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Synthetic Tubulysin Derivative, Tubugi-1, Against Invasive Melanoma Cells: The Cell Death Triangle

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Abstract. *Background/Aim: Tubugi-1 is a more stable and accessible synthetic counterpart of natural tubulysins. This study aimed to evaluate its cytotoxic potential against anaplastic human melanoma cells. Materials and Methods: The viability of A-375 cells was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet assay. The type of cell death and proliferative rate were investigated using flow cytometry and fluorescent microscopy, while the molecular background was evaluated by western blot. Results: Tubugi-1 reduced the viability of A-375 cells, inducing massive micronucleation, followed by augmented expression of inhibitor of nuclear factor-κB and caspase-2, typical of a mitotic catastrophe. Disturbed proliferation and G_2M block with prominent caspase activity, weakened the expression of B-cell lymphoma 2 and B-cell lymphoma 2-associated X transient up-regulation, coexisted with intensive autophagy. Specific inhibition of autophagy by chloroquine resulted in conversion from mitotic catastrophe to rapid apoptosis. Conclusion: Multilevel anticancer action of tubugi-1 is extended by co-application of an autophagy inhibitor, giving a new dimension in further preclinical advancement of this potential agent.*

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Cancer is an enormous health problem, growing at an alarming pace all over the world. Invasive melanoma is the key cause of skin cancer-related deaths regardless of its low prevalence of less than 5% of all skin cancer cases (1). Its incidence has risen over the past five decades (1), indicating weak progress in terms of desirable and effective therapy regimens. Over 60% of anticancer medications brought to the market in past 25 years come from the nature or are modeled on it (2). Natural compounds also form the backbone of traditional systemic chemotherapy today, and are commonly used in the treatment of a wide variety of cancer types. Over 25% of new agents a step from clinical use target the cellular protein tubulin and disturb mitotic spindle function (2).

Tubulysins represent a family of tetrapeptides originating from the genus *Myxobacterium* and recognized as highly active metabolites regarding their antiproliferative capacity. This was demonstrated in several human cancer cell lines (3, 4), even including multidrug-resistant clones (3, 5), with 50% inhibitory concentration (IC_{50}) values ranging from nano- to picomolar concentrations. The cytotoxic activity of tubulysin A, one representative of the family, is ascribed to the inhibition of tubulin polymerization, as well as induction of depolymerization of microtubules (6). Kaur *et al.* reported that tubulysin A induced apoptosis of cancer cells, but not normal cells (4). For reasons of limited availability of natural metabolites and their intrinsic sensitivity to hydrolysis (*e.g.* to form cyclo-tubulysins), tubulysin analogs, named tubugis, have been synthesized to overcome both limitations. Tubugi-1 was the first such analog to be developed. A cytotoxic study of tubugis on PC-3 prostate and HT-29 colon cancer cell lines confirmed that they retain the extraordinarily high antitumor activity of tubulysins (7). Our recent study on a B16 melanoma model revealed atypical apoptosis as the main mechanism of tubugi-1 action, with classical morphological features present, but without the externalization of phosphatidylserines (PS) (8). The significance of this result is the disclosure of a PS-independent pathway in eradication

of apoptotic cells from the tissue, since tubugi-1-induced apoptotic bodies were efficiently engulfed by primary macrophages regardless of the absence of PS on the outer side of a dying cell's membrane. Additionally, that study showed that tubugi-1 suppressed melanoma growth *in vivo* (8). The genesis of PS-free apoptotic cells may explain the observation that macrophages from tubugi-1-treated animals established an M1 phenotype, which represents the most significant result of this study. Thus, in addition to the extraordinary direct tumoricidal effect of tubugi-1, it also appears to potentiate the innate immune response against tumor cells. Consequently, this contributes to the drug's overall treatment outcome and is probably the first antimitotic agent for which such an effect has been reported.

The specificity of the mode of action of microtubule-affecting agents makes them prone to inducing mitotic catastrophe (MC), a form of cell death that arises after failed mitosis (9). Compromised cell-cycle checkpoints as the main feature of a malignant cell, coupled with DNA or mitotic spindle damage, create the background for the appearance of mitotic cell death (10). Induction of MC may represent a promising approach in the therapy of cancer. Some experimental data indicated that even mixed cell death, apoptosis and MC can occur in response to paclitaxel or docetaxel (11). Low doses of paclitaxel led to aberrant mitosis and micronucleation culminating in apoptotic cell death of cervical and breast cancer cells (12, 13). However, at higher concentrations, terminal mitotic arrest and necrosis were induced (14). Several reports show that this feature of mitotic spindle poisons can be useful for overcoming resistance to apoptosis in numerous types of cancer. It was found that triggering MC by vincristine surmounted the resistance to apoptosis of colon adenocarcinoma cells (15).

Herein we report the efficacy of tubugi-1 against A-375 human melanoma cells. The complex interplay between autophagy, MC and apoptotic cell death can be defined as a base for the successful application of this experimental agent against human melanoma, where MC represents a key determinant of cell destiny. Our results have special importance in light of the fact that the A-375 cell line originated from a highly metastatic form of melanoma with constitutive expression of inducible nitric oxide synthase (iNOS), making it highly aggressive and resistant to chemotherapy (16, 17).

Materials and Methods

Reagents and cells. RPMI-1640 culture medium was obtained from Biowest (Riverside, MO, USA). Fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin, dimethyl sulfoxide, crystal violet (CV), carboxyfluorescein diacetate succinimidyl ester (CFSE), propidium iodide (PI), 3-methyladenine (3-MA) and chloroquine were from Sigma–Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were bought from AppliChem (St. Louis, MO, USA). Paraformaldehyde (PFA) was purchased from Serva (Heidelberg, Germany). Annexin V-fluorescein isothiocyanate (FITC) was from BD Pharmingen (San Diego, CA, USA) and Apostat from R&D Systems (Minneapolis, MN, USA). Acridine orange (AO) was from LaboModerne (Paris, France). Penicillin/streptomycin solution was from Biological Industries (Cromwell, CT, USA). A-375 human melanoma cell line was obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany).

A-375 cells were cultivated in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate and antibiotics (penicillin 100 units/ml and streptomycin 100 μg/ml). Cells were grown in a humidified atmosphere at 37˚C with 5% CO_2. For viability tests, cells were seeded in 96-well plates at 5×10^3 cells/well and for flow cytometric analyses and immunoblotting in 6-well plates at 2.5×10^5 cells/well.

Tubugi-1 was synthesized according to a published procedure by Professor Wessjohann's group at the Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany (7). Compound was dissolved in dimethyl sulfoxide and stored at –20˚C until usage. Before the treatment, working solutions were prepared in culture medium.

Viability tests. Cells were treated with different concentrations of tubugi-1 for 48 h. MTT and CV tests were performed as described elsewhere (8). To determine the outcome of induced autophagy, cells were treated with IC_{50} dose (0.3 nM) of tubugi-1 concomitantly with 3-MA (1 mM) or chloroquine (20 μM) and cellular viability was assessed by CV test after 48 h.

Annexin V-FITC/PI, apostat and AO staining. For apoptosis detection, cells were exposed to the IC_{50} dose (0.3 nM) of tubugi-1 for 6, 12 or 24 h. After incubation, cells were harvested, washed and stained with annexin V-FITC and PI (15 μg/ml) according to the manufacturer's procedure for 15 min at room temperature. To check whether apoptosis was mediated by caspase activation, cells were incubated with tubugi-1 (0.3 nM) for 48 h, collected and afterward stained with apostat, in compliance with the manufacturer's protocol. For the detection of autophagosomes, A-375 cells were stained with a solution of 10 μM AO for 15 min at 37˚C after exposure to tubugi-1 (0.3 nM) for 6, 12, 24 or 72 h. Cells were then washed and resuspended in PBS, and finally analyzed with

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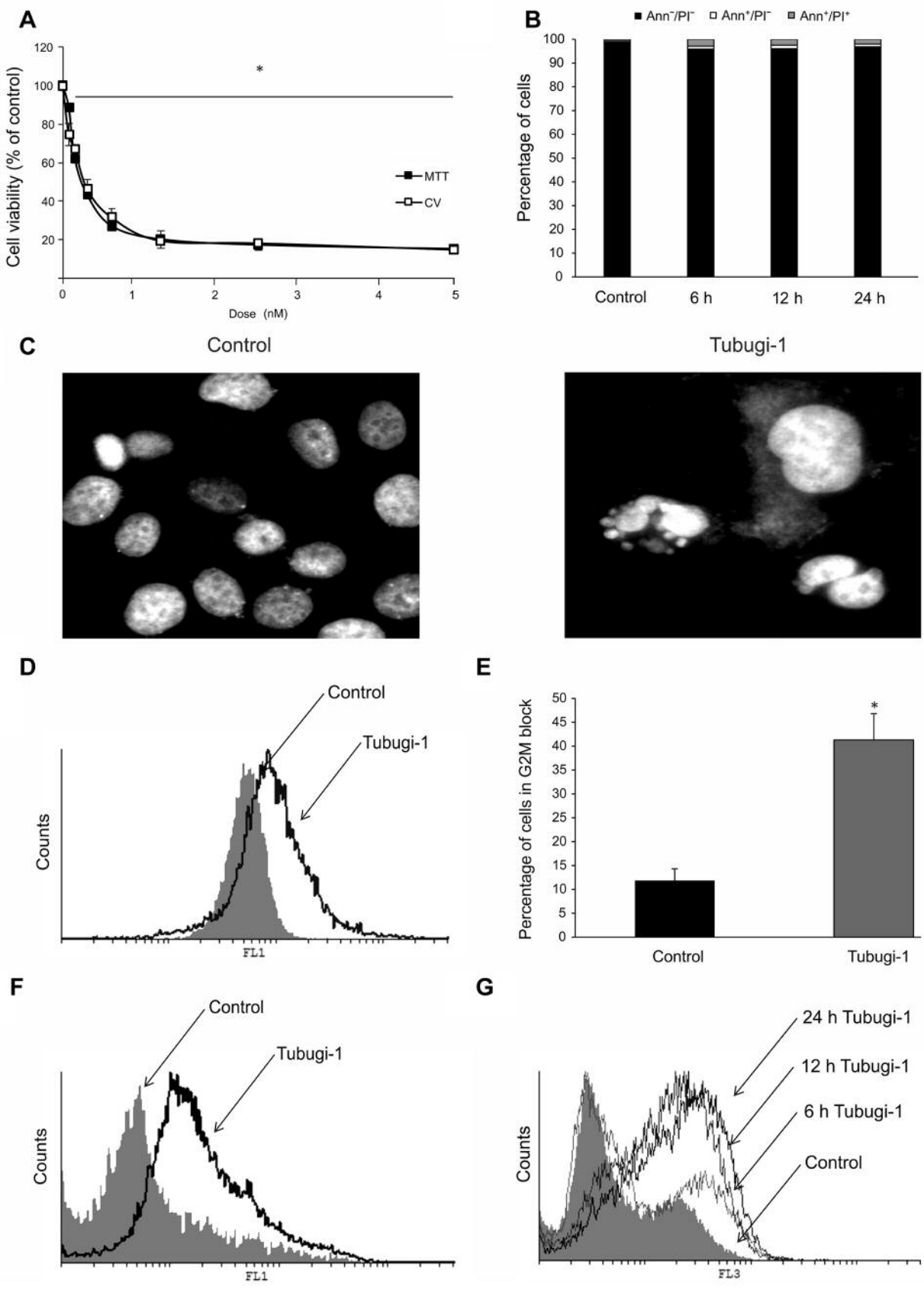
Figure 1. *Tubugi-1 provokes micronucleation, inhibits cell proliferation and induces autophagy. A: A-375 cells were treated with a range of concentrations of tubugi-1. 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and crystal violet (CV) assays were performed after 48 h. The results are presented as a percentage relative to that of the control (mean±SD) from one representative out of three independent experiments. *Significantly different at p<0.05 from untreated cultures. B-G: Cells were treated with 0.3 nM tubugi-1 (50% inhibitory concentration for A-375 cells after 48 h), then stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (Ann/PI) at three time points up to 24 h (B), or with 4',6-diamidino-2-phenylindole (DAPI) after 48 h (C), or analyzed for cellular proliferation after 72 h (D), cell cycle distribution after 48 h (E), apostat staining after 48 h (F) and acridine orange staining after 6, 12 and 24 h (G). Original magnification for (C), ×400.*


A
Cell viability (% of control)
Dose (nM)
MTT
CV
*
B
Ann⁻/PI⁻
Ann⁺/PI⁻
Ann⁺/PI⁺
Percentage of cells
Control
6 h
12 h
24 h
C
Control
Tubugi-1
D
Control
Tubugi-1
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Percentage of cells in G2M block
Control
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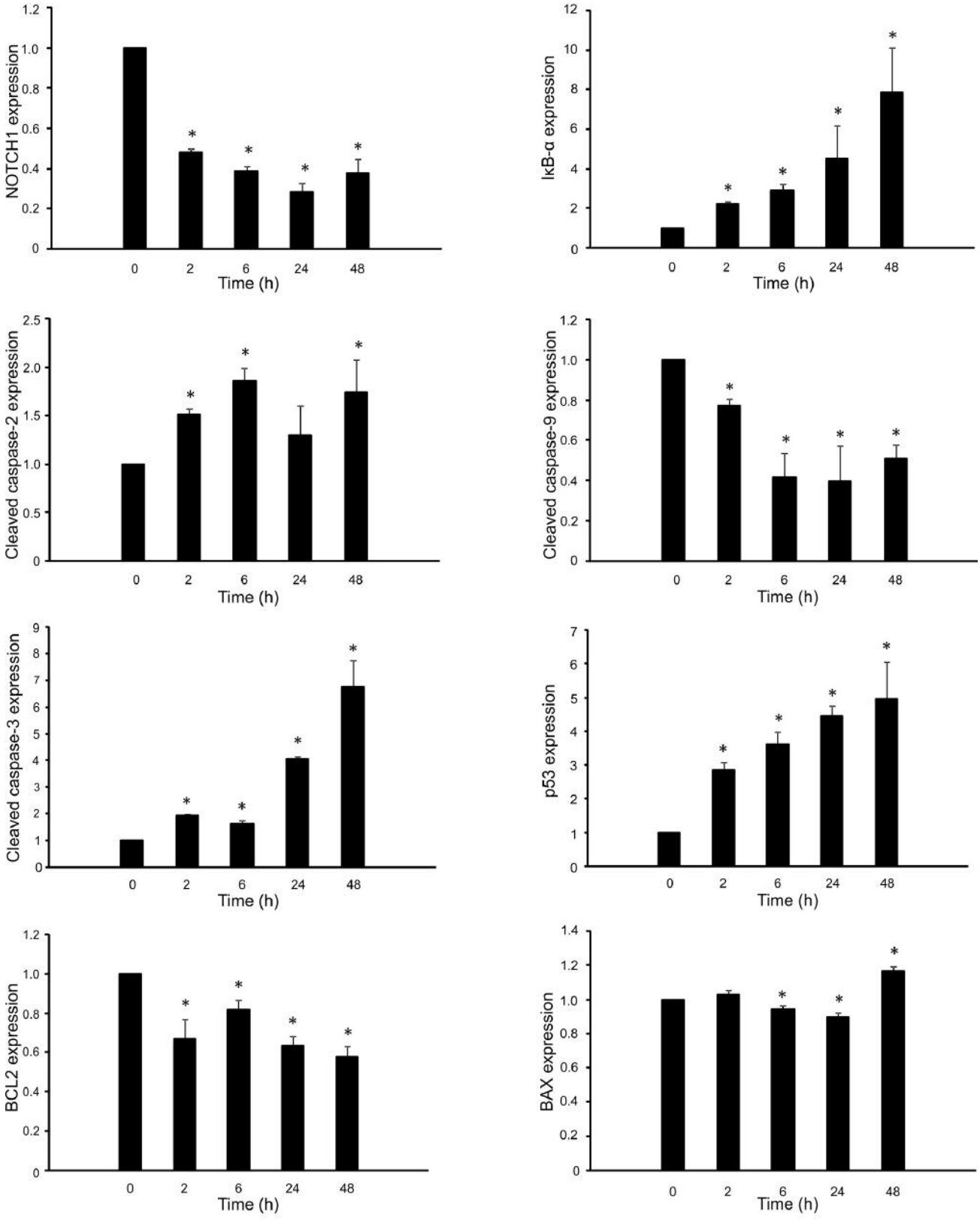

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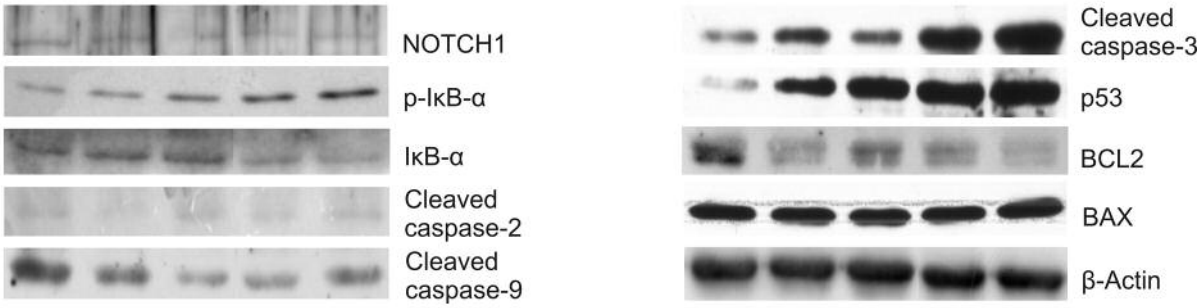

Figure 2. *Tubugi-1 affects proteins relevant for both mitotic catastrophe (MC) and apoptosis at the intracellular level. A-375 cells were incubated with 0.3 nM tubugi-1. The expression of NOTCH1, inhibitor of NF-κB (IκB-α), caspases-2, -3 and -9, p53, B-cell lymphoma 2 (BCL2) and BCL2-associated X (BAX) was evaluated over 48 h by western blotting.*

CyFlow® Space Partec using the PartecFloMax® software (Partec GmbH, Münster, Germany).

CFSE staining. For the analysis of the impact of tubugi-1 on cellular proliferation, A-375 cells were pre-stained with 1 μM CFSE for 10 min, washed and then treated with 0.3 nM tubugi-1 for 72 h. At the end of incubation cells were analyzed as described elsewhere (8).

Cell-cycle analysis. To examine cell distribution among cell-cycle phases, cells were incubated with 0.3 nM tubugi-1 with or without chloroquine (20 μM) for 48 h and then stained with DAPI (1μg/μl) in 1% tritonX-100/PBS or PI (20 μg/ml) in the presence of RNase (0.1 mg/ml) for 45 min at 37˚C, after fixation with 70% ethanol and overnight incubation at 4˚C. Cells were then analyzed with BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) and CyFlow® Space Partec using BD FACS Diva™ and PartecFloMax® software, respectively.

DAPI staining on chamber slides. In order to detect morphological changes of nuclei following MC and apoptosis, A-375 cells were seeded, treated with 0.3 nM tubugi-1 with or without 20 μM chloroquine for 48 h and stained according to the previously described procedure (8).

Western blot analysis. Cells were incubated with 0.3 nM tubugi-1 for 2, 6, 12, 24 and 48 h. Samples and method of protein extraction were performed as already described (8). Membranes were incubated overnight at 4˚C with specific antibodies to: NOTCH1 (Santa Cruz Biotechnology, Dallas, TX, USA), phospho-IκB-α (Ser32), IκB-α, caspase-9, caspase-3, beclin-1, phospho-p44/p42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase (ERK1/2)) (Thr202/Tyr204), p44/p42 MAPK (ERK1/2), protein kinase B (AKT), phospho-AKT, phospho-S6 ribosomal protein (Ser240/244), S6 ribosomal protein, phospho-p70 S6 kinase(Thr389), p70 S6 kinase (Cell Signaling Technology, Danvers, MA, USA), caspase-2, p53, B-cell lymphoma 2 (BCL2), BCL2-associated X (BAX), β-actin (Abcam, Cambridge, UK), p62/sequestosome 1 (SQSTM1) (Novus Biologicals, Littleton, CO, USA) and microtubule-associated protein light chain 3B (LC3B) (Sigma–Aldrich, St. Louis, MO, USA). As a secondary antibody, goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA) was used. Bands were detected using a chemiluminescence detection system (ECL; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK), while the protein levels were determined by densitometry using ImageJ software (NIH, Bethesda, MD, USA), and expressed relative to β-actin or total protein expression in the case of IκB-α, ERK1/2, AKT, p70 S6K and S6. The results are presented as fold variation in signal intensity relatively to the control that was arbitrarily set to a value of 1.

Statistical analysis. Cellular viability data are expressed as the mean±SD from three independent experiments. Significant differences were calculated according to the analysis of variance (ANOVA) or a Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Tubugi-1 induces MC synchronized with strong autophagy in A-375 cells. To evaluate the cytotoxic potential of tubugi-1, A-375 cells were exposed to different concentrations for 48 h. The cell viability was determined by measurement of respiration and the number of adherent cells. The obtained data revealed remarkable activity of tubugi-1 and with a dose-dependent decrease in the number of viable cells in culture (Figure 1A). Cells were subsequently exposed to an IC_{50} dose of tubugi-1 (0.3 nM) and apoptotic cell death was determined using annexin-V/PI double staining. Despite reduced viability, there was no significant apoptosis detectable at this concentration, neither in the early nor in the late phase (Figure 1B). Having in mind results obtained earlier in B16 mouse melanoma cells showing a specific apoptotic pattern upon tubugi-1 treatment, the absence of annexin-V^+ cells does not explicitly mean the absence of apoptotic cell death, but rather of some of its typical markers. For that purpose, cells were stained with DAPI and the morphology of the nuclei was evaluated by fluorescent microscopy. As shown in Figure 1C, the appearance of multiple micronucleation was found in cultures cultivated in the presence of tubugi-1. This specific phenomenon is

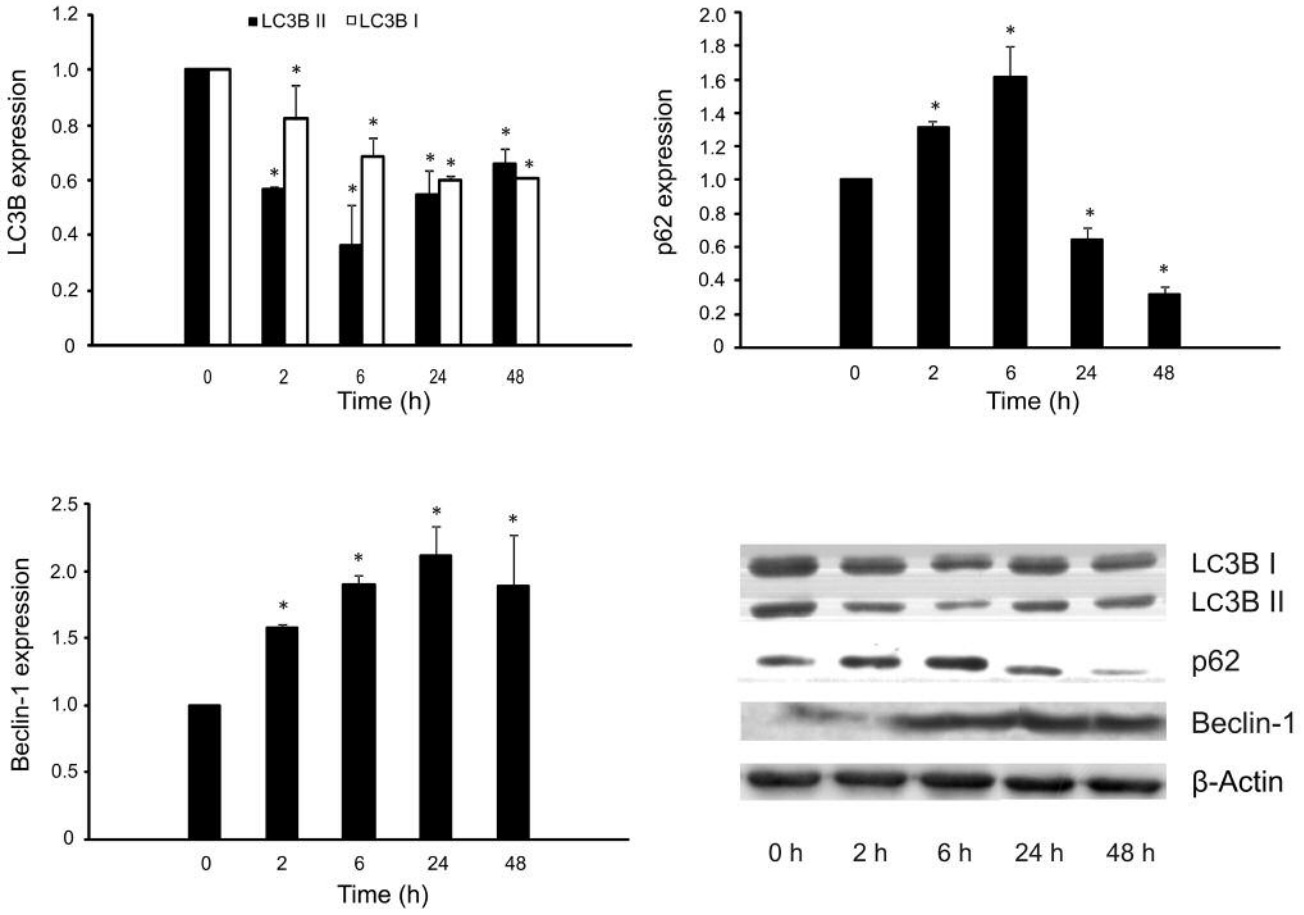

Figure 3. *Tubugi-1 differentially regulates proteins involved in autophagy. A-375 cells were cultivated in the presence of 0.3 nM tubugi-1. Microtubule-associated protein light chain 3B (LC3B), p62 and beclin-1 were analyzed by western blotting over 48 h incubation.*

recognized as MC, a process caused by aberrant mitosis. To confirm this, analyses of the proliferative rate, as well as cell-cycle distribution were performed. As presented in Figure 1D and E, tubugi-1 suppressed cell division and promoted the accumulation of cells in the G_2M phase. Moreover, as MC is defined as a stage driving a cell to death *via* apoptosis, caspase activation was determined in cells treated with tubugi-1. Judging from apostat staining, tubugi-1 induced strong caspase activity (Figure 1F). Since the involvement of autophagy in MC was recently reported (18), it was important to analyze the presence of autophagy upon tubugi-1 treatment. Intensified red fluorescence due to the elevated presence of autophagosomes in the cytoplasm of AO-stained cells persistent in time indicated the coexistence of autophagy and MC (Figure 1G). Taken together, treatment of aggressive A-375 cells by tubugi-1 resulted in massive MC concomitant with an autophagic process and caspase activation.

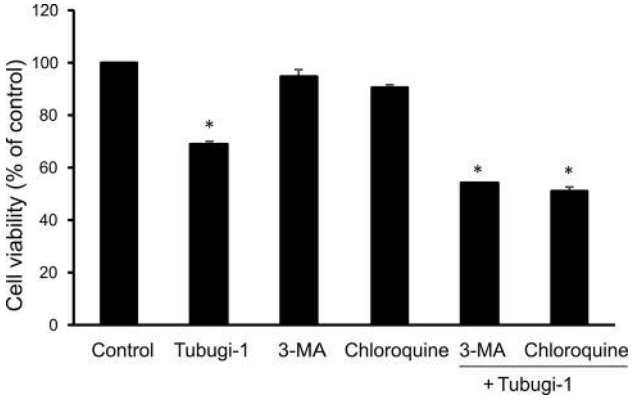

Figure 4. *Tubugi-1 provokes cytoprotective autophagy in A-375 cells. Cells were treated with 0.3 nM tubugi-1 and in combination with the autophagy inhibitor 3-methyladenine (3-MA) (1 mM) or chloroquine (20 μM) for 48 h. Cellular viability was assessed by crystal violet test after 48 h. *Significantly different at p<0.05 from untreated cultures.*

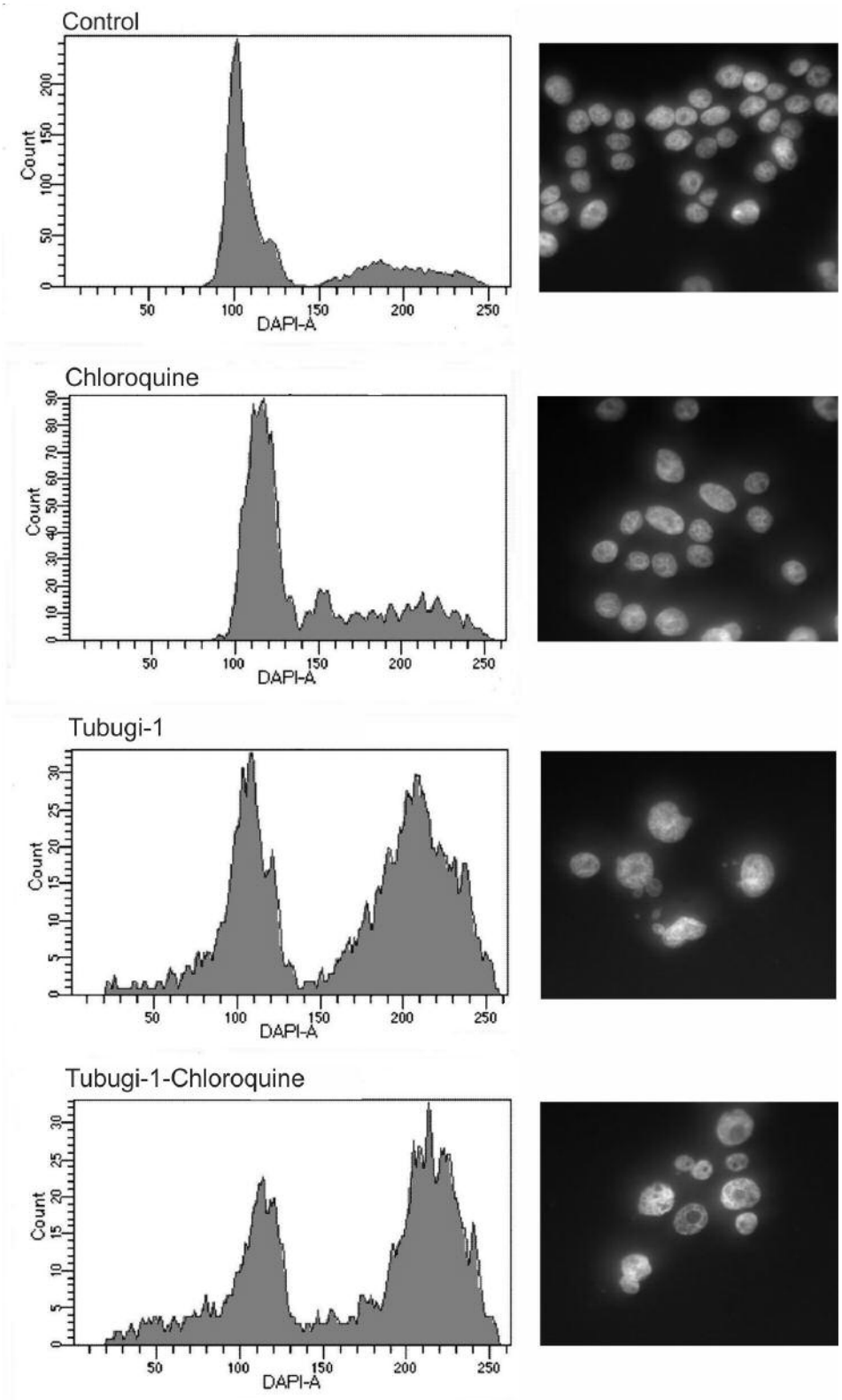

Figure 5. *Neutralization of autophagy potentiates apoptotic cell death in response to tubugi-1. A-375 cells were treated with 0.3 nM tubugi-1 and simultaneously with chloroquine (20 μM) for 48 h, then cell-cycle analysis (left panel) and DAPI staining (right panel) were carried out. Original magnification, ×400.*

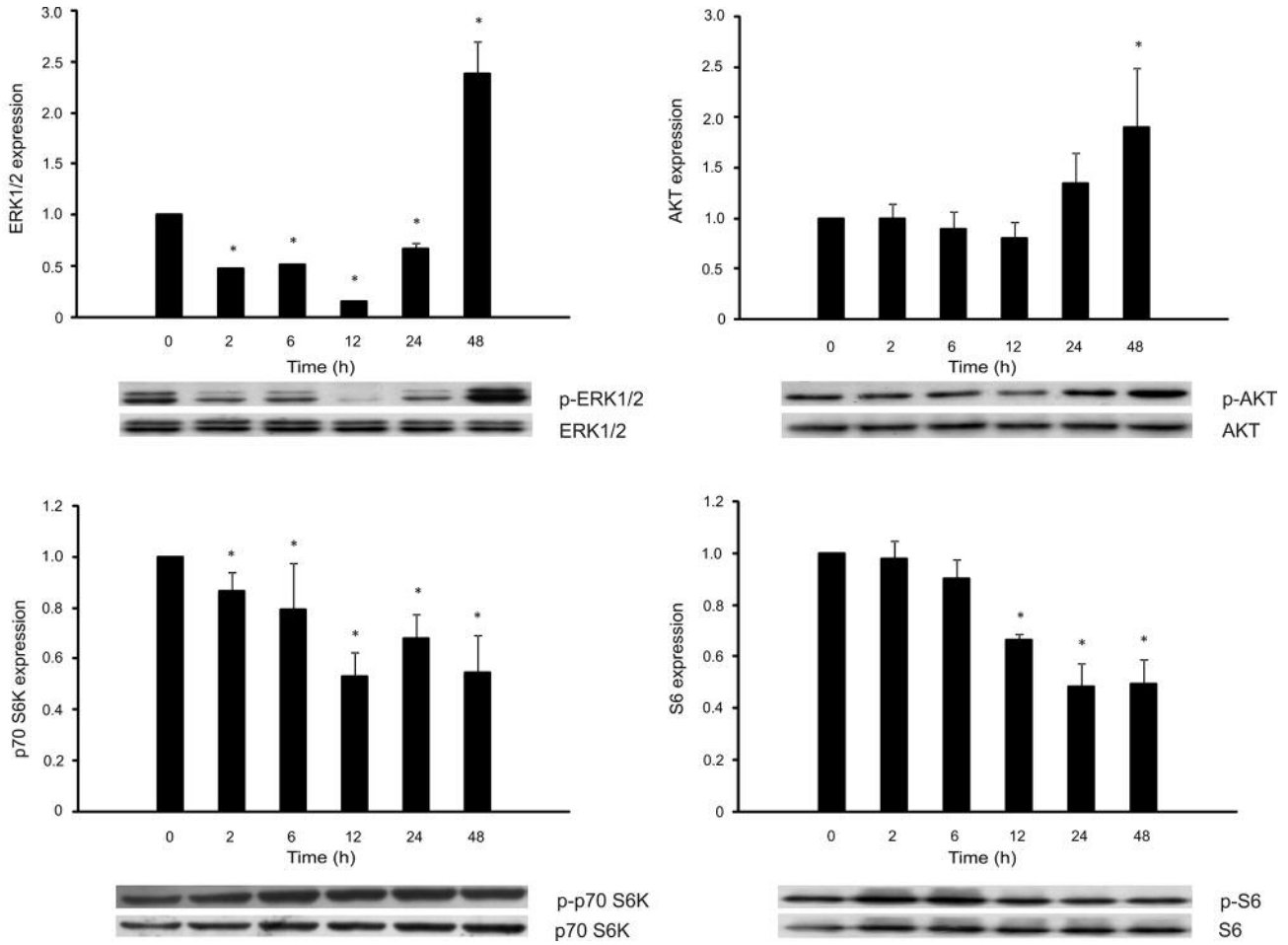

Figure 6. *Tubugi-1 treatment influences key proteins of cell division and death. Cells were exposed to 0.3 nM tubugi-1 during 48 h and the expression of extracellular signal-regulated kinase (ERK1/2), protein kinase B (AKT), p70 S6K and S6 protein was determined by western blotting.*

Tubugi-1 affects intracellular mediators of MC. Since the presence of MC was morphologically proven upon tubugi-1 treatment, it was essential to delineate the intracellular signature behind it. Bearing in mind that MC is usually accompanied by a specific signaling pattern culminating in apoptosis, cells were incubated in the presence of tubugi-1, and proteins relevant for both MC and apoptosis were explored by western blot analysis (Figure 2). While NOTCH1 expression was progressively inhibited over time, two main proteins involved in MC, namely nuclear factor-κB (NF-κB) and caspase-2 were strongly activated. In detail, the analysis of phosphorylation, inhibitory subunit of NF-κB (IκB-α), revealed sustained, intensified up-regulation confirming the activation of NF-κB. According to recent data about the connection between caspase-3 and -9 in the activation of caspase-2, these two proteins were explored. The increase in the level of cleaved caspase-3 confirmed induction of apoptosis. Since an amplified presence of the 37 kDa fragment of caspase-9 correlates with the apoptotic process, its sustained inhibition observed upon treatment with tubugi-1 supports the hypothesis about the involvement of MC. In parallel, the expression of tumor-suppressor protein p53 was significantly up-regulated. BAX expression was not significantly changed until the last time point (48 h). Its late activation, concordantly with diminished expression of BCL2, indicated apoptosis. In summary, the intracellular molecular signature detected adequately corroborates the results obtained with nuclear morphology and flow cytometric analyses.

Tubugi-1 triggers protective autophagy. Treatment with tubugi-1 promoted a strong autophagic response, as described above. To demonstrate this at the intracellular level, LC3B I and LC3B II, p62 and beclin-1 expression

were determined by western blot (Figure 3). Beyond 6 h of incubation with tubugi-1, p62 expression showed a strong tendency to decline. This pattern is typical for autophagic processes and is in concordance with the results of AO staining (see Figure 1G). Moreover, up-regulation of expression of beclin-1, a protein involved in the formation of autophagosomes, also supported the presence of autophagy. In parallel, expression of both LC3B I and LC3B II decreased, but with different dynamics. While that of cytoplasmic protein LC3B I continuously decreased over time, expression of membrane-bound LC3B II declined during the first 6 h, followed by a typical enhancement as a signature of the autophagic process. To determine the role of intensive autophagy, cells were exposed to two different inhibitors of autophagy, chloroquine and 3-MA, in parallel to tubugi-1 treatment. Chloroquine inhibits autophagy by increasing the lysosomal pH, and thereby it stops fusions of autophagosomes and lysosomes, and consequently further proteolytic degradation in autophagosomes. 3-MA blocks the formation of autophagosomes through the phosphatydilinositol-3-kinase/AKT signaling pathway.

The concomitant treatment of cells with tubugi-1 and inhibitors of autophagy resulted in a further decline of cellular viability indicating that autophagy has a cytoprotective role (Figure 4). Thus, it was expected that the elimination of autophagy would increase the cytotoxicity of tubugi-1 treatment. To investigate this, cells were exposed to nontoxic concentrations of chloroquine in parallel with tubugi-1, and cell-cycle distribution analysis and DAPI staining were performed. The combined treatment with an autophagy inhibitor led to the accumulation of cells in the subG compartment, indicating an enhancement of apoptotic cell death (Figure 5 left panel). In parallel, the presence of micronucleation as a hallmark of MC was replaced by the typical morphological changes of apoptotic nuclei. Chromatin condensation intensified and nuclear shrinkage appeared, confirming that tubugi-1 in the absence of autophagy induces extensive apoptosis (Figure 5 right panel).

Signaling network response to tubugi-1 treatment. To clarify the influence of tubugi-1 on the main signaling pathways involved in the regulation of cell division and death, cells were exposed to IC_{50} dose (0.3 nM) and protein expression was determined at 5 time points up to 48 h (Figure 6).While the expression of ERK1/2 declined during the first 12 h, additional incubation revealed a rapid enhancement of its activation. Although AKT was not significantly affected during the first 24 h by tubugi-1, phosphorylation of downstream proteins such as p70 S6 kinase (activity domain T389) and S6 was strongly and sustainedly reduced. This pattern of expression can be recognized as characteristic for prolonged and transient autophagy in combination with induction of apoptosis.

Discussion

The problem of limited quantities of mitotic spindle poisons derived from nature can be surmounted by the development of synthetic analogs with improved properties. Here, we present one such example: the synthetic tubulysin analog, tubugi-1 (7). In prostate and colon cancer cell lines, tubugi-1 induced apoptosis after a prolonged period of incubation (L.A. Wessjohann *et al*., unpublished data). A revolutionary discovery regarding tubugi-1 and a peculiar form of induction of apoptosis has recently emerged and might change the general scientific statements and therapeutic protocols based on this and related drug candidates (19). Namely, the induction of apoptosis deprived of PS inversion in the past was considered undesirable because phagocytic receptors are not addressed (20). Despite this, we showed that phagocytosis of tubugi-1-induced apoptotic cells occurs even in the absence of PS-mediated signaling and that this process can be even more efficient in eliminating cancer cells by stimulating cytotoxic activities of macrophages (8). One of the most serious problems of advanced cancer treatments is the different sensitivity and frequent resistance of metastatic cells derived from primary tumor counterparts (21). In this study, we show that tubugi-1 was equally efficient against the highly invasive anaplastic and amelanotic A-375 metastatic melanoma cell line. These cells exhibit low differentiation with the unique feature of producing nitric oxide as an endogenous growth factor (16, 22). This kind of cell line is representative of high-grade melanoma unresponsive to almost all available therapeutic strategies. Tubugi-1, differently from previously described effects against primary melanoma cells, in the case of A-375 cells, induced a cell death triangle with autophagy, MC and apoptosis at each corner, conferring the ability to enhance the drug's efficacy by manipulating the relative ratios of these processes.

According to cell death classification, MC is a type of death of a cell occurring during mitosis (23). As an irreversible process, MC manifests as apoptosis, necrosis, or senescence. It was recently found that autophagy largely influences cell fate (24). During MC, cells that undergo nuclear alteration possess small, independent micronuclei apart from the main one before terminal arrest and subsequent death. These micronuclei form spontaneously as a consequence of chromosome mis-segregation aggravated by genotoxic stress (25, 26). All together these result from mitotic arrest triggered by treatment with inhibitors of correct microtubule formation or maintenance. It was reported that impairment leading to MC can be provoked by agents that influence microtubule formation, depolymerization or DNA damage, such as taxanes, epothilones, laulimalide, vinca alkaloids and colchicines (25-27). Tubugi-1 appears to be an agent that affects both synthesis and polymerization of microtubules (L.A. Wessjohann *et al*., unpublished data).

Treatment of A-375 cells with tubugi-1 led to a suppression of proliferation as expected, and a strong accumulation of cells in the G_2M phase of the cell cycle. The appearance of micronuclear structures was confirmed by DAPI staining, indicating that the anticancer activity of this synthetic analog is realized through a well-recognized cascade of events typical for this kind of drug.

MC is regulated by numerous molecular mediators such as cell-cycle specific kinases (cyclin B1-dependent kinase, polo-like kinases and aurora kinases), cell-cycle checkpoint proteins, inhibitor of apoptosis proteins, p53, caspases and BCL2 family proteins (9). Prolonged G_2M arrest can be associated with NOTCH inhibition and its role in MC (28). NOTCH proteins are highly conserved transmembrane receptors that serve as transcriptional activators. This signaling is implicated in stem cell development and typical of high-grade tumors such as breast, lung, brain and sarcomas (29, 30). Its inhibition can trigger abnormal mitosis concerning NF-κB activation that is responsible for cyclin B1 increase and cell death through MC (29). NF-κB regulates a long list of genes controlling cellular proliferation and survival. In concordance with all these aspects, MC triggered by tubugi-1 in A-375 cells was followed by strong inhibition of NOTCH1 and intensified activity of NF-κB.

The hallmark of MC is activation of caspase-2 at the pre-mitochondrial level (31). Caspase-2 is predominantly located in the nucleus and its activation is caused by DNA damage, which further leads to mitochondrial membrane permeabilization and the subsequent release of caspase-3. Interestingly, it was found that in the response to UV or tumor necrosis factor activation of caspase-2 depends on effector caspases-3 and -9 (32). An explicit connection to caspase-9 was found in the case of the intrinsic apoptotic pathway, but not the extrinsic one (33). Therefore, it is not surprising that under our conditions, remarkable activation of caspase-2 was not accompanied by caspase-9 expression. Moreover, the enhanced expression of the active fragment of caspase-2 might be responsible for the observed NF-κB up-regulation, as judged by the expression of inhibitory subunit IκBα (34). The signaling pathways affected by tubugi-1 in A-375 cells are those with the typical pattern of MC. One of the consequences of activation of caspase-2 is elevated p53 expression, since caspase-2 is responsible for murine double minute 2-mediated p53 ubiquitination (31). Subsequently, BCL2 down-regulation would be expected. The amplification of MC occurs under BCL2-depleted conditions (35). While for BCL2, a connection with MC is well confirmed, this type of cell death is also possible in p53-deficient tumor cells, underlining the irrelevance of p53 for MC (9, 36). On the other hand, in cell lines with wild-type *p53*, this tumor suppressor must be involved in the signaling network triggered by the treatment. The observed enhancement of total p53 in tubugi-1-treated cells combined with activation of caspase-2 and BCL2 down-regulation indicates that this protein is involved in the outcome of the treatment. In concordance with this, chromosomal breaks during MC were shown to promote p53-mediated apoptosis (37). Tubugi-1 treatment resulted in a strong and sustained autophagic process which was 'switched off' after 72 h (data not shown). The phenomenon of spontaneous disappearance of autophagy might be related to the previously mentioned up-regulation of caspase-2 and inhibition of caspase-9 expression. Namely, it is known that caspase-2 is an endogenous suppressor of autophagy (38), while caspase-9 inhibition obstructed cytoprotective autophagy through modulation of lysosomal pH and acid-dependent activities of cathepsin (39).

The complex interplay between autophagy, MC and apoptosis was recently described by Sorokina *et al*. (18). The authors claimed that MC induction in colorectal carcinoma is a crucial step defining the route of cell death by autophagy or consequent apoptosis. Under our circumstances, and since the elimination of autophagy by chloroquine and 3-MA resulted in even more profound toxicity, autophagy can be considered as a primarily cytoprotective mechanism. Similarly, paclitaxel elicited protective autophagy in A549 cells (40). Therefore, it is not surprising that autophagy elimination resulted in an increased percentage of cells in the subG compartment and the appearance of apoptotic bodies. Combining the stable synthetic tubulysin derivative, tubugi-1, with inhibitors of autophagy might therefore provide a promising strategy to combat metastatic tumors. This is in concordance with the previously observed sensitizing effect of inhibitors of autophagy on antitumor activity of paclitaxel in endometrial carcinoma (41). In prolonged treatment with tubugi-1, the spontaneous 'turning off' of autophagy can be observed and, in parallel with this, rapid enhancement of proapoptotic signaling is detected (up-regulated p53, caspase-3 and BAX at the end time point).

It is obvious that the dialog between MC, autophagy and apoptosis defines the success of such treatment. Under these circumstances, many proteins interfere with each other and it is hard to define an explicit or primary influence on MAPK and PI3K/AKT signaling (42). For example, on one hand, the key autophagic proteins p62 and LC3B II serve as a platform regulating ERK phosphorylation. Mice with knockout p62, a protein responsible for labeling of protein for autophagic degradation, developed ERK phosphorylation (43), indicating a complex crosstalk between ERK and autophagy. Therefore, it is not surprising that ERK and p62 expression under tubugi-1 treatment had opposing patterns. On the other hand, the connection of ERK to the apoptotic process is well defined (44). Frequently, ERK activation is associated with apoptosis execution in response to cell damage triggered by different agents. ERK activity has been found to affect mitochondrial behavior, inhibiting their respiration and disturbing the membrane potential (45, 46). Active ERK is also able to localize to the mitochondrial

membrane, to promote cytochrome c release by influencing expression of the BCL2 family (44). Thus, an intensified activity of ERK after 12 h of treatment confirms that a transient autophagic process is replaced by apoptosis. AKT was not remarkably affected during the first 24 h of incubation with tubugi-1, while downstream proteins like p70 S6K and S6 were significantly down-regulated. This indicates that the upper part of this signaling pathway was not directly affected by tubugi-1. Late up-regulation of AKT might have been feedback of persistent inhibition of p70 S6K (47). Blommaart *et al*. found autophagy and protein synthesis to be regulated oppositely to the degree of p70 S6K phosphorylation (48). Our data show a similar relation in the dynamic interplay of autophagy, apoptosis, cancer cell proliferation and p70 S6K activity.

Induction of MC by the next-generation tubulysin, tubugi-1, is a central point on the autophagy–apoptosis seesaw. Interference with cytoprotective autophagy that characterizes advanced tumors might be a valuable alternative strategy for overcoming their resistance to treatment with mitotic spindle poisons.

Conflicts of Interest

The Authors declare no competing interests in regard to this study.

Authors' Contributions

Conceptualization: SM, DMI; Formal analysis: DD, SM, DMI, GNK; Investigation: DD, TK, GNK; Writing – original draft preparation: DD, SM, GNK, LAW, DMI; Writing – review and editing: DD, SM, GNK, LAW, DMI; Supervision: SM, DMI; Funding acquisition: LAW, DMI.

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