Research Article

Sensitization of human melanoma cells for TRAIL-induced apoptosis by a selective aurora kinase A inhibitor

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Abstract

Different therapeutic strategies in metastatic melanoma focused on signalling pathways controlling cell proliferation, cell cycle and apoptosis. While TRAIL (TNF-related apoptosis-inducing ligand) has been shown to be an interesting candidate for inducing apoptosis in cancer cells without affecting normal cells, the ability of cancer cells to develop resistance, limits its therapeutic potential. Using a recently established experimental A-375 melanoma cell model for investigating TRAIL resistance, we could demonstrate that the aurora kinase A inhibitor Alisertib (MLN 8237) enhances the proapoptotic effects of TRAIL and sensitizes TRAIL-selected melanoma cells with acquired resistance, associated with an activation of intrinsic mitochondrial apoptotic pathways. In course of this activation, an upregulation of p53 in the nuclear fraction was shown. Thus, the aurora kinase A inhibitor Alisertib is able to overcome TRAIL-induced resistance in melanoma cells suggesting the combination of TRAIL and Alisertib as a promising therapeutic strat-

Introduction

Therapy of metastatic melanoma is still a challenge because of its high resistance against traditional chemotherapeutic drugs as well as against new therapeutic approaches. Recently, BRAF inhibitors and immune-checkpoint modulators have shown promising results in melanoma therapy (Shtivelman *et al*. 2014), however due to frequent tumor relapse, the development of new ideas for improved therapy is still needed. We have shown in several experimental models that triggering apoptotic cascades by overexpression of proapoptotic factors provides an efficient targeting strategy (Bektas *et al*. 2005, Eberle et al. 2007, Fecker et al. 2005, Hossini et al. 2003). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/APO2L) belongs to the tumor necrosis factor (TNF) superfamily and binds to its death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 (Pan *et al*. 1997, Walczak *et al*. 1997). The particular significance of TRAIL for clinical strategies results from its ability to selectively target tumor cells, whereas most non-malignant cells are largely protected (Yagita *et al*. 2010).

Over recent years, high-throughput analyses have provided important insights into molecular pathways that play a crucial role in the progression from early to advanced melanoma - thereby identifying aurora kinases as possible key regulators. Aurora kinases are members of a serine/threonine kinase family, which are essential for cell division and are therefore potential targets for cancer therapy. It was shown that aurora kinases are upregulated to high levels during melanoma progression from *in situ* to primary and metastatic melanoma (Jeng *et al*. 2004, Liu *et al*. 2013, Wang *et al*. 2010).

Systemic treatment of human melanoma xenografts with an aurora kinase small-molecule inhibitor or with aurora kinase targeting vectors impairs cell proliferation and cell cycle progression and induces apoptosis (Wang *et al*. 2010). Recently, it was shown in a mouse xenograft model for metastatic melanoma that inhibition of aurora kinase A by Alisertib impairs mitosis, induces senescence and markedly blocks cell proliferation (Liu *et al*. 2013).

The aim of the present experimental study was to clarify the role of aurora kinase A for TRAIL-induced apoptosis in melanoma. Using an established experimental melanoma cell culture model comparing TRAIL-selected A-375 and wild type A-375 cells (Kurbanov *et al.* 2007), we investigated the role of aurora kinase A in TRAIL resistance. We demonstrate that inhibition of aurora kinase A by the selective inhibitor Alisertib not only sensitizes human melanoma cells for TRAIL-induced apoptosis but also overcomes induced TRAIL resistance. This may indicate a new promising strategy in melanoma therapy.

Materials and Methods

Cell culture

TRAIL-sensitive human melanoma cell lines A-375 and Mel-HO as well as induced TRAIL-resistant A-375-TS and intrinsic TRAIL-resistant MeWo were investigated. A-375-TS cells have been selected by continuous treatment with 5 ng/ml TRAIL (Kurbanov *et al.* 2007, 2010). All cell lines were cultivated at 37° C, 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium; 4.5 g/l glucose; Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (fetal calf serum) and antibiotics (Biochrom, Berlin, Germany). For selection, A-375-TS cells were continuously kept under TRAIL treatment (5 ng/ml). For induction of apoptosis, soluble human TRAIL (Alexis, Gruenberg, Germany; 20 ng/ml, 16 - 24 h) was applied. Treatment with aurora kinase A inhibitor (Alisertib, MLN 8237, Selleckchem, Munich, Germany) was set up 2 h before TRAIL, whereas control cells received DMSO (Dimethyl sulfoxide). Treatment with QVD-Oph (Quinoline-Valine-Aspartic-CH2-O-Phenyl) was set up 2 h before Alisertib.

Real-time cell analysis

For obtaining growth curves, cell confluence was continuously monitored by real-time cell analysis (RTCA, xCELLigence, ACEA Biosciences, San Diego, CA, USA). The technique is based on microelectrodes integrated in the bottom of each well of special 96-well E-plates. The cell index corresponds to electric resistance, which itself is largely proportional to cell density. 1,250 cells were seeded per microtiter well, and treatment started after 24 h.

Quantification of apoptosis, cytotoxicity and cell viability

For quantification of apoptosis, cell cycle analyses were carried out after propidium iodide staining (Riccardi & Nicoletti 2006). Cells were harvested by trypsinization, stained with PBS buffer (phosphate-buffered saline) containing Triton-X 100, sodium citrate and propidium iodide (Sigma, Taufkirchen, Germany; 40 µg/ml). After centrifugation, cells were washed with PBS and analyzed by flow cytometry in a FACS (fluorescence-activated cell sorting analyses) Calibur. Cytotoxicity was determined in parallel by measuring LDH (lactate dehydrogenase) activity in culture fluids applying a cytotoxicity detection assay (Roche Diagnostics).

For quantification of cell viability, trypsinized cells were stained with calcein-AM viability dye (eBioscience; San Diego, CA; 2.5 µg/ml in PBS, 37°C, 1 h), washed with PBS and analyzed by flow cytometry (Berger *et al.* 2013).

Mitochondrial membrane potential

For determination of the mitochondrial membrane potential, the fluorescent dye TMRM (tetramethylrhodamine methyl ester perchlorate) was used. Cells were harvested by trypsinization, stained with TMRM (Sigma Aldrich, Taufkirchen, Germany; 1 µM, 20 min; 37°C), washed once with PBS buffer and analyzed in PBS by flow cytometry (Berger *et al.* 2013).

Western blot analysis

For Western blotting, total protein extracts were obtained by cell lysis in 150 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetate), 2 mM PMSF (phenylmethylsulfonylfluoride), 1 mM leupeptin, 1 mM pepstatin, 0.5% SDS (sodium dodecylsulfate), 0.5% NP-40 (Nonidet-40), and 10 mM Tris-HCl (Tris (hydroxymethyl) aminomethane hydrochloride), pH 7.5. For p53 detection, cytosolic and nuclear cell fractions were separated (Berger *et al.* 2013). Western blotting on nitrocellulose membranes was performed as described previously (Eberle *et al.* 2003).

The following primary antibodies were used: cleaved caspase-3 (Cell Signaling, 3664, rabbit, 1:1000), caspase-3 (Cell Signaling, 9662, rabbit, 1:10,000), caspase-8 (Cell Signaling, 9746, mouse, 1:1000), caspase-9 (Cell Signaling, 9505, rabbit, 1:1000), p53 (Santa Cruz, sc-126, mouse, 1:500), p21 (Santa Cruz, sc-6246, mouse, 1:200), Bax (Santa Cruz, sc-20067, mouse, 1:200), Bak (Santa Cruz, sc-832, rabbit, 1:200), Bcl-2 (Santa Cruz, sc-509, mouse, 1:200), Mcl-1 (Santa Cruz, sc-12756, mouse, 1:200), AURKA (Leica Biosystems, Newcastle Upon Tyne, UK; JLM28, 1:750), GAPDH (Santa Cruz, sc-32233, mouse, 1:200) and TATA-box-binding protein (Abcam, 1TBP18, mouse, 1:2000).
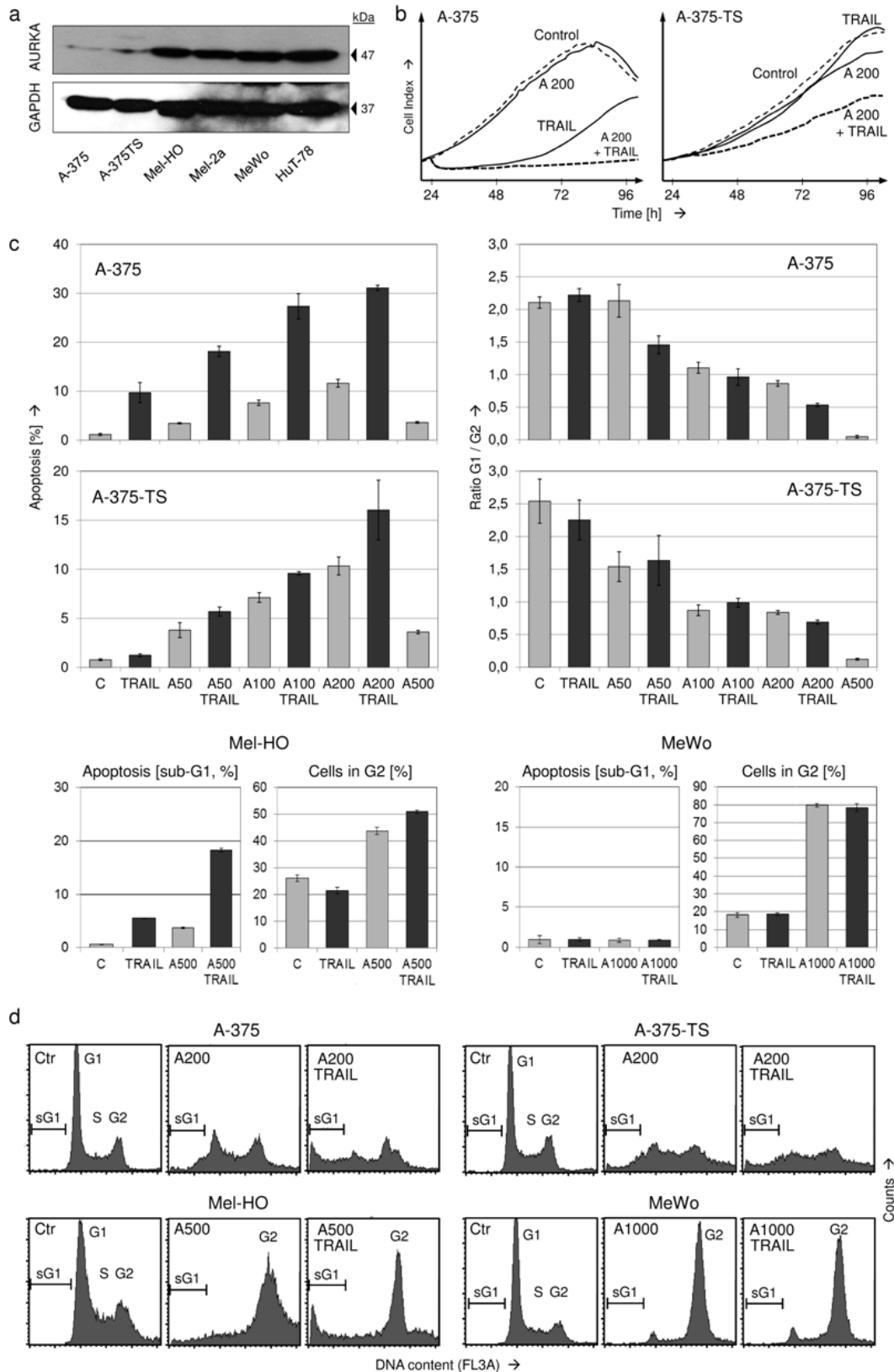
Figure 1. Enhanced TRAIL-induced apoptosis and G2 cell cycle arrest by Alisertib. **(a)** Expression of aurora kinase A was investigated by Western blot analysis in melanoma cell lines A-375, A-375-TS, Mel-HO, Mel-2a, and MeWo and was compared to a positive control (HuT-78, cutaneous lymphoma cell line). **(b)** Growth curves of A-375 and A-375-TS were determined by real-time cell analysis after treatment with 200 nM Alisertib (A200), TRAIL and the combination. Cell index corresponds to cell confluence. **(c)** Cell cycle analyses were performed in A-375, A-375-TS, Mel-HO and MeWo in response to increasing concentrations of Alisertib (50 nM – 1000 nM, A50 – A1000) +/- TRAIL. They based on flow cytometry after propidium iodide staining and were evaluated for percentages of apoptotic, sub-G1 (sG1) cells, for cell populations in G2 or for ratios of cells in G1/G2. **(d)** Examples of cell cycle analyses are given. At least two independent experiments (each one with triplicates) showed highly comparable results.

Results

For evaluating the significance of aurora kinase inhibitors in melanoma, the effects of the selective aurora kinase A inhibitor Alisertib on cell proliferation, apoptosis and cell cycle progression were determined in TRAIL-sensitive melanoma cell lines A-375 and Mel-HO as well as in intrinsically TRAIL-resistant MeWo. The ability to overcome inducible resistance to the death ligand TRAIL was further evaluated in a TRAIL resistance model based on TRAIL-selected A-375-TS cells. Melanoma cell lines showed general protein expression of aurora kinase A, as determined by Western blotting (Figure 1a). Whereas the effects of Alisertib alone (200 nM) on A-375 growth remained rather lim-

ited, a significant reduction of cell proliferation was recorded when it was used in combination with TRAIL. This also applied to A-375-TS, which revealed complete resistance to TRAIL single treatment (real-time cell analysis, Figure 1b).

Inhibition of cell proliferation correlated with significant induction of apoptosis by the combination Alisertib/TRAIL, as shown in a concentration series by cell cycle analysis. Thus, TRAIL-induced apoptosis was clearly enhanced in sensitive A-375 and Mel-HO, and TRAIL resistance was overcome in TRAIL-selected A-375-TS (Figure 1c). In contrast, cytotoxicity was not enhanced by the combination treatment vs. TRAIL alone, as determined by release of lactate dehydrogenase (data not shown). Alisertib also resulted
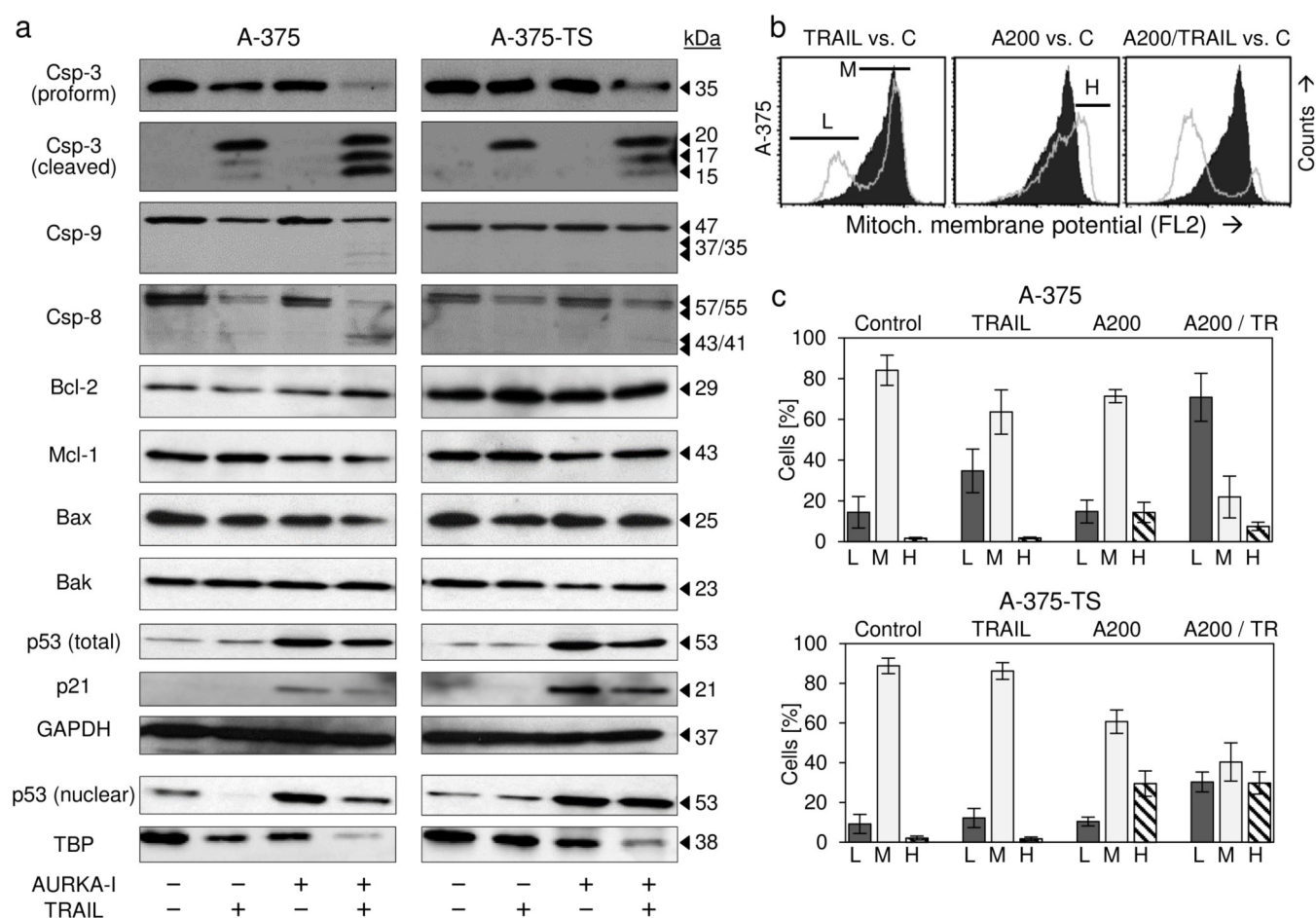

Figure 2. Enhanced TRAIL-induced caspase processing as well as activation of proapoptotic mitochondrial and p53-dependent pathways. **(a)** Expression of regulators of apoptosis pathways and of cell cycle control was investigated by Western blotting in A-375 and A-375-TS in response to 200 nM Alisertib (A200) +/- TRAIL. Analyses included effector caspase-3 and initiator caspases -8 and -9, pro- and antiapoptotic Bcl-2 proteins (Bcl-2, Mcl-1, Bax, Bak), p53 and its target p21. Glycerin aldehyde 3 phosphate (GAPDH) served as loading control for total protein extracts. Nuclear extracts were analyzed for nuclear p53, and TATA-binding protein was used as control. (b) Activation of proapoptotic mitochondrial pathways was monitored by determination of the mitochondrial membrane potential (Dym). Histogram examples are given and cell populations with low (L), medium/normal (M) and high (H) Dym are indicated. (c) Cell numbers with different Dym (L/M/H) were evaluated for A-375 and A-375-TS in response to treatment with 200 nM Alisertib (A200) +/- TRAIL. Mean values and SDs correspond to three independent experiments (each one with triplicate values).
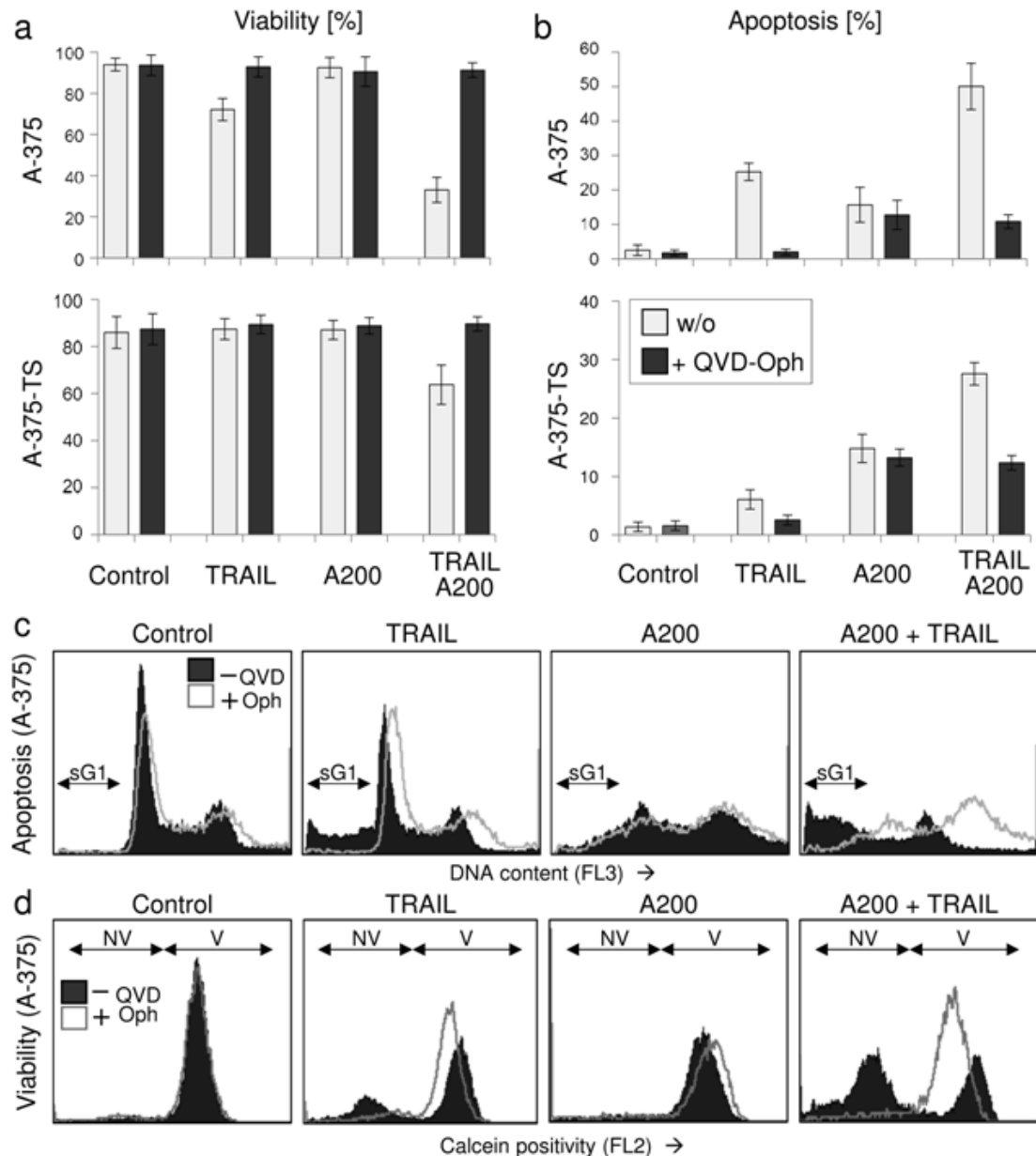
Figure 3. Enhancement of TRAIL-induced apoptosis by Alisertib depends on caspase activity in contrast to the effects of Alisertib alone. **(**a,b) Dependency of the proapoptotic effects of Alisertib (200 nM) +/- TRAIL on caspase activity was investigated in A-375 and A-375-TS cells treated in addition with the pancaspase inhibitor QVD-Oph (dark bars, 1 h pretreatment). (a) cell viability as determined by flow cytometry after calcein-AM staining; (b) apoptosis (sub-G1 cells). (c,d) examples of apoptosis (c) and cell viability (d) are given for A-375 cells. Two independent experiments (each one with triplicates) showed highly comparable results. Abbreviations: sG1, sub-G1 cells; NV, non-viable cells; V, viable cells.

in dose-dependent G2 cell cycle arrest in all cell lines, which was most pronounced at higher concentrations, when almost no G1 cells remained. In contrast, intrinsically TRAIL-resistant MeWo could not be sensitized for TRAIL, but this cell line was also clearly responsive as seen by massive G2 cell cycle arrest after treatment with 1000 nM Alisertib (Figure 1c, d).

To investigate the mechanisms of apoptosis induction in more detail, activation of the caspase cascade was examined by Western blotting. Caspase 3 processing due to TRAIL was largely blocked in A-

375 and was completely blocked in A-375-TS at its premature cleavage product of 20 kDa. This blockage was decisively overcome in both cell lines by the combination of TRAIL and Alisertib, resulting in mature caspase-3 cleavage products of 17 and 15 kDa. It further correlated with significant degradation of the caspase-3 proform. Similarly, initiator caspases -8 and -9 were activated (processed) in A-375 (Figure 2a).

No characteristic regulation of antiapoptotic (Bcl-2, Mcl-1) or of the two multidomain proapoptotic Bcl-2 proteins (Bax, Bak) was observed in course of

200 nM Alisertib treatment at 24 h. But significant activation of the p53 pathway was found. Whereas p53 and p21 were not upregulated by TRAIL, Alisertib resulted in marked upregulation/stabilization of the levels of total p53 and its characteristic target p21. By examining the subcellular distribution of p53, it was furthermore shown that p53 was clearly upregulated by Alisertib in nuclear fractions, an essential step in the p53 pathway (Figure 2a).

Loss of the mitochondrial membrane potential ($\Delta\psi$m) indicates an activation of mitochondrial apoptosis pathways. As published by our group, TRAIL led to mitochondrial membrane potential breakdown in A-375 whereas A-375-TS was resistant. Alisertib itself did not decrease Dym, but resulted in a hyperpolarization, its role in apoptosis regulation is not clearly defined (Figure 2b). In combination with TRAIL, however, a dramatic loss of Dym was observed: 71% +/- 12% and 30% +/- 5% for A-375 and A-375-TS, respectively (Figure 2c). This suggests that the combination treatment overruled TRAIL resistance by triggering mitochondrial proapoptotic pathways. While A-375-TS are resistant to TRAIL-induced mitochondrial membrane potential breakdown, Alisertib is able to induce its effect also in this cell line.

Furthermore, induction of apoptosis and decreased cell proliferation correlated with decreased cell viability after Alisertib/TRAIL treatment, as shown by calcein-AM staining (Figure 3a, light grey bars). The role of caspase-dependent pathways was further elucidated by the use of a pan-caspase inhibitor (QVD-Oph). Thus, QVD-Oph completely abrogated apoptosis induced by TRAIL itself, and it prevented the enhancing effect of Alisertib on TRAIL-induced apoptosis. In contrast, Alisertib-induced apoptosis was not blocked upon caspase inhibition, indicating that the enhancing effect and direct induction of apoptosis by Alisertib were mediated by distinct mechanisms (Figure 3b, c). A similar effect of QVD-Oph was observed on cell viability, namely the loss of cell viability due to treatment with TRAIL alone or TRAIL/Alisertib was prevented (Figure 3a, 3d). This underlined the significance of the caspase pathway for apoptosis sensitization by Alisertib.

Discussion

The ability of tumor cells to undermine checkpoints of cell cycle control results in enhanced cell proliferation and induced apoptosis resistance. This understanding has led to the identification of new antitumor targets. Resistance to TRAIL was related to upregulation of Bcl-2 and cIAPs, inhibited Bax activation as well as downregulation of death receptors and caspases

(Eberle *et al*. 2007, Kurbanov *et al*. 2007) Aurora kinases have come into focus in recent years because of their crucial role in cell cycle regulation. Aurora kinase A is located at the spindle pole and controls transition from G2 phase to mitosis (Andrews *et al*. 2003, Vader & Lens 2008). Although constitutively expressed in all cells, aurora kinase A was shown to be upregulated in a variety of cancer cells (Jeng *et al*. 2004).

In this study we also demonstrate strong expression of aurora kinase A in melanoma cells. The inhibition of aurora kinase A by the selective inhibitor Alisertib was able to overcome inducible TRAIL resistance in melanoma cells, whereas an intrinsically TRAIL resistant cell line (MeWo) was not sensitized. Indeed, inducible TRAIL resistance, as seen in A-375-TS, appears as a major problem in melanoma TRAIL sensitivity. Thus, we had previously shown expression of TRAIL receptor 1 (DR4) in >90% of melanoma sections, which is characteristic for intrinsic sensitivity and inducible resistance, whereas MeWo is DR4-negative (Kurbanov *et al*. 2005).

Proapoptotic effects were associated with activation of the p53/p21 pathway, also seen by nuclear accumulation of p53. Interestingly, apoptosis induced by Alisertib itself does not enclose activation of the proapoptotic caspase cascade nor of mitochondria, whereas the combination correlated with activation of caspases and loss of mitochondrial membrane potential. This indicates distinct activities leading to direct induction of apoptosis and enhanced TRAIL sensitivity.

A correlation between aurora kinase A overexpression and suppression of p53 activity has already been discussed previously (Katayama *et al*. 2004). Thus, Aurora kinase A overexpression results in genomic instability, aneuploidy, cell cycle arrest as well as dysregulation of NF-kB and AP-2α, which trigger tumor growth, chemotherapy and apoptosis resistance. Suppression of p53 eliminates important checkpoints in cell cycle and apoptosis control (Meraldi *et al*. 2002). It is known that p53 and aurora kinase A are involved in a strictly regulated, negative feedback loop, which is frequently interrupted in malignant cells (Katayama *et al*. 2004). The important role of p53 for epithelial tumors is well described whereas its role in melanoma is still controversial.

Very recently, Liu and coworkers reported that combining aurora kinase inhibitors and TRAIL or TRAIL agonistic antibodies triggers apoptosis in melanoma cells and melanoma xenografts (Liu *et al*. 2015). In conclusion, both studies using different experimental models support the strategy to combine aurora kinase inhibitors with TRAIL for targeting melanoma.

Competing interests

Authors' contributions

Acknowledgements

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