combination. (A, C) Experiments were performed 2-3 times, each time with triple values, leading to highly comparable results. (D) Apoptosis was determined in parallel with C). Means and SDs of

triple values of a representative experiment are shown. Statistical significance is indicated by ** ($p < 0.005$), when comparing cells treated with the combination to indirubin alone.

Figure 6: Release of mitochondrial factors

(A) Mitochondrial fractions (Mito) and cytosolic fractions (Cyto) were isolated from A-375-TS and A-375 cells treated for 8 h with indirubin (10 µM), TRAIL (20 ng/ml) or the combination. Equal amounts of cytosolic cell extracts (20 µl) were separated by SDS-PAGE. Consistent blotting was proven by Ponceau staining and by evaluation of GAPDH expression. Molecular weights of identified protein bands are indicated. Mitochondrial extracts served as controls, and analysis of the mitochondrial protein VDAC ruled out any contamination of cytosolic extracts with mitochondria. The experiment was repeated once, which revealed largely comparable results.

Figure 7: Effect of Indirubin on Bcl-2-overexpessing cells

(A, B) A-375 cell clones stably transfected with a Bcl-2 expression plasmid (A-375-Bcl-2) and cells transfected with the empty plasmid (A-375-Mock) were treated for 8 h with indirubin (5 µM or 10 µM), TRAIL (20 ng/ml), CH-11 (50 ng/ml) or combinations, and apoptosis was determined. Bars represent mean values +/- SD of a representative of three independent experiments, each one consisting of triple values. Independent experiments revealed comparable results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.005$), when the combination treatment was compared with indirubin alone. Inset shows Bcl-2 overexpression in A-375-Bcl-2. (C) Loss of $\Delta\psi$ was determined by flow cytometry after TMRM staining for A-375-Bcl-2 and A-375-Mock. Cells were treated for 8 h with indirubin (10 µM), TRAIL (20 ng/ml) or the combination and were compared to non-treated controls (grey). The experiment was repeated twice, which revealed largely comparable results.

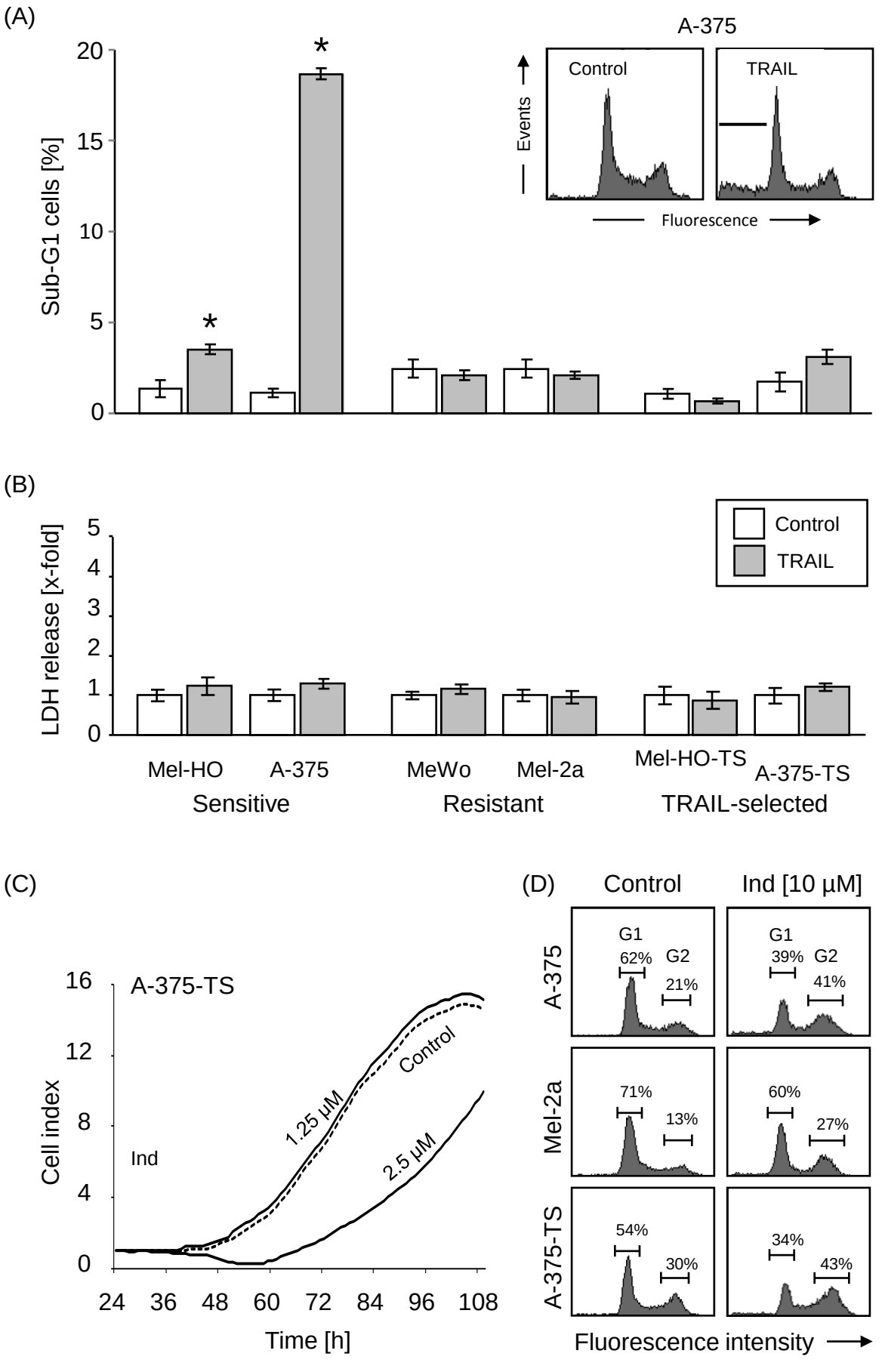
Figure 8: Expression of Bcl-2 proteins and cIAPs

The expression of apoptosis regulators was determined in A-375-TS and A-375 cells treated for 8 h with indirubin (10 µM) and/or TRAIL (20 ng/ml) by Western blot analysis. Equal protein amounts (30 µg per lane) were separated by SDS-PAGE, and consistent blotting was proven by Ponceau staining as well as by evaluation of GAPDH expression. Molecular weights of identified protein bands are indicated. Experiments were performed at least twice, which revealed largely comparable results.

Figure 9: Expression of death receptors and p53.

(A) Surface expression of DR4 and DR5 was determined by flow cytometry after antibody staining for A-375-TS and A-375 cells treated for 8 h with indirubin (10 µM), TRAIL (20 ng/ml) or the combination (open graphs, continuous line). Non-treated controls were used for comparison (grey filled graphs). Cells stained with control mouse IgG1 served as controls (open graphs, punctured line). (B) Expression of DR4, DR5 and of p53 in total protein extracts is shown for A-375-TS and A-375 cells, as determined by Western blot analysis. Equal protein amounts (30 µg per lane) were separated by SDS-PAGE, and consistent blotting was proven by Ponceau staining as well as by evaluation of GAPDH expression. Molecular weights of identified protein bands are indicated. Cells were treated for 8 h with indirubin (10 µM) and/or TRAIL (20 ng/ml), as indicated. Two independent experiments were performed, which revealed largely comparable results.

Figure 1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LDH release [x-fold]
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Mel-2a
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A-375-TS
Sensitive
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A-375-TS
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96
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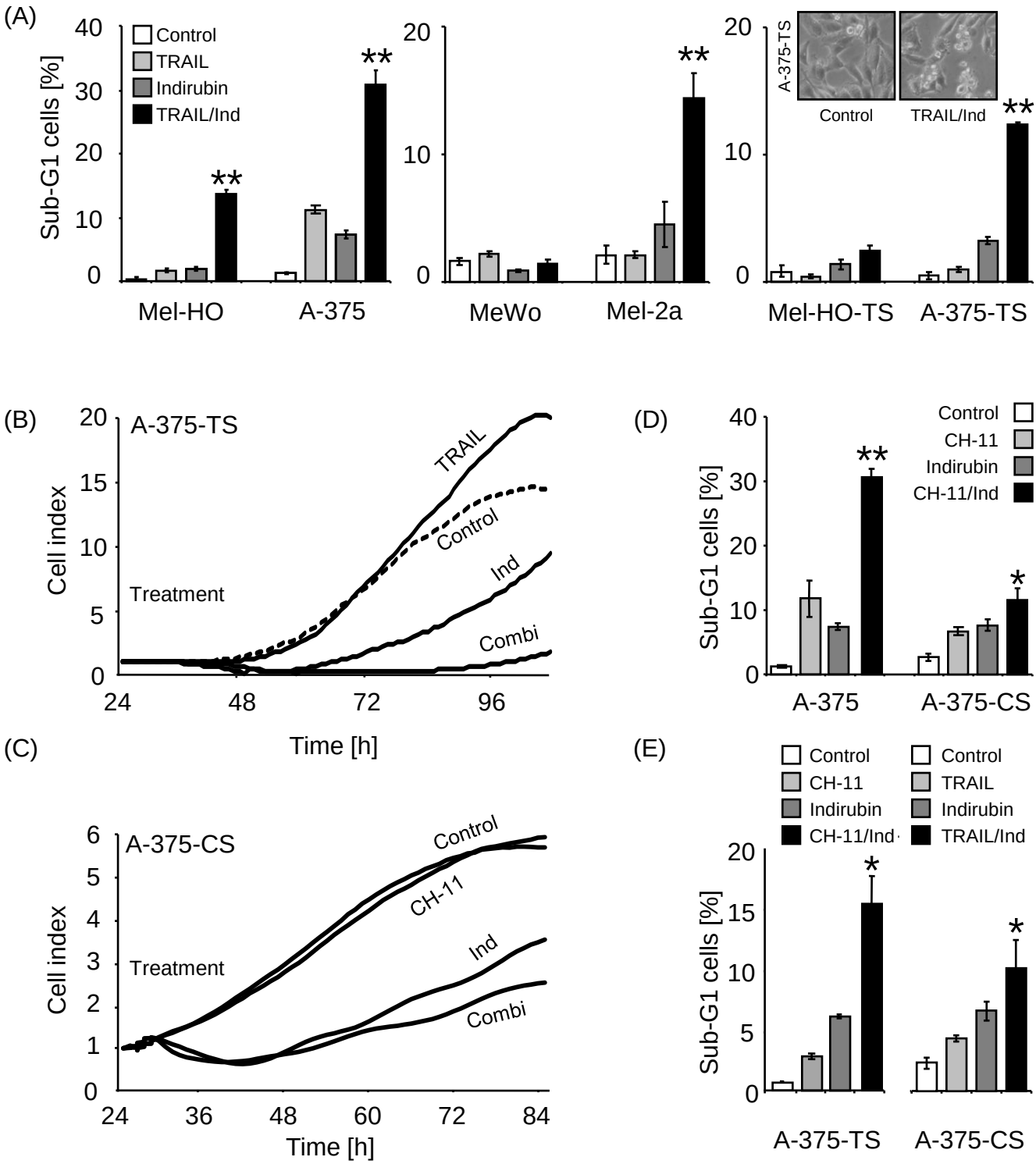
Fluorescence intensity

Figure 2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Sub-G1 cells [%]
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Mel-HO
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Control
TRAIL/Ind
Mel-HO-TS
A-375-TS
(B)
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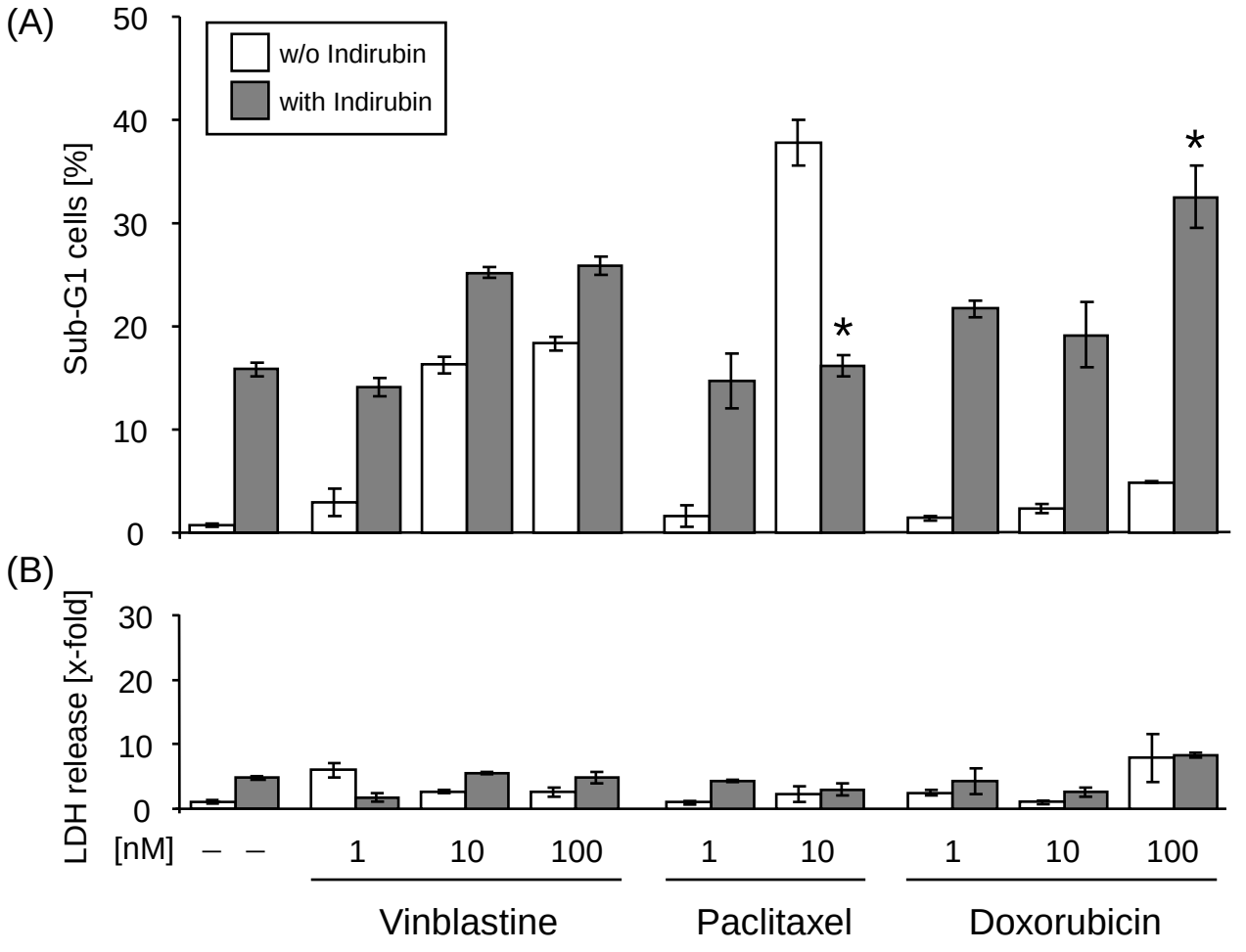
Figure 3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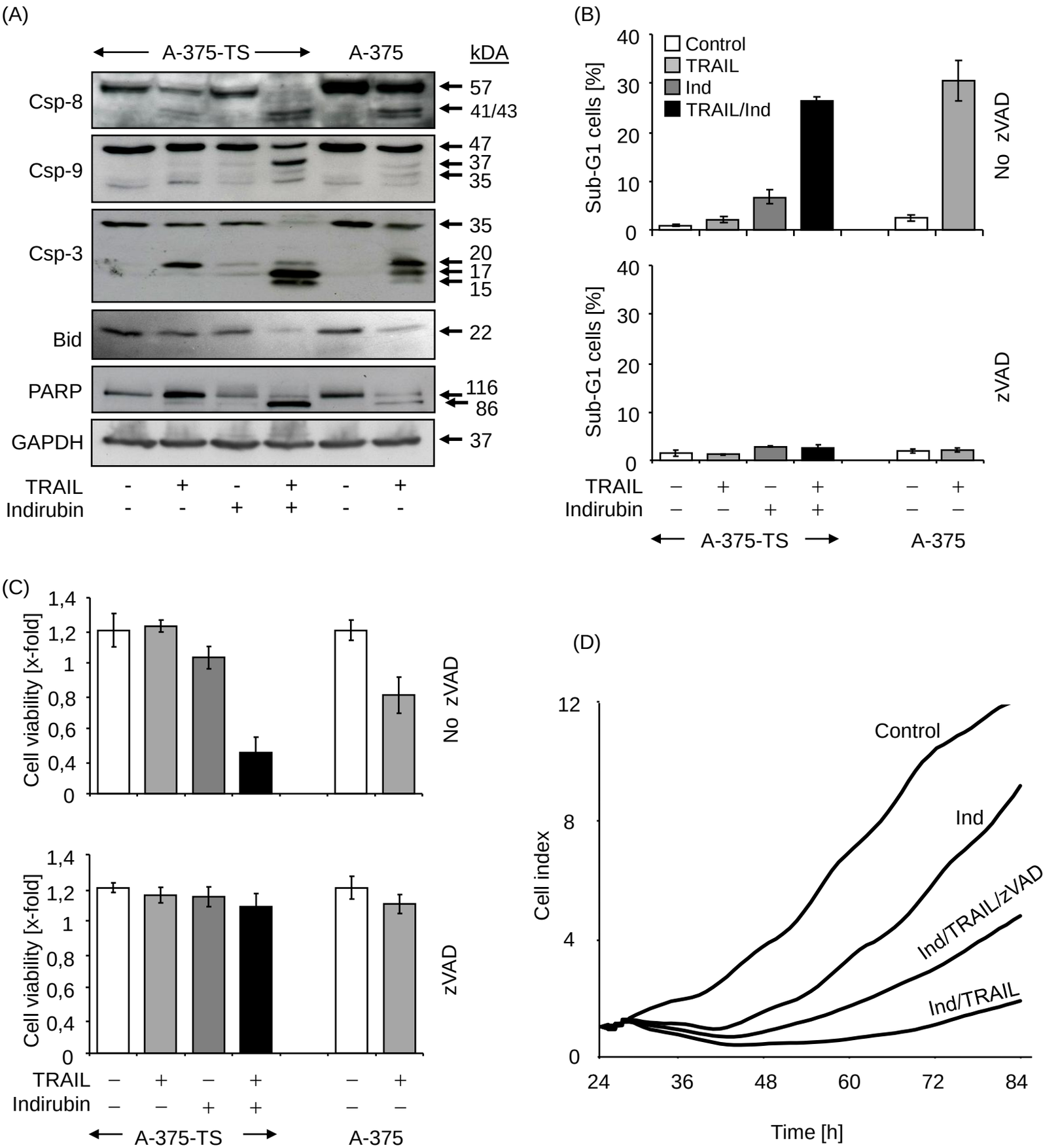
Vinblastine
Paclitaxel
Doxorubicin

Figure 4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Bid
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PARP
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GAPDH
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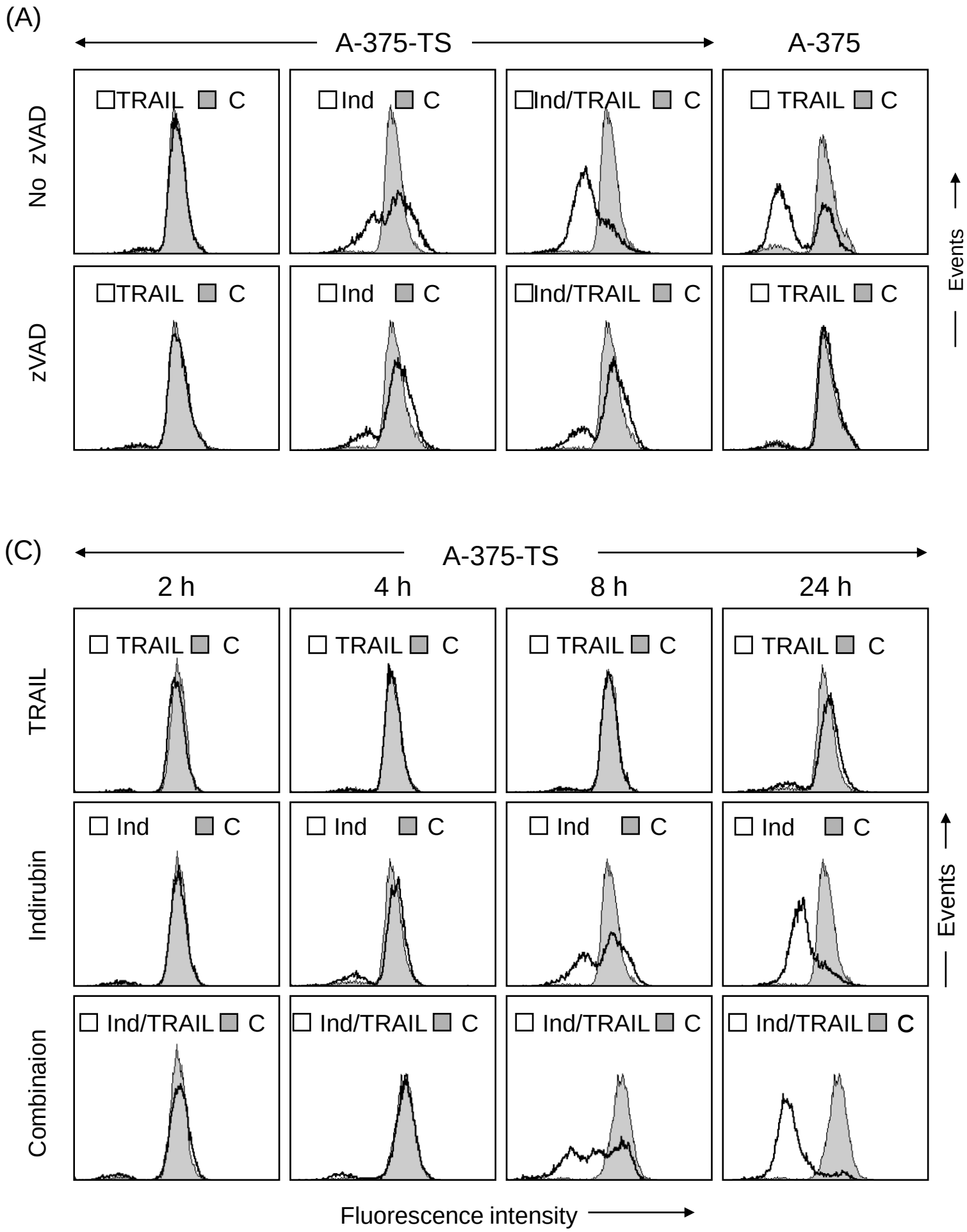
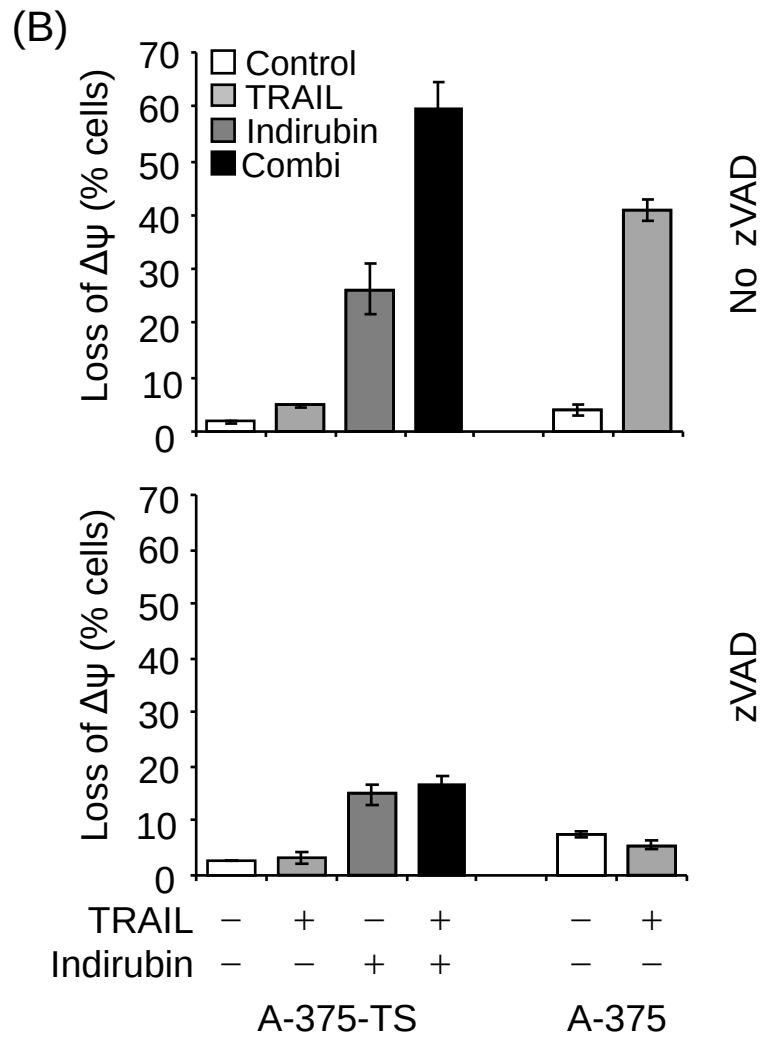
Ind/TRAIL/zVAD
Ind/TRAIL

Figure 5


(A)
A-375-TS
A-375
No zVAD
zVAD
TRAIL C
Ind C
Ind/TRAIL C
Events
(C)
A-375-TS
2 h
4 h
8 h
24 h
TRAIL
Indirubin
Combinaion
Fluorescence intensity


(B)
Control
TRAIL
Indirubin
Combi
Loss of Δψ (% cells)
No zVAD
zVAD
TRAIL
Indirubin
A-375-TS
A-375

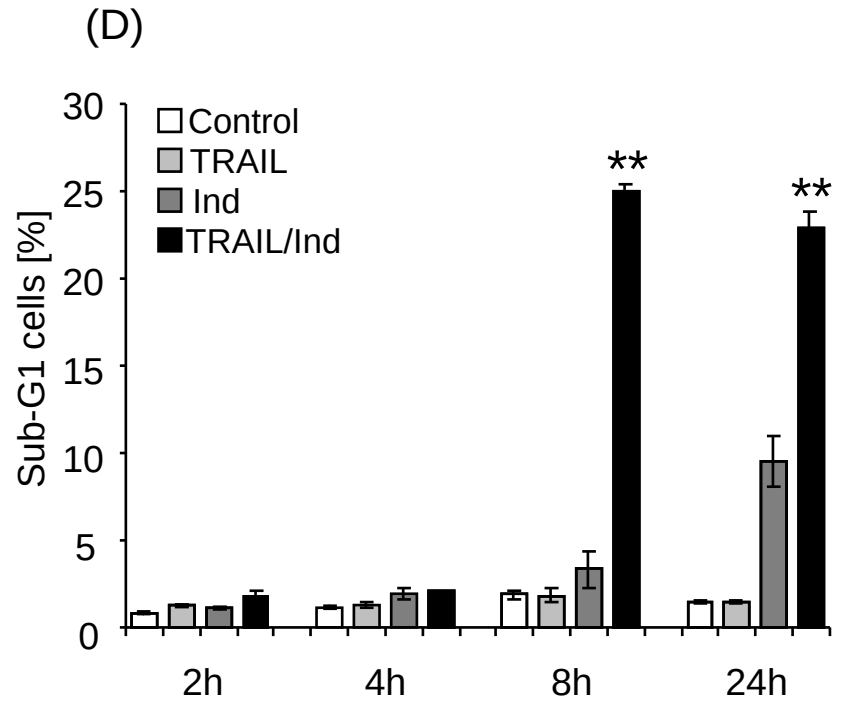

(D)
Control
TRAIL
Ind
TRAIL/Ind
Sub-G1 cells [%]
**
2h
4h
8h
24h

Figure 6

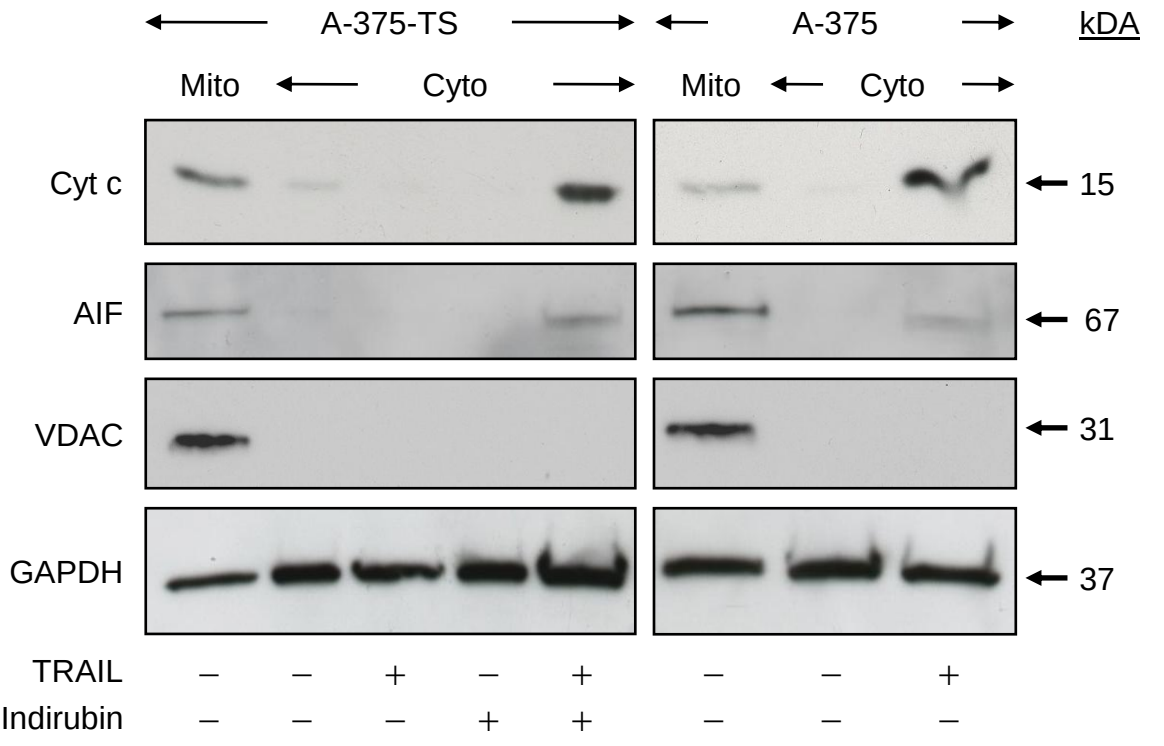

A-375-TS
A-375
kDA
Mito
Cyto
Mito
Cyto
Cyt c
AIF
VDAC
GAPDH
15
67
31
37
TRAIL
Indirubin

Figure 7

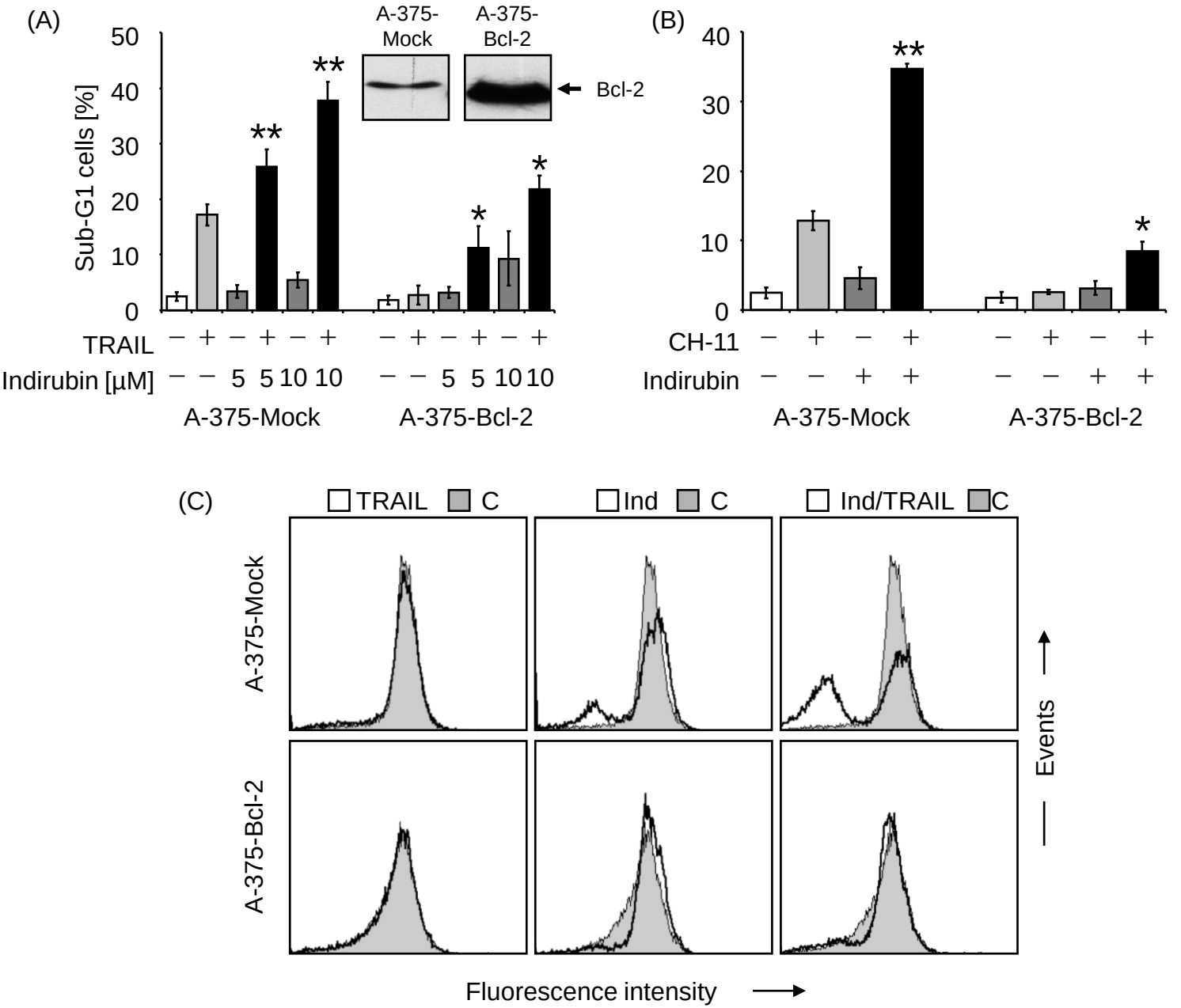

(A)
A-375-Mock
A-375-Bcl-2
Bcl-2
Sub-G1 cells [%]
50
40
30
20
10
0
**
**
*
*
TRAIL
Indirubin [μM]
5 5 10 10
5 5 10 10
A-375-Mock
A-375-Bcl-2
(B)
40
30
20
10
0
**
*
CH-11
Indirubin
A-375-Mock
A-375-Bcl-2
(C)
TRAIL
C
Ind
C
Ind/TRAIL
C
A-375-Mock
A-375-Bcl-2
Events
Fluorescence intensity

Figure 8

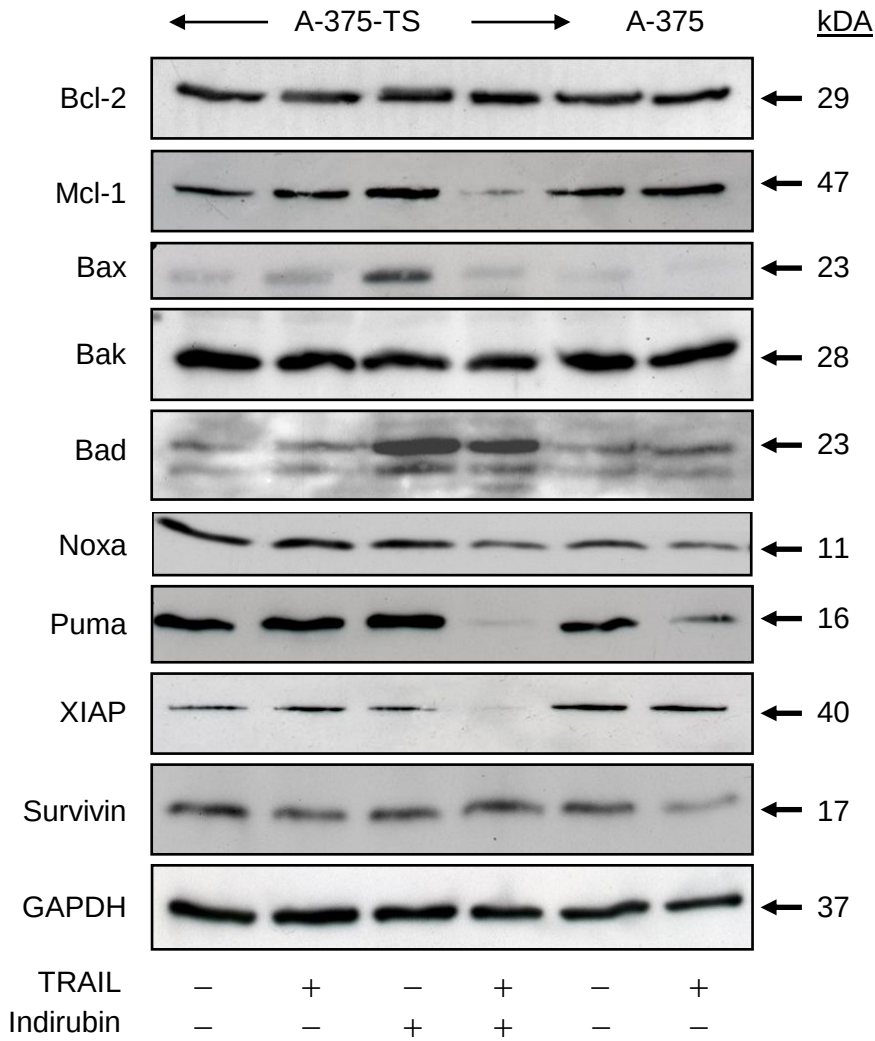
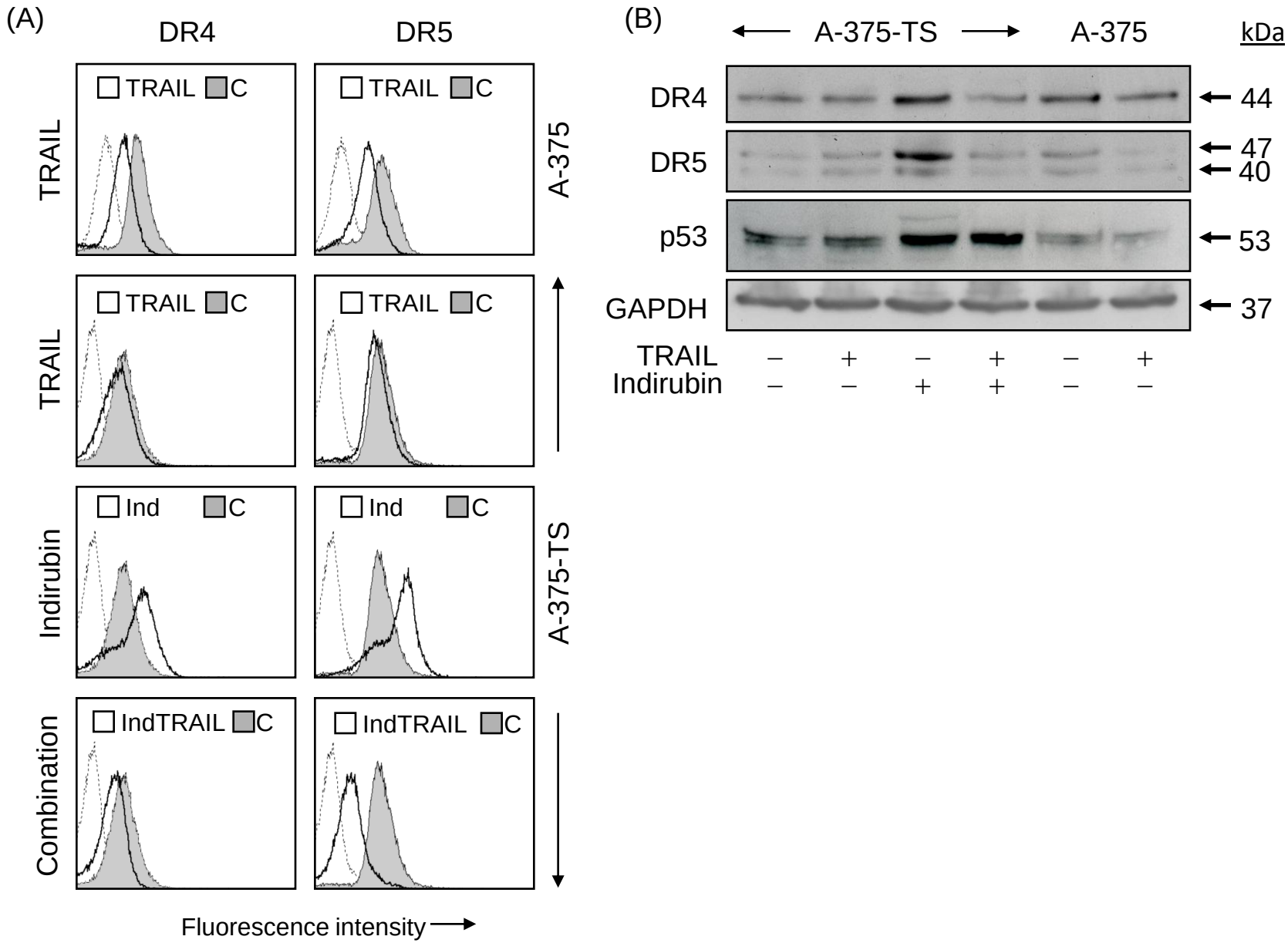
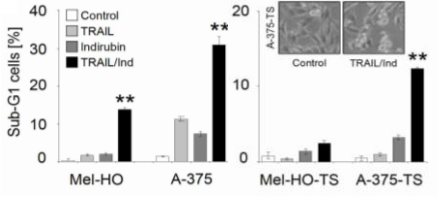

A-375-TS
A-375
kDA
Bcl-2
29
Mcl-1
47
Bax
23
Bak
28
Bad
23
Noxa
11
Puma
16
XIAP
40
Survivin
17
GAPDH
37
TRAIL
Indirubin

Figure 9

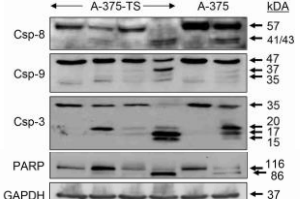

(A)
DR4
DR5
TRAIL
C
Ind
IndTRAIL
A-375
A-375-TS
Indirubin
Combination
Fluorescence intensity
(B)
A-375-TS
A-375
kDa
DR4
DR5
p53
GAPDH
44
47
40
53
37
TRAIL
Indirubin

*Graphical Abstract

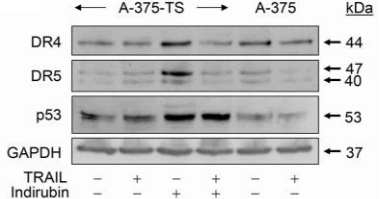
Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative – Enhancement of both extrinsic and intrinsic apoptosis pathways



Induction of TRAIL-mediated apoptosis by 8-Rha-β



Enhanced caspase activation



Upregulation of death receptors and p53