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

4
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1 Abstract

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

22
23 Running title: Indirubin derivative sensitizes for death ligands

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

25

1 **1. Introduction**

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

6 Two main proapoptotic pathways (extrinsic and intrinsic) have been described. The extrinsic
7 pathway is initiated by the binding of death ligands to cell surface death receptors leading to
8 the formation of a death inducing signaling complex (DISC), where initiator caspases 8 and
9 10 are activated [4]. On the other hand, the intrinsic apoptosis pathway is triggered by
10 intracellular signals such as cellular and DNA damage, also when caused by chemotherapy.

11 Key events are the depolarization of the mitochondrial membrane potential and the release of
12 mitochondrial factors such as cytochrome c into the cytosol, which results in activation of
13 initiator caspase-9 [5;6]. Both pathways may meet at the mitochondria due to caspase-8-
14 mediated activation of the proapoptotic Bcl-2 protein Bid [7]. Mitochondrial activation is
15 critically controlled by the family of Bcl-2 proteins, which consists of antiapoptotic proteins
16 (e.g. Bcl-2, Bcl-x_L and Mcl-1), proapoptotic multidomain proteins (Bax and Bak) and
17 proapoptotic BH3-only proteins (e.g. Bid, Bad, Puma and Noxa) [8]. According to these
18 pathways, caspase cascades appear as central in apoptosis regulation. Initiator caspases cleave
19 and activate downstream effector caspases as caspase-3, which then target a number of death
20 substrates to set apoptosis into work [5;9].

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

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

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

1 2. Materials and methods

2 2.1 Cell culture

3 Four human melanoma cell lines were investigated: death ligand sensitive Mel-HO and A-375
4 as well death ligand resistant Mel-2a and MeWo [15]. TRAIL-resistant subclones of Mel-HO
5 and A-375 (Mel-HO-TS; A-375-TS) derived from a selection with 5 ng/ml TRAIL [15].
6 CD95-resistant subclones of A-375 (A-375-CS) were generated by selection with 20 ng/ml
7 CH-11 agonistic CD95 antibody. Selected cells were continuously cultured with the
8 respective death ligand. As previously described, other subclones of A-375 resulted from
9 stable transfection with a pIRES-Bcl-2 construct (A-375-Bcl-2) or the pIRES empty plasmid
10 (A-375-Mock) [21]. The pIRES plasmid originated from Clontech (Palo Alto, CA, USA).
11 Melanoma cells were cultivated at 37°C, 5% CO₂ in DMEM (4.5 g/l glucose; Gibco,
12 Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and antibiotics (Biochrom,
13 Berlin, Germany). Experiments were performed in 6-well, 24-well or 96-well plates (200,000,
14 50,000 or 1,250 seeding density per well, respectively). For induction of apoptosis, soluble
15 human killerTRAIL (Alexis, Gruenberg, Germany ALX-201-073-C020; 20 ng/ml) or the
16 agonistic monoclonal CD95 antibody CH-11 (Beckman Coulter, Marseille Cedex, France PN
17 IM 1504; 50 ng/ml) were applied. As indicated, incubation with death ligands took place for
18 8-24 h. Treatment with indirubin 8-Rha- β , described previously [20], was set up in parallel
19 with death ligands, whereas control cells received only the solvent DMSO (Applichem,
20 Darmstadt, Germany). For inhibition of caspases, cells were preincubated for 1 h with 10 μ M
21 of the pancaspase/panprotease inhibitor zVAD-fmk (R&D Systems, Wiesbaden, Germany),
22 which binds the active sites of caspase-like proteases.
23 In further experiments, the chemotherapeutic substances doxorubicin, paclitaxel and
24 vinblastine (Sigma, Taufkirchen, Germany) were applied in concentrations of 1, 10 and 100

1 nM. Simultaneous treatments were used for combinations of chemotherapeutics and indirubin
2 8-Rha- β (24 h).

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9 4 **2.2 Real-time cell analysis and cell viability assay**

10 5 For obtaining growth curves, cell confluence was continuously monitored by real-time cell
11 6 analysis (RTCA, xCELLigence, Roche diagnostics; Penzberg, Germany). The technique is
12 7 based on microelectrodes integrated in the bottom of each well of special 96-well E-plates.
13 8 The electric impedance corresponds to the cell density. 1,250 cells were seeded per microtiter
14 9 well, and treatment started after 24 h. The impedance was determined up to 120 h after
15 10 seeding.

16 11 For monitoring cell viability and proliferation, a colorimetric assay (WST-1, Roche
17 12 diagnostics, Mannheim, Germany) was used. A number of 1,250 cells per well was seeded in
18 13 96-well plates and treated the next day for 24 h with 20 ng/ml TRAIL, 2.5 μ M indirubin or
19 14 the combination. For 100 μ l culture medium per well, 10 μ l WST-1 reagent were added,
20 15 following by incubation of the cells at 37°C between 0.5 to 4 h, until color changes were seen.
21 16 The optical density of triplicated samples was determined several times at 450 nm in an
22 17 ELISA reader. For background correction, the values of untreated controls were subtracted.

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44 19 **2.3 Quantification of apoptosis and cytotoxicity**

45 20 For quantification of apoptosis, cell cycle analyses were carried out after propidium iodide
46 21 staining, according to Nicoletti et al. [22]. Cells were harvested by trypsinisation, stained with
47 22 PBS buffer containing triton-X 100, sodium citrate and propidium iodide (Sigma,
48 23 Taufkirchen, Germany; 200 mg/ml), centrifuged, washed with PBS and analyzed by flow
49 24 cytometry in a FACS Calibur. Cytotoxicity was determined in parallel by measuring LDH
50 25 activity in culture fluids applying a cytotoxicity detection assay (Roche Diagnostics).

1 2.4 Western blot analysis

2 For protein analysis, cells were lysed in 10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM
3 EDTA; 2 mM PMSF; 1 mM leupeptin; 1 mM pepstatin; 0.5% SDS and 0.5% Nonidet P-40.

4 For cytochrome c, AIF and VDAC, cytosolic and mitochondrial cell fractions were separated
5 by a mitochondria/cytosol fractionation kit (Alexis, Gruenberg, Germany). Protocols for
6 protein extraction and Western blot analysis were described previously [23].

7 The following primary antibodies were used: cleaved caspase-3 (Cell Signaling, rabbit,
8 1:10000), caspase-3 (Cell Signaling, rabbit, 1:1000), caspase-8 (Cell Signaling, mouse,
9 1:1000), caspase-9 (Cell Signaling, rabbit, 1:1000), PARP (Biomol, mouse, 1:5000), Bid (Cell
10 Signaling, rabbit, 1:1000), Mcl-1 (Santa Cruz, mouse, 1:200), Bcl-2 (Santa Cruz, mouse,
11 1:200), Bax (Santa Cruz, rabbit, 1:200), Bak (Dako, rabbit, 1:500), Bad (Cell Signaling,
12 rabbit, 1:1000), Puma (Epitomics, rabbit, 1:1000), Noxa (Pro Sci, rabbit, 1:500), cytochrome
13 c (BD Biosciences, mouse, 1:1000), AIF (Santa Cruz, goat, 1:200), anti-Porin 31 HL (VDAC)
14 (Calbiochem, mouse, 1:5000), XIAP (Cell Signaling, rabbit, 1:1000), Survivin (Santa Cruz,
15 mouse, 1:500), DR4 (abcam, rabbit, 1:500), DR5 (abcam, rabbit, 1:500), p53 (Santa Cruz,
16 rabbit, 1:500) and GAPDH (Santa Cruz, mouse, 1:200).

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18 2.5 Mitochondrial membrane potential

19 For determination of mitochondrial membrane potential, the fluorescent dye TMRM
20 (Tetramethylrhodamine methyl ester perchlorate) was used. Cells were harvested by
21 trypsinisation, stained with TMRM (Sigma-Aldrich, Taufkirchen, Germany; 1 nM, 15 min;
22 37°C), washed once with PBS buffer and analyzed in PBS by flow cytometry.

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1 2.6 Determination of ROS

2 For measurement of intracellular ROS levels, the fluorescent dye H₂DCFDA (20,70-
3 dichlorodihydrofluoresceindiacetate) was used. Cells were harvested by trypsinisation, stained
4 with H₂DCFDA (Molecular Probes, Invitrogen, Eugene, Oregon, USA; 15 mM, 30 min),
5 washed once with PBS buffer and analyzed in PBS by flow cytometry.

7 2.7 Surface Expression of DR4 and DR5

8 Cells were seeded in 6-well plates (2x10⁵ cells/well) and treated for 8 h with indirubin or
9 TRAIL, alone or in combination. Then, cells were harvested with 500 µM EDTA, stained
10 with the primary mouse monoclonal antibodies for TRAIL-R1/DR4 (Alexis, clone HS101,
11 ALX-804-297-C100, 1:100), or TRAIL-R2/DR5 (Alexis, clone HS 201, ALX-804-298-100,
12 1:100), or for control with mouse IgG1 (Ansell, # 278-010). After 15 min of incubation at
13 4°C and 15 min at room temperature, cells were centrifuged, washed twice with PBS/1% BSA
14 and resuspended in 50 µl PBS/BSA containing the secondary antibody for mouse IgG1
15 (Alexis, ALX 211-201-050, 1:100). After 30 min incubation at room temperature, cells were
16 centrifuged, washed twice, resuspended in 200 µl of PBS and analyzed by flow cytometry.

18 2.8 Statistical analyses

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

1 3. Results

2 3.1 Melanoma cell culture models of TRAIL resistance

3 We have previously described pre-existing and inducible TRAIL resistance in human
4 melanoma cell lines [15]. MeWo and Mel-2a (DR4-, DR5+) revealed resistance, whereas A-
5 375 (DR4+, DR5+) and Mel-HO (DR4-, DR5+) were sensitive. In addition, A-375 and Mel-
6 HO developed an inducible resistance upon continuous cultivation with TRAIL leading to
7 selected resistant cell populations (Mel-HO-TS, A-375-TS). The apoptotic response to TRAIL
8 of these six cell culture models is demonstrated by determination of sub-G1 cell populations
9 at 24 h of TRAIL treatment (20 ng/ml). At this time, A-375 revealed strongly increased
10 number (18%) and Mel-HO revealed moderately increased (4%) number of apoptotic cells,
11 whereas the other four cell lines remained resistant (Fig 1A). Cytotoxicity was unaffected at
12 this time, as determined by LDH in the cell culture supernatants (Fig 1B).

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

19 3.2 Indirubin sensitizes melanoma cells for death ligand-induced apoptosis

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

1 375 (32%), Mel-2a (14%) and A-375-TS (12%). Also, visible effects such as reduced cell
2 numbers, rounded cells and cell detachment were clearly evident. In contrast, MeWo and
3 Mel-HO-TS remained resistant (Fig 2A).

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

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

14 Both selected A-375 cell populations revealed cross-resistance for the respective other death
15 ligand. Thus A-375-TS was largely resistant to CH-11, and A-375-CS was largely resistant to
16 TRAIL, suggesting parallel pathways to death ligand resistance. These cross-resistances were
17 also overcome by combinations with indirubin (Fig 2E). For understanding how general the
18 apoptosis sensitization by indirubin was, it was combined with the chemotherapeutics
19 vinblastine, paclitaxel and doxorubicin, respectively. Therefore, the cell line A-375 was
20 simultaneously treated with 8-Rha- β (10 μ M) and chemotherapeutics in concentrations of 1,
21 10 and 100 nM (vinblastine, doxorubicin) or 1 and 10 nM (paclitaxel). For vinblastine, some
22 enhancement of apoptosis was seen in the combinations, however less than additive effects
23 (Fig 3A). Paclitaxel at 10 nM revealed a strong proapoptotic effect on A-375, which was
24 however diminished by the combination with 8-Rha- β (seen in two independent experiments,
25 $p < 0.005$). Only the combination with 100 nM doxorubicin showed a significant

1 enhancement of apoptosis by about two-fold, when compared to 8-Rha- β single treatment ($p <$
2 0.005). Cytotoxicity was unaffected for all treatments at this time (24 h) (Fig 3B). A complete
3 series of these combination experiments was also performed with A-375-TS. These cells
4 revealed essentially a similar response to chemotherapeutics as the parental cell line, namely
5 enhancement in the combination with 100 nM doxorubicin but antagonistic effects with 10
6 nM paclitaxel (data not shown).

7 3.3 Activation of the full caspase cascade by combination with indirubin with TRAIL

8 For understanding the way of indirubin-mediated apoptosis sensitization, activation of
9 initiator and effector caspases was investigated in A-375-TS cells by Western blot analysis.
10 The extrinsic caspase cascade with typical cleavage products of the initiator caspase-8 and the
11 main effector caspase-3 was seen in A-375 parental cells upon TRAIL treatment. Further loss
12 of the proform of Bid and processing of caspase-9 were indicative for an amplification loop
13 through the mitochondrial/intrinsic pathway. As a result of caspase-3 activity, cleavage of its
14 characteristic substrate PARP (poly-ADP-ribose polymerase) was seen (Fig 4A).
15 Interestingly, A-375-TS revealed also visible processing of caspase-8 as well as a 20 kDa
16 cleavage product of caspase-3 upon TRAIL treatment. This 20 kDa form, however, appears as
17 not completely active, as PARP was not cleaved (Fig 4A).
18 In contrast, at combined treatment with indirubin stronger processing of caspase-8 was seen in
19 A-375-TS, as well as the 17 and 15 kDa cleavage products of caspase-3 representing its
20 completely processed large subunit. This coincided with significant processing of PARP.
21 Characteristically, Bid and caspase-9 were now also activated (Fig 4A).
22 In order to confirm caspase involvement, the pancaspase inhibitor zVAD-fmk was used.
23 Preincubation for 1 h with zVAD completely abolished apoptosis induction in parental A-375
24 as well as in A-375-TS cells treated with the combination (Fig 4B). Caspase inhibition also

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

8 **3.4 Activation of mitochondrial apoptosis pathways**

9 A clear involvement of the mitochondrial apoptosis pathway became evident after measuring
10 the mitochondrial membrane potential $\Delta\psi$, which is characteristically decreased in apoptotic
11 cells. This effect was completely blocked in A-375-TS, whereas TRAIL strongly decreased
12 $\Delta\psi$ in about 40% of parental A-375 cells. Indirubin, however, resulted in some decrease of
13 $\Delta\psi$ in A-375-TS, which was further strongly enhanced by the combination with TRAIL.
14 Thus, indirubin was able to restore also this TRAIL-mediated effect (Fig 5A; 5B).

15 Pre-incubation with zVAD only partly prevented the $\Delta\psi$ loss induced of indirubin but
16 completely abrogated any further TRAIL effect, both in parental and selected cells. Thus, the
17 mitochondrial response by TRAIL appeared as downstream of the early caspase cascade (Fig
18 5A; 5B).

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

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

8 **3.5 Indirubin overrides apoptosis resistance by Bcl-2**

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

15 In particular, the TRAIL and CH-11 resistance of A-375-Bcl-2 cells was significantly
16 diminished by the combination with indirubin, resulting in 12% of apoptotic cells (TRAIL,
17 5% indirubin), 22% (TRAIL, 10% indirubin) and 8% (CH-11, 10% indirubin), respectively
18 (Fig 7A; B). Thus Bcl-2 was able to reduce but not to block apoptosis through indirubin and
19 death ligands. In contrast, the decrease of $\Delta\psi$ was completely blocked in A-375-Bcl-2,
20 proving the complete abrogation of the mitochondrial apoptosis pathway (Fig 7C). Thus, the
21 remaining apoptosis appeared as independent of mitochondria.

23 **3.6 Effects of indirubin on Bcl-2 proteins and cIAPs**

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

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

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

15 **3.7 Effects of indirubin on death receptors and p53**

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

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

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

7 **4. Discussion**

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

13 New hope may be set in the death ligand TRAIL, due to its induction of apoptosis in a variety
14 of human cancer cells while normal cells were largely spared [10]. In mouse and primate
15 models, suppression of tumor growth was reported, when applying TRAIL as monotherapy or
16 in combination with chemotherapy [25;26]. Recombinant TRAIL derivatives and agonistic
17 monoclonal antibodies have been developed enabling selective activation of DR4 or DR5
18 [27;28]. Clinical trials were also performed in patients with advanced colorectal carcinoma
19 and breast cancer, which revealed only little side effects, however, the response on TRAIL
20 monotherapy was also rather limited [29;30].

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

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

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

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

21 Indirubin, the red isomer of indigo, is the active ingredient of a traditional Chinese medicine.
22 Indirubin and its substituted derivatives are potent kinase inhibitors, and inhibition of GSK-
23 3β , cyclin dependent kinases (CDKs), c-Src and FGF-R1 have been reported [17-19]. In
24 contrast, other kinases appear as activated, in particular a long-term activation of p38 MAP

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

3 Another effect is the induction of apoptosis, that has been attributed to indirubins [37;38].

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

12 In melanoma cells, we present clear evidence that the tumor suppressor p53 was activated by
13 indirubin, seen in enhanced levels of p53, which is usually regulated by its stability [40].
14 Furthermore, Bax, Bad and both agonistic TRAIL receptors were found upregulated upon
15 indirubin 8-Rha- β treatment, which are characteristic targets of p53 [41;42]. Indeed, enhanced
16 activity of p38 could explain the activation of the p53 pathway in melanoma, as p38
17 activation has been reported as a major indirubin effect [19], and p53 can be activated by p38
18 [43;44]. Effects on p53 have also been stated for other indirubin derivatives, such as in
19 cervical cancer and lung cancer cells, which had been further related to upregulation of the
20 cell cycle inhibitor p21 [45;46]. Thus, activation of p53 may be a critical issue in indirubin-
21 mediated cell cycle inhibition and induction of apoptosis.

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

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

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

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

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

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

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

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

24 **Acknowledgment**

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1 **Figure legends**

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5 **Figure 1: Effects of TRAIL and indirubin alone**

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7 4 (A) Sub G1 cell populations (in %) were determined by flow cytometry in six melanoma cell
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9 5 lines, 24 h after starting treatment with 20 ng/ml TRAIL. (B) LDH-release indicative for
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11 6 cytotoxicity (fold change vs. control) was determined parallel. Values of untreated cells were
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13 7 set to 1. (A, B) Bars represent mean values and SDs of a representative of two independent
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15 8 experiments, each consisting of triplicate values. Independent experiments revealed largely
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17 9 comparable results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.005$),
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19 10 when comparing TRAIL-treated with control cells. (C) Real-time cell analysis (RTCA) is
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21 11 shown for A-375-TS cells treated with 1.25 and 2.5 μM indirubin. Treatment started at 24 h.
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23 12 The determined cell index, normalized to the time of treatment, gives a relative measurement
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25 13 of cell numbers. The experiment was performed two times, each time with triple values,
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27 14 which revealed largely comparable results. (D) Cell cycle analysis by flow cytometry after PI
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29 15 staining is shown for Mel-2a, A-375 and A-375-TS treated with 10 μM indirubin for 24 h, as
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31 16 compared to control cells. The numbers of cells in G1 and G2 (%) are given within the
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33 17 histograms. G1/G2 ratios were also determined for Mel-HO, MeWo and Mel-HO-TS, but not
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35 18 reveal a difference with indirubin treatment (data not shown).
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46 **Figure 2: Sensitization for death ligand-induced apoptosis**

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48 21 (A) Apoptosis values are given as percentage of sub-G1 cell populations, as determined by
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50 22 flow cytometry after PI staining. Cells were treated with indirubin (10 μM) and/or TRAIL (20
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52 23 ng/ml), as indicated. (B) Real-time cell analysis (RTCA) of A-375-TS cells treated with 2.5
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54 24 μM indirubin, 20 ng/ml TRAIL or the combination. (C) RTCA of A-375-CS cells treated with
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56 25 indirubin and/or 50 ng/ml CH-11. (B, C) Seeding density was 1,250 cells per microtiter well.
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1 The cell index was normalized at 24 h, when treatment had started. The experiment was
2 performed twice (each time with triple values), which revealed largely comparable results.
3 (D) Induction of apoptosis by CH-11 (50 ng/ml), indirubin (10 μ M) or the combination is
4 shown for A-375 and A-375-CS cell. (E) Cross resistance for the death ligands TRAIL and
5 CH-11. Left, A-375-TS were treated with CH-11 (50 ng/ml), indirubin (10 μ M) or the
6 combination; right, A-375-CS cells were treated with TRAIL (20 ng/ml), indirubin (10 μ M)
7 or the combination. (A, D, E) Means and SDs of triple values of representative experiments
8 are shown. All experiments were repeated three times, which revealed largely comparable
9 results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p < 0.005$), when comparing
10 combined treatment with indirubin alone.

12 **Figure 3: Combination of indirubin with chemotherapeutics**

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

23 **Figure 4: Enhanced caspase activation**

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

1 control cells. Equal protein amounts (30 μg per lane) were separated by SDS-PAGE, and
2 consistent blotting was proven by Ponceau staining as well as by evaluation of GAPDH
3 expression. Molecular weights (in kDa) of identified protein bands are indicated. (B)
4 Induction of apoptosis was determined for A-375 and A-375-TS cells without addition of
5 zVAD-fmk (above) or with preincubation of zVAD-fmk (10 μM) for 1 h (below). Further
6 treatments: indirubin (10 μM) and/or TRAIL (20 ng/ml). (C) Cell viability (fold change vs.
7 control) for A-375 and A-375-TS cells was determined by WST-1 assay. Cells were
8 preincubated for 1 h with zVAD-fmk (10 μM , below) before indirubin (2.5 μM) and/or
9 TRAIL (20 ng/ml) treatment started. The comparison was to cells without zVAD (above). (D)
10 RTCA of A-375-TS cells treated with indirubin (2.5 μM) alone, in combination with TRAIL
11 (20 ng/ml) or in triple combination with TRAIL and zVAD-fmk (10 μM). Seeding density
12 was 1,250 cells per microtiter well. The cell index was normalized at 24 h, when treatment
13 had started. The experiment was performed twice (each time with triple values), which
14 revealed largely comparable results.

16 **Figure 5: Response of mitochondria to indirubin**

17 (A) Decreased mitochondrial membrane potential ($\Delta\psi$) was determined by flow cytometry
18 after TMRM staining in A-375 and A-375-TS cells treated with TRAIL (20 ng/ml), indirubin
19 (10 μM) or the combination (above). Histograms of non-treated controls, C, are in grey. A
20 comparison is shown with cells preincubated with zVAD-fmk for 1 h (below). (B)
21 Quantification of A). (C) Loss of $\Delta\psi$ is shown in a time kinetic for A-375-TS cells treated for
22 2, 4, 8 and 24 h with 10 μM indirubin, 20 ng/ml TRAIL or the combination. (A, C)
23 Experiments were performed 2-3 times, each time with triple values, leading to highly
24 comparable results. (D) Apoptosis was determined in parallel with C). Means and SDs of

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

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7 **Figure 6: Release of mitochondrial factors**

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10 (A) Mitochondrial fractions (Mito) and cytosolic fractions (Cyto) were isolated from A-375-
11 TS and A-375 cells treated for 8 h with indirubin (10 μ M), TRAIL (20 ng/ml) or the
12 combination. Equal amounts of cytosolic cell extracts (20 μ l) were separated by SDS-PAGE.
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14 Consistent blotting was proven by Ponceau staining and by evaluation of GAPDH expression.
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16 Molecular weights of identified protein bands are indicated. Mitochondrial extracts served as
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18 controls, and analysis of the mitochondrial protein VDAC ruled out any contamination of
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20 cytosolic extracts with mitochondria. The experiment was repeated once, which revealed
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22 largely comparable results.
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31 **Figure 7: Effect of Indirubin on Bcl-2-overexpressing cells**

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33 (A, B) A-375 cell clones stably transfected with a Bcl-2 expression plasmid (A-375-Bcl-2)
34 and cells transfected with the empty plasmid (A-375-Mock) were treated for 8 h with
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36 indirubin (5 μ M or 10 μ M), TRAIL (20 ng/ml), CH-11 (50 ng/ml) or combinations, and
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38 apoptosis was determined. Bars represent mean values \pm SD of a representative of three
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40 independent experiments, each one consisting of triple values. Independent experiments
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42 revealed comparable results. Statistical significance is indicated by * ($p < 0.05$) or ** ($p <$
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44 0.005), when the combination treatment was compared with indirubin alone. Inset shows Bcl-
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46 2 overexpression in A-375-Bcl-2. (C) Loss of $\Delta\psi$ was determined by flow cytometry after
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48 TMRM staining for A-375-Bcl-2 and A-375-Mock. Cells were treated for 8 h with indirubin
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50 (10 μ M), TRAIL (20 ng/ml) or the combination and were compared to non-treated controls
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52 (grey). The experiment was repeated twice, which revealed largely comparable results.
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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 μ M) and/or TRAIL (20 ng/ml) by Western blot analysis. Equal protein amounts (30 μ g 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.

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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 μ M), 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 μ g 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 μ M) and/or TRAIL (20 ng/ml), as indicated. Two independent experiments were performed, which revealed largely comparable results.

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Figure 1

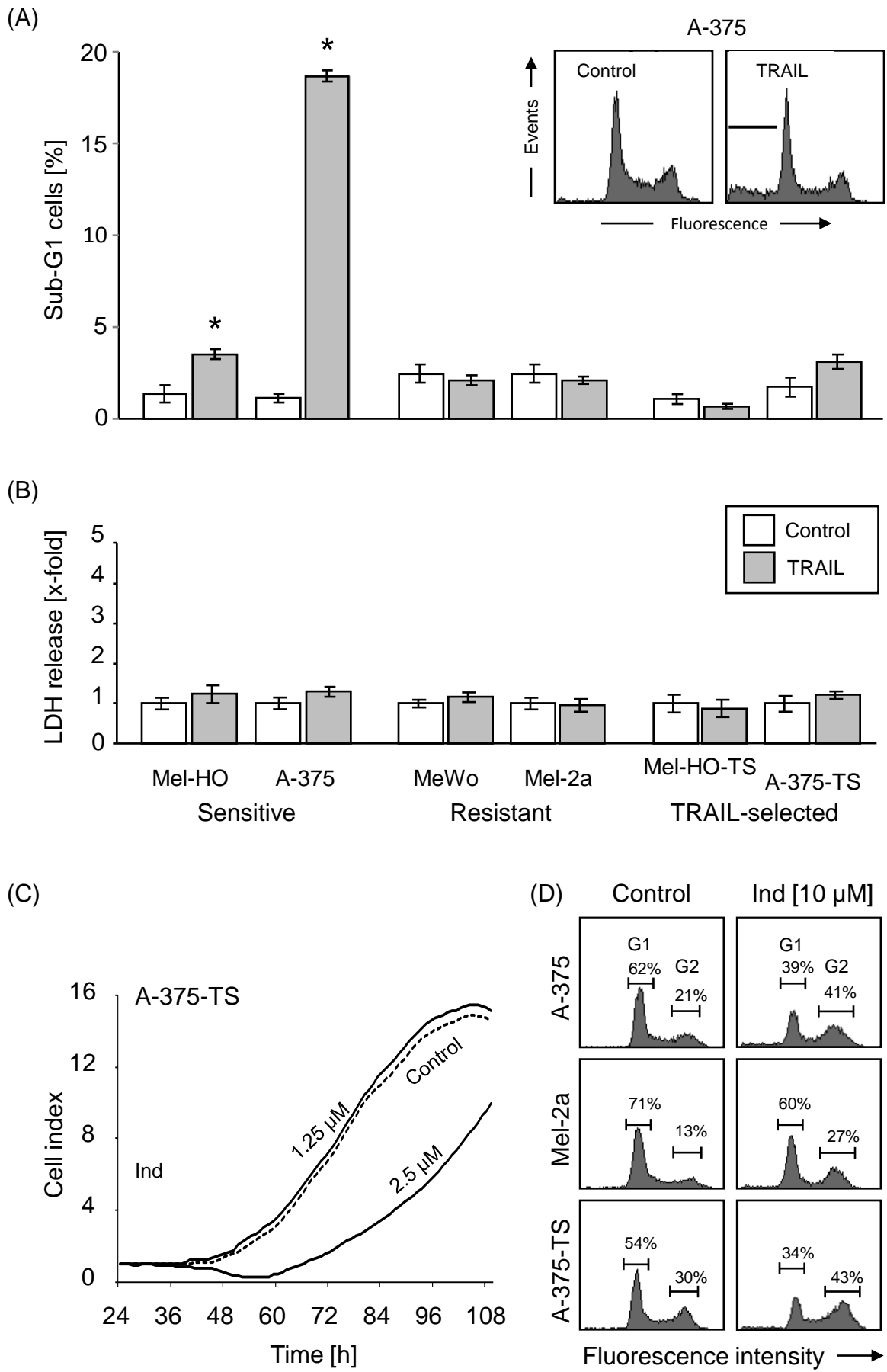


Figure 2

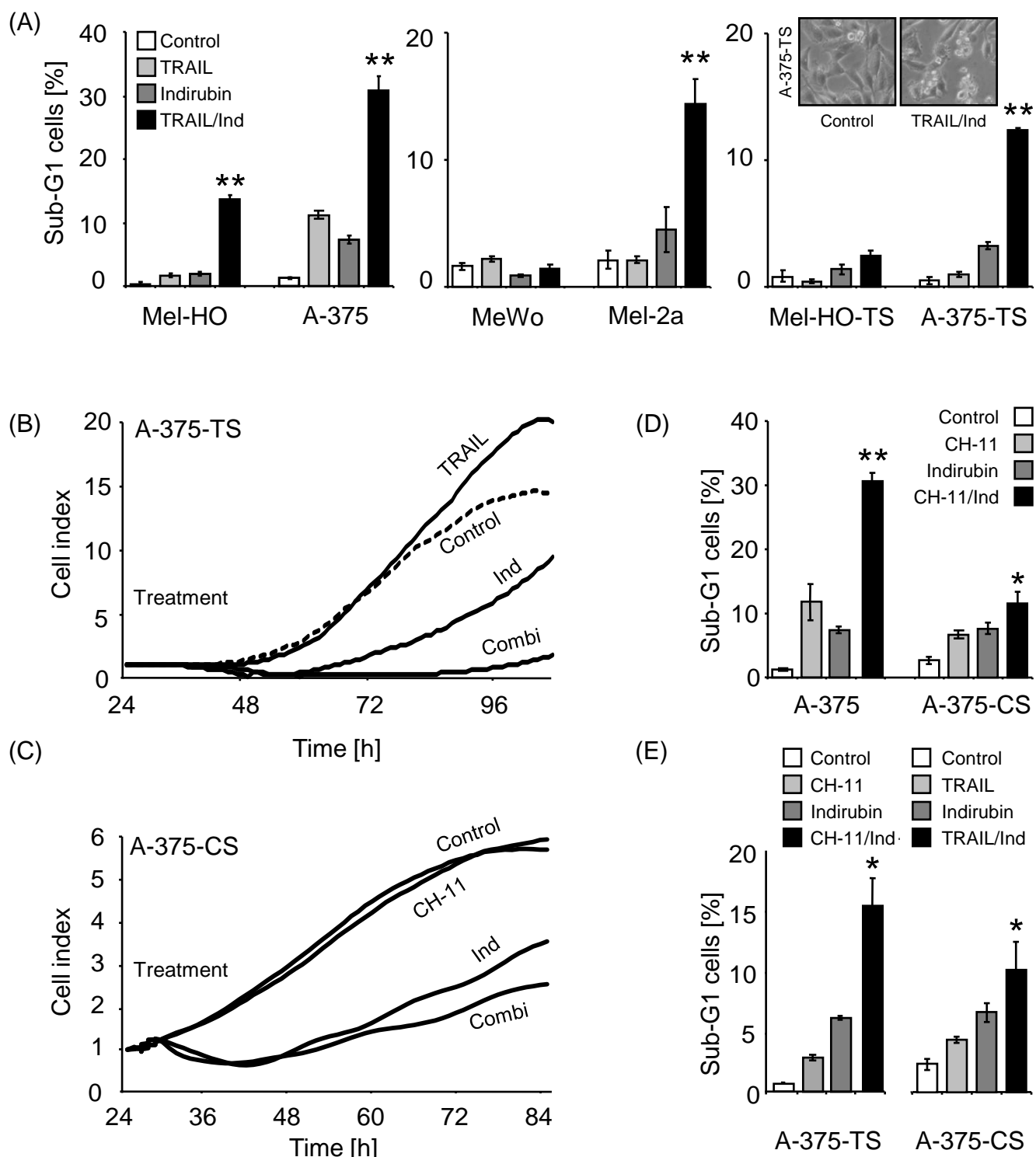


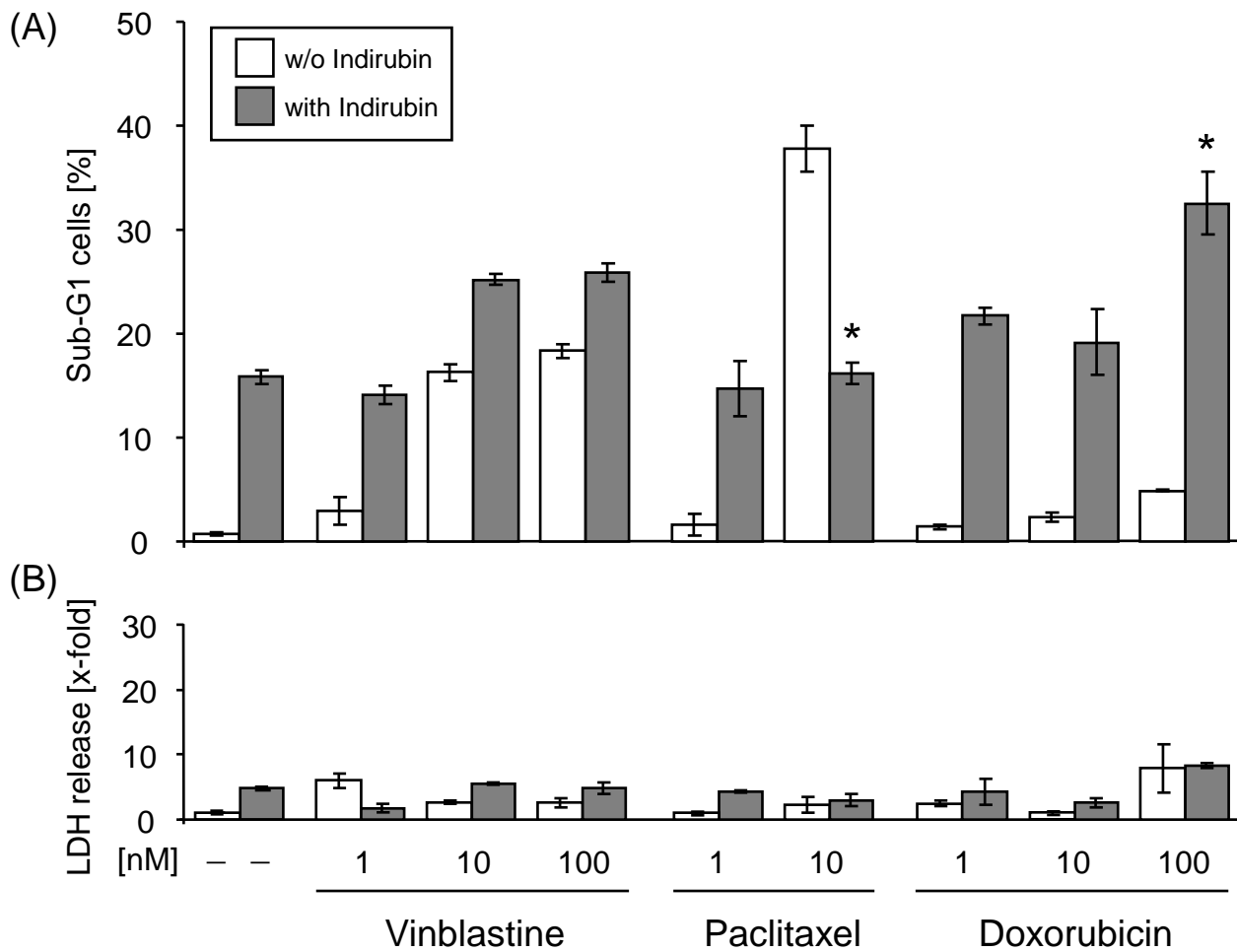
Figure 3

Figure 4

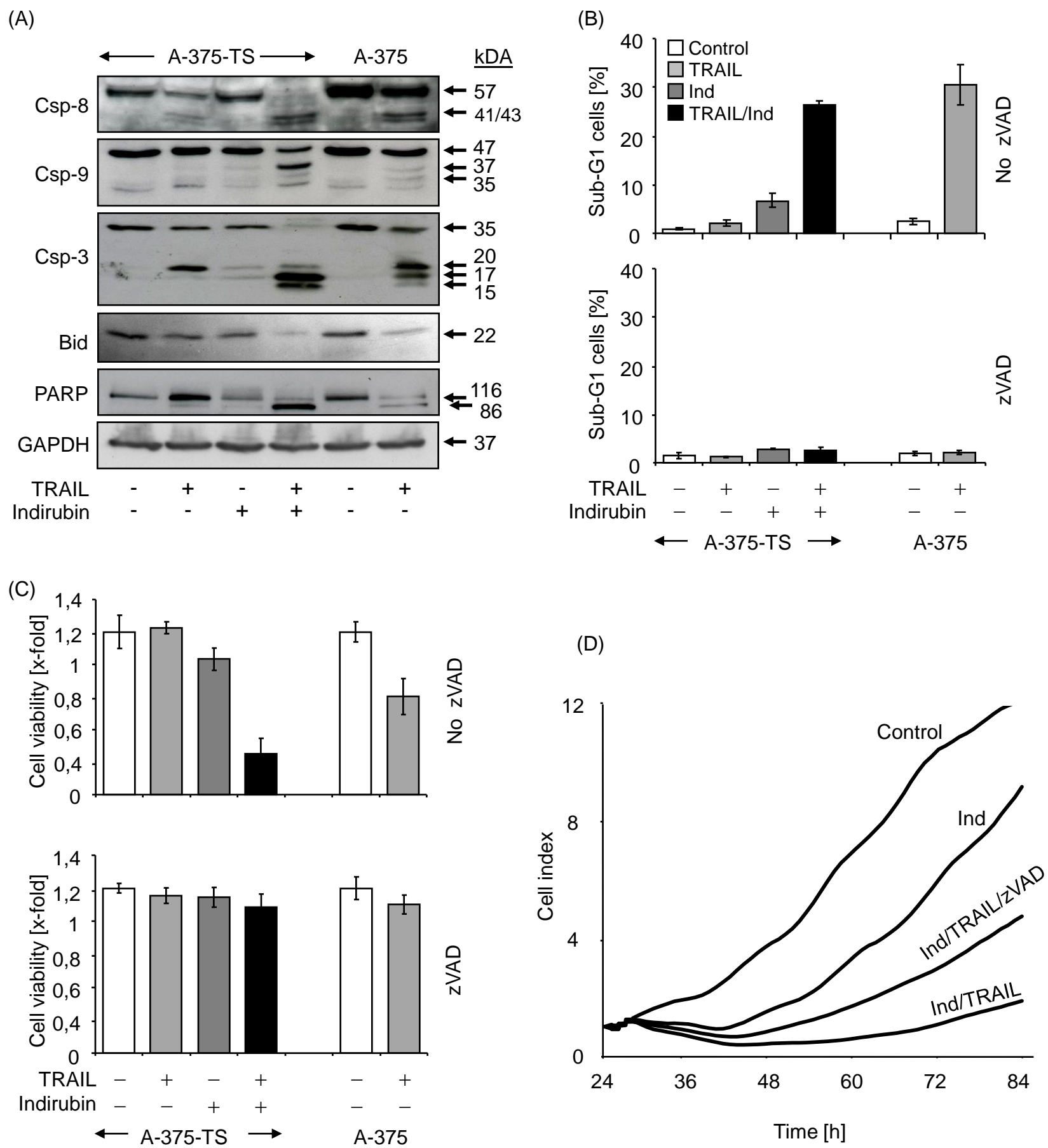


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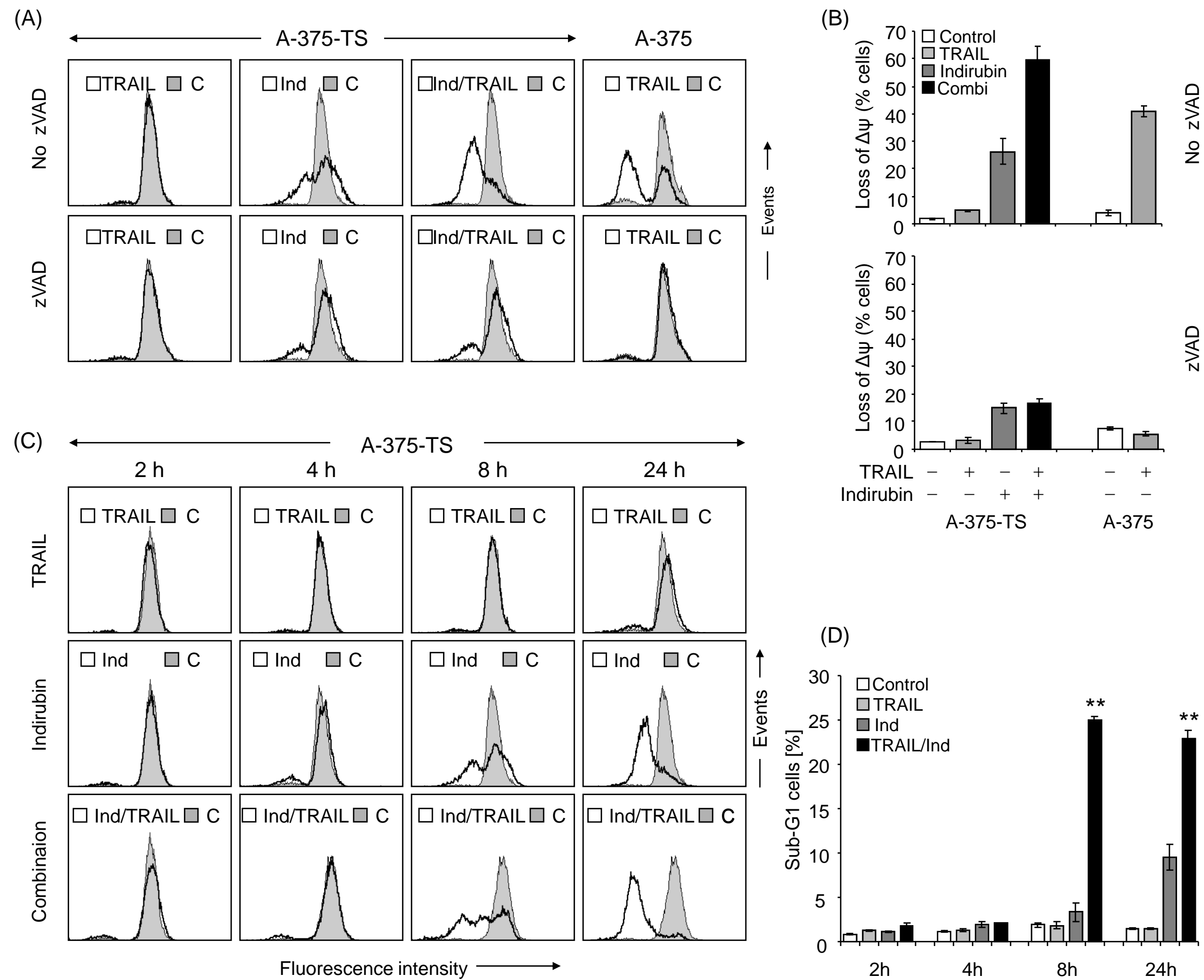


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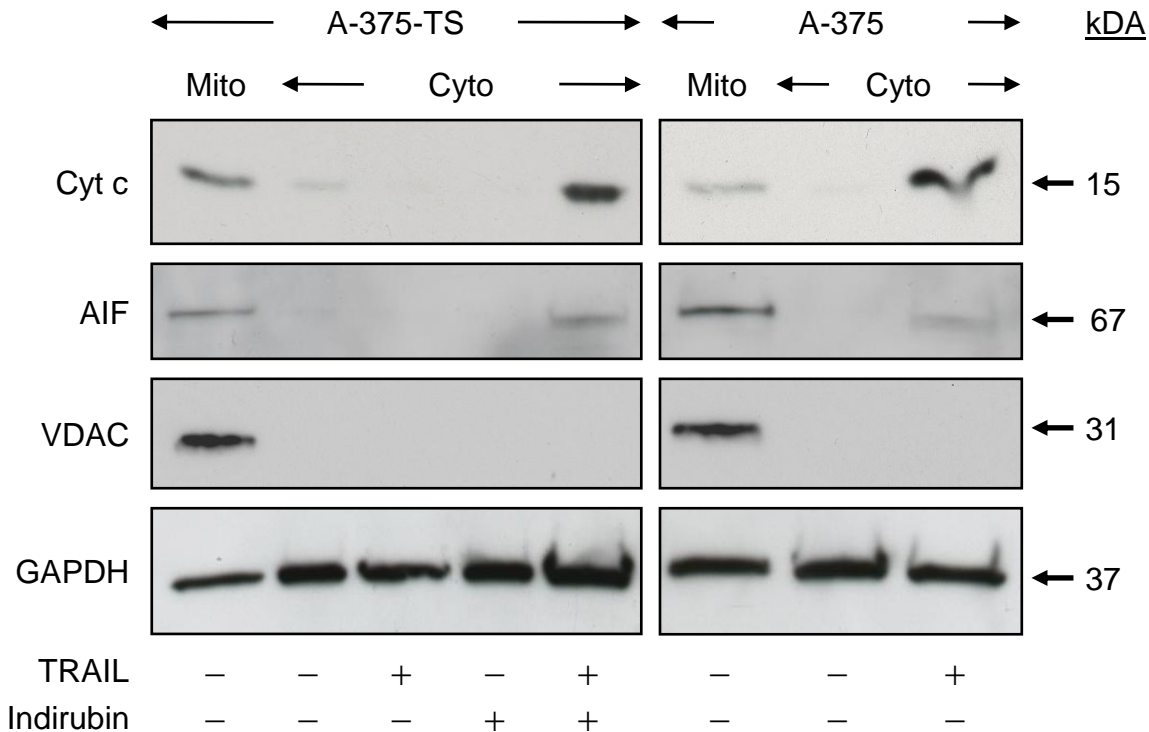


Figure 7

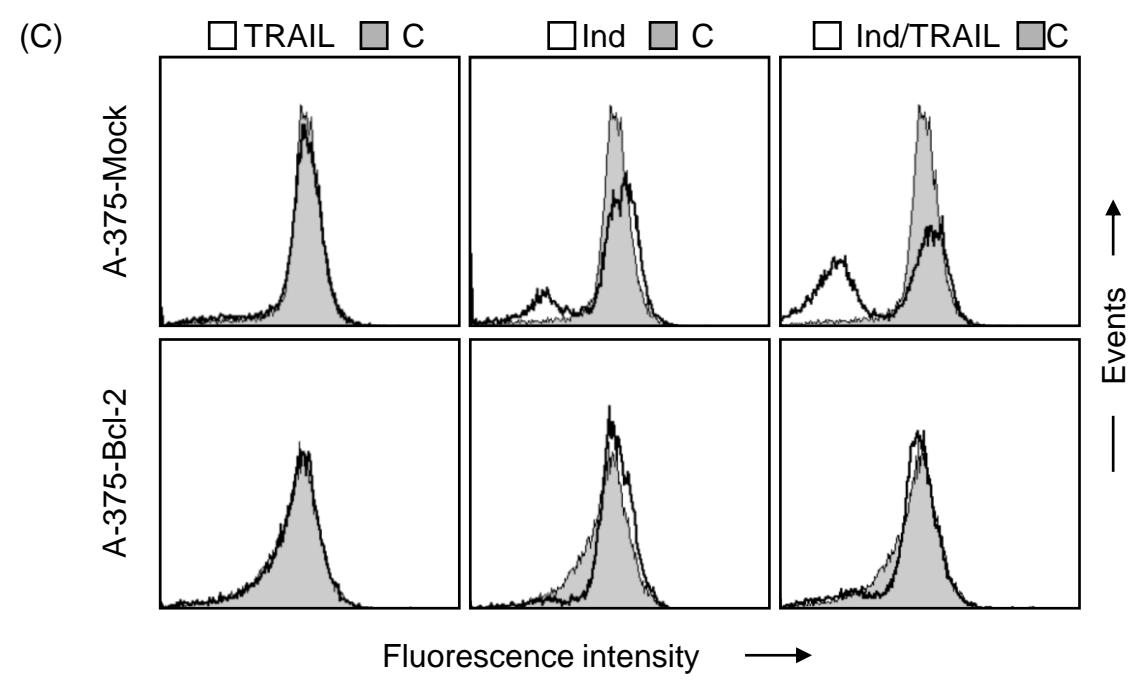
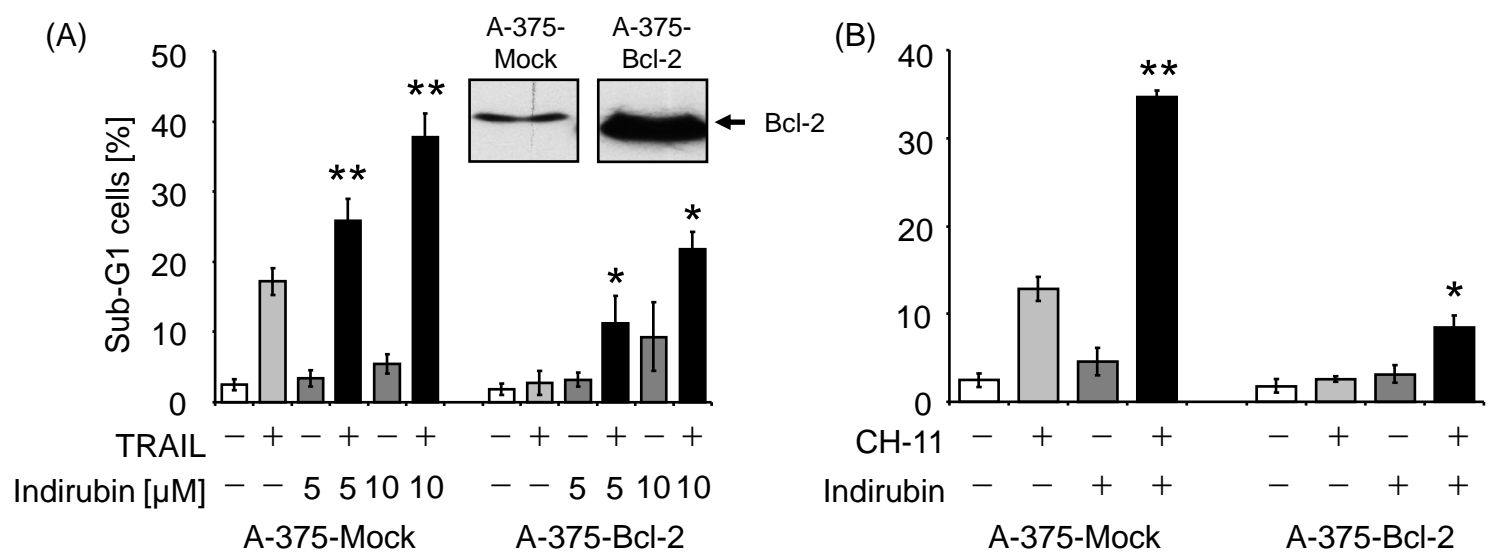


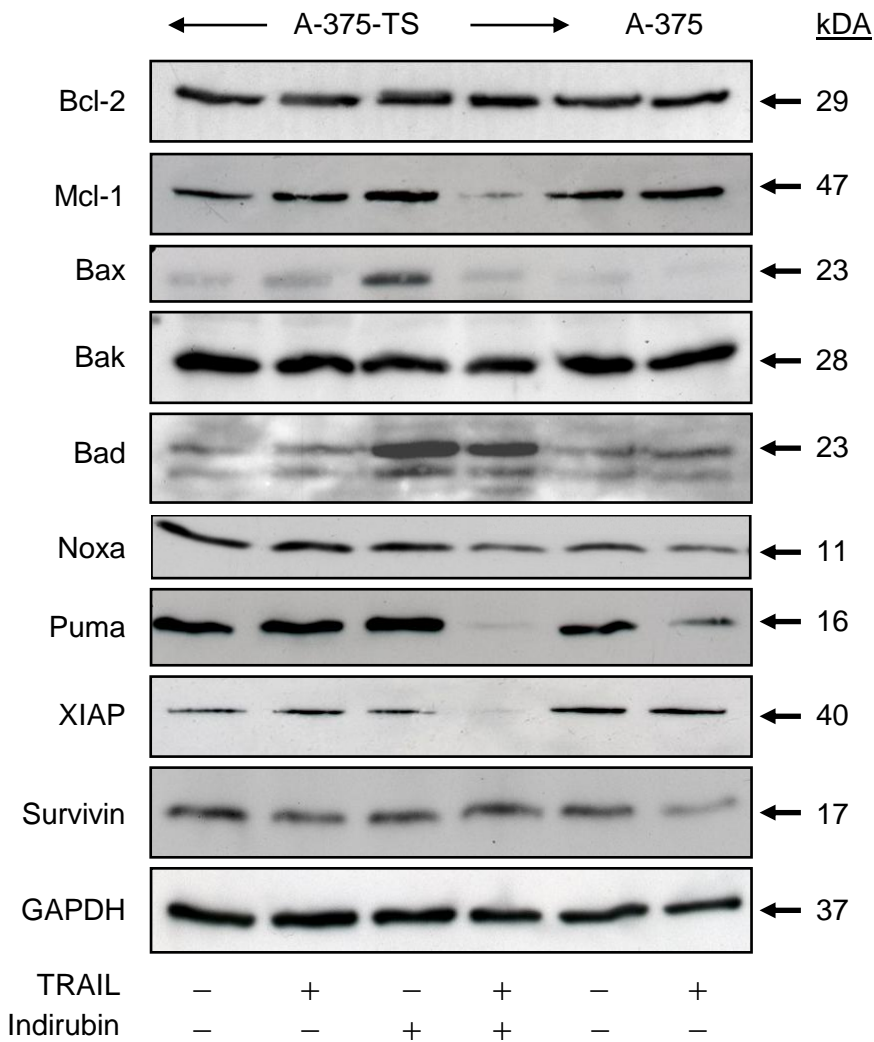
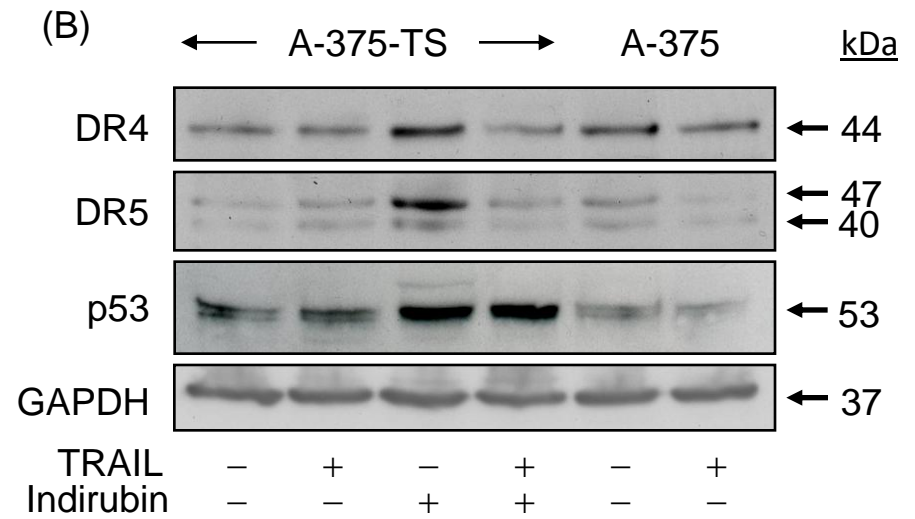
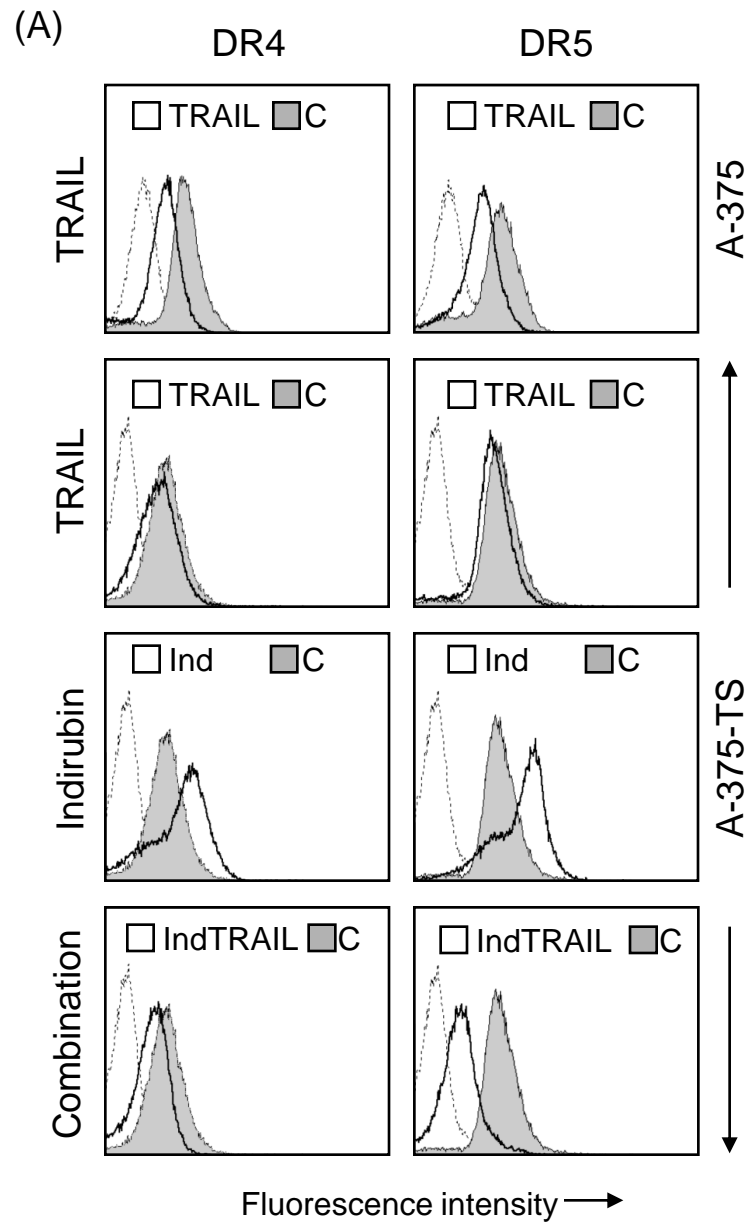
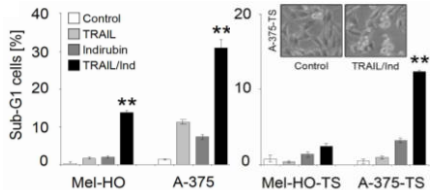
Figure 8

Figure 9

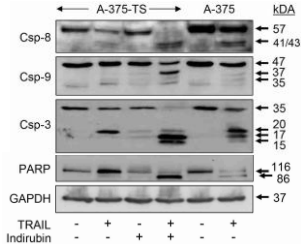


*Graphical Abstract

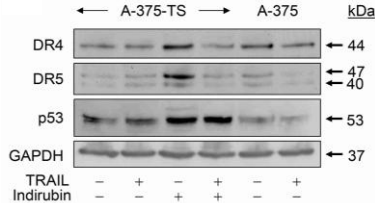
Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative – Enhancement of both extrinsic and intrinsic apoptosis pathways



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



Enhanced caspase activation



Upregulation of death receptors and p53