Toddaculin, a natural coumarin from *Toddalia asiatica*, induces differentiation and apoptosis in U-937 leukemic cells

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Abstract:

Chemotherapeutics represent the main approach for the treatment of leukemia. However, the occurrence of adverse side effects and the complete lack of effectiveness in some cases make it necessary to develop new drugs. As part of our screening program to evaluate the potential chemotherapeutic effect of natural coumarins, we investigated the anti-leukemic activities of a series of six prenylated coumarins isolated from the stem bark of *Toddalia asiatica* (Rutaceae). Among these, 6-(3-methyl-2-butenyl)-5,7-dimethoxycoumarin (toddaculin) displayed the most potent cytotoxic and anti-proliferative effects in U-937 cells. To determine whether these effects resulted from induction of cell death or differentiation, we further evaluated the expression of several apoptosis and maturation markers. Interestingly, while toddaculin at 250 µM was able to induce apoptosis in U-937 cells, involving decreased phosphorylation levels of ERK and Akt, 50 µM toddaculin exerted differentiating effects, inducing both the capacity of U-937 cells to reduce NBT and the expression of differentiation markers CD88 and CD11b, but no change in p-Akt or p-ERK levels. Taken together, these findings indicate that toddaculin displays a dual effect as a cell differentiating agent and apoptosis inducer in U-937 cells, suggesting it may serve as a pharmacological prototype for the development of novel anti-leukemic agents.
Keywords:

Toddalia asiatica, toddaculin, apoptosis, differentiating activity, leukemia.

Abbreviations:

Introduction

Natural products, such as plant extracts and microbial compounds, have achieved high prominence in the development of commercial drugs. In this regard, approximately 74% of the chemotherapeutics currently employed in the treatment of cancer are either natural products or natural product-derived drugs (Tan, 2006). Occurring widely throughout the Plant Kingdom, coumarins (known as 1,2-benzopyrones) constitute one of the most representative families of plant secondary metabolites, displaying a broad spectrum of biological activities. These include antibacterial (Laurin, 1999), oral anti-coagulant (Olderburg, 2007), anti-mutagenic (Pillai, 1999) and anti-inflammatory properties (Fylaktakidou, 2004), as well as capacity to inhibit human platelet aggregation (Roma, 2003), reactive oxygen species scavenging capacity (Kancheva, 2010) and anti-HIV activity (Kirkiacharian, 2002).

Our group and others have identified novel coumarin compounds with anti-proliferative and/or cytotoxic activity on cancer cells, depending on their substitution pattern (Riveiro, 2010). Specifically, in previous publications, we reported the significant inhibitory activity of certain coumarins on the proliferation of leukemic cell lines (Riveiro, 2004, 2008a, b and 2009). In addition, we described that such inhibitory effects could be related to either differentiating (Riveiro, 2004, 2009) or pro-apoptotic activities (Riveiro, 2008a, b) of the compounds, depending on the distribution of their substituents in the coumarin ring.

Toddalia asiatica (Rutaceae) is a woody liana widely distributed in Southeast Asia, South Africa and tropical Africa, including Madagascar. The genus Toddalia is known to produce prenylated coumarins and phenanthridine alkaloids and derivatives. Of interest, the antineoplastic activity of an alkaloid extracted from T. asiatica has been described previously (Iwasaki, 2006 and 2010), but there is no published report on the anti-cancer activity of prenylated coumarins. Thus, aiming to extend our previous research, in the present work we performed a screening of six natural prenylated coumarins isolated from Toddalia asiatica for potential effects on U-937 cell proliferation, viability and differentiation, as well as for pro-apoptotic activity. Table 1 shows the structures of these coumarins (compounds 1 – 6).
Among the evaluated compounds, the 6-(3-methyl-2-butenyl)-5,7-dimethoxycoumarin, toddaculin (6) was the most potent in terms of anti-proliferative and cytotoxic activities. What is more, this coumarin was able to promote apoptosis and partial differentiation in a concentration- and time-dependent manner. In this regard, at higher concentrations toddaculin (6) induced U-937 cell death mediated by caspase cascade activation and down-regulation of ERK 1/2 and serine/threonine protein kinase Akt phosphorylation, whereas at lower concentrations it induced NBT reduction and expression of CD11b and CD88 monocytic differentiation markers.

Collectively, the findings presented herein constitute the first report to demonstrate the pro-apoptotic and differentiating activities of the natural coumarin toddaculin (6) on U-937 cells, pharmacological properties that may be useful for the development of novel chemotherapeutic agents for the treatment of leukemia.

Materials and Methods

Reagents and antibodies

RPMI 1640 medium, gentamicin antibiotic, bovine serum albumin, db-cAMP, PBS, Fura 2-AM, rhC5a, RNase A, ATP, HO, PI, NBT and PMA were obtained from Sigma Chemical Co. (St. Louis, USA). DMSO was obtained from Baker (Deventer, The Netherlands). FCS was purchased from PAA Laboratories GmbH (Austria). Anti-caspase-3, anti-PARP, anti-p-Akt, anti-Akt, anti-p-ERK 1/2, anti-ERK 1/2, anti-p-JNK, anti-JNK, anti-p-p38, anti-p38, anti-rabbit and anti-mouse-HRP IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). All commercial chemicals and solvents were of reagent grade and used without further purification unless otherwise specified.
Compounds

Toddalia asiatica (Rutaceae) was collected in the Blyde Nature Reserve, Mpumalanga, South Africa, and a voucher specimen retained (N. Crouch & O. Grace 991, NH). The combined dried and milled stem wood and bark were extracted sequentially with hexane, dichloromethane, ethyl acetate and methanol using a Soxhlet apparatus. Repeated column chromatography over silica gel (Merck 9385) and solvent systems yielded the six prenylated coumarins shown in Table 1: 6-(2,3-epoxy-3-methylbutyl)-5,7-dimethoxycoumarin (aeculeatin, 1); 6-(3-methyl-2-butenyloxy)-5,7-dimethoxycoumarin (2); 8-(3-methyl-2-butenyl)-6,7-dimethoxycoumarin (O-methylcedrelopsin, 3); 6-(2-hydroxy-3-methyl-3-butenyl)-5,7-dimethoxycoumarin (toddanol, 4); 6-(2,3-dihydroxy-3-methylbutyl)-5,7-dimethoxycoumarin (toddalolactone, 5) and 6-(3-methyl-2-butenyl)-5,7-dimethoxycoumarin (toddaculin, 6).

The structures of these compounds were determined using 2D NMR and EIMS techniques, and confirmed by comparison of spectroscopic data for the compounds isolated against literature data (Sharma, 1981).

The coumarin 5,7-dimethoxy-4-methylcoumarin (7; Table 1) was obtained from INDOFINE Chemical Company, Inc. (Boston, USA).

Both commercial and natural coumarins listed on Table 1 were dissolved in DMSO and stored at -20 °C.

Cell culture

The U-937 cell line (American Type Culture Collection, Rockville, MD) was cultured at 37 °C in a humidified atmosphere with 5% CO₂ in RPMI 1640 medium, supplemented with 10% FCS and 50 µg/ml gentamicin. Cells in exponential growth were used for all experiments.

Before seeding, viability of U-937 cells was tested by Trypan Blue assay. Cells were used only when viability was higher than 90%.
Cell growth inhibition assays (IC\textsubscript{50})

Exponentially growing U-937 cells were seeded at a density of 10\(^4\) cells in 100 \(\mu\)l of RPMI 1640 in a 96-well culture plate and incubated under a 5% CO\(_2\) atmosphere. These were then exposed to different concentrations of coumarins, ranging from 0.15 \(\mu\)M to 2.0 mM, or 0.6% (v/v) DMSO (vehicle control group) and later processed as previously described by Riveiro et al. in order to determine the IC\textsubscript{50} value (Riveiro 2004).

To evaluate the time course of U-937 cell proliferation, the number of cells was determined using a cell counter Coulter Z-1. Briefly, 10\(^5\) cells/ml were seeded in 24 wells plates and treated with different concentrations of the compounds to be tested, 400 \(\mu\)M db-cAMP (positive control), or 0.1% (v/v) DMSO (vehicle control group) for 3 days. Cells were then collected at different times according to each experiment, and the number was determined using a Coulter Z-1.

Measurement of the cytotoxic concentration 50 (CC\textsubscript{50})

Cells growing in exponential phase were seeded at 3.5 \(\times\) 10\(^5\) cells in 1 ml of RPMI 1640 in a 48 well culture plate and incubated in a 5% CO\(_2\) atmosphere. They were then exposed to different coumarin concentrations (0.15 \(\mu\)M to 2.0 mM) or 0.6% (v/v) DMSO (vehicle control group) for 48 h, after which the CC\textsubscript{50} values were determined as previously reported by our group (Riveiro, 2004)

Cell cycle analysis

U-937 cells growing in exponential phase were treated with different concentrations of toddaculin (6) or 2% (v/v) DMSO (positive control for apoptosis group) for 24 h or 48 h. Then, cells were harvested and centrifuged at 1000 rpm for 5 min. Cell suspensions were fixed and permeabilized by vigorous addition of nine volumes of ice-cold 70% (v/v) ethanol and stored at -20 °C for a minimum of 24 h, prior to analysis. Cells at a density of approximately 10\(^6\) were re-suspended in 800 \(\mu\)l of PI staining solution (20 \(\mu\)g/ml propidium iodide and 200 \(\mu\)g/ml RNase A in PBS, pH 7.4) and incubated
in the dark at room temperature for 30 min. The percentages of cells in the sub-G0/G1, G0/G1, S and G2/M cell cycle phases were determined over a range of concentrations and time points, and data from at least three independent experiments were analyzed using Cyflogic free software (www.cyflogic.com).

Clonogenic assay

U-937 cells were treated with different concentrations of toddaculin (6) or 0.1% (v/v) DMSO (vehicle control group). After 24 h of incubation, 2.5 x 10^3 treated and control cells were washed extensively and resuspended in 1.5 ml of mixture containing 0.4% (w/v) agar select and 15% FCS-RPMI 1640 medium. Each cell suspension was seeded over a bottom layer containing 0.7% (w/v) agar select and 15% FCS-RPMI 1640 medium in a 6 well culture plate. Plates were incubated in a 5% CO_2 atmosphere for 4 weeks. The colonies were stained with crystal violet (0.5% w/v), and colonies containing at least 50 cells were scored using a stereomicroscope.

Evaluation of morphological changes

Cells in exponential growth were seeded at a density of 7.5 x 10^5 cells in 2 ml of RPMI 1640 in a 24 well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed to different concentrations of toddaculin (6; 0.05 mM – 1.0 mM) or 0.1% (v/v) DMSO (vehicle control group). The morphology of treated and untreated cells was examined after 24 h of incubation by triplicate in at least three independent experiments, as reported by Riveiro et al., 2008a.

Western blot assays

Cells were lysed in 50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue, and sonicated to shear DNA. Total cell lysates were resolved by SDS-PAGE, and probed with the indicated primary antibodies. In the case of the MAPKs and Akt
pathways, membranes were first incubated with p-Akt, p-ERK 1/2, p-JNK and p-p38 primary antibodies, then stripped and re-probed for total Akt, ERK 1/2, JNK and p38 antibodies to verify equal loading of protein. All blots were incubated with horseradish peroxidase conjugated anti-rabbit or anti-mouse antibodies and developed by enhanced chemiluminescence (ECL) following the manufacturer's instructions (Amersham Life Science, England).

Caspase-3 activity

U-937 cells growing in exponential phase (3.5 x 10^5 cells/ml) were treated with different concentrations of test compounds, 2% (v/v) DMSO (positive control) or 0.1% (v/v) DMSO (Vehicle control group) during 24 h or 48 h. After that, cells were harvested and processed according to CASP3C caspase-3 colorimetric assay kit provided by Sigma Chemical Co. (St. Louis, USA).

Surface myeloid CD11b and CD14 antigens assay

U-937 cells (3.5 x 10^5 cells/ml) were treated with different concentrations of the compounds to be tested, 0.1% (v/v) DMSO (vehicle control group) or 400 µM db-cAMP (positive control group) for 72 h. Treated and control cells were washed twice in PBS and incubated with a saturating concentration of phycoerythrin (PE) anti-CD11b or anti-CD14 antibody (BD Pharmingen) at 4 ºC for 30 min. In all cases isotype-matched control monoclonal antibodies were used, and a gate (R1) was defined in the analysis to exclude all nonviable cells and debris, based on size and propidium iodine staining. Analysis was performed using a FACS flow cytometer and CellQuest software (BD Biosciences). The results are expressed as mean fluorescence intensity with respect to control (non-treated cells).
Determination of CD88 expression, NBT reduction capacity and chemotactic response

U-937 cells (3.5 x 10^5/ml) were treated with the compounds to be tested, 400 µM db-cAMP or 1 µM ATRA (positive control), or 0.1% (v/v) DMSO (control group) for 72 h. Cells from each experimental group were processed in order to determine the expression of the C5a complement factor receptor (CD88), NBT reduction capacity and chemotactic response, according to well-described methods previously reported by our group (Riveiro et al., 2004).

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by the Student–Newman–Keuls (SNK) *a posteriori* test using InfoStat software (2009). A p-value of 0.05 or less was considered statistically significant. In order to satisfy the requirements of the one-way ANOVA test, logarithmic transformation of data was applied to achieve homoscedasticity of the variable when necessary.

Results

Evaluation of the cytotoxic and anti-proliferative effects of prenylated coumarins isolated from *T. asiatica* in U-937 cells

As depicted in Table 1, toddaculin (6) showed the highest anti-proliferative activity in U-937 cells among the natural coumarins tested, with an *IC*_{50} value of 51.38 ± 4.39 (Table 1, *IC*_{50} value for toddaculin (6) vs. *IC*_{50} values for coumarins 1 – 5, *p* < 0.01). Trypan blue exclusion assay was then performed to examine the cytotoxic effects of these natural coumarins. Toddaculin (6) was the most potent inducer of cytotoxicity, with a *CC*_{50} value of 138.90 ± 3.50 (Table 1, *CC*_{50} value of toddaculin (6) vs. *CC*_{50} values for coumarins 1 – 5, *p* < 0.01).
The synthetic compound 5,7-dimethoxy-4-methylcoumarin (7) was also tested in order to evaluate the influence of the 5,7-dimethoxy substitution pattern in the anti-proliferative and cytotoxic activities of the coumarins under study herein. As shown in Table 1, 5,7-dimethoxy-4-methylcoumarin (7) exhibited neither growth inhibitory nor cytotoxic activities in U-937 cells after 48 h of treatment, indicating that the activities of coumarins 1, 3, 4, 5 and 6 cannot be attributed to the methoxyl residues in their structures.

Subsequently, a series of experiments were conducted in order to better characterize the anti-proliferative effects observed for toddaculin (6). Figure 1 shows that the coumarin inhibited cell proliferation and induced cytotoxicity in U-937 cells in a concentration-dependent manner. Finally, the survival capability of U-937 cells after toddaculin (6) treatment was evaluated by means of clonogenic survival assays (Franken, 2006). Significant differences were found between the colony forming rates of cells subjected to a 24-h treatment with either 100 or 250 µM toddaculin (6) and control cells (Figure 2), pointing out that toddaculin (6) induces irreversible loss of U-937 cell viability.

Toddaculin (6) induces apoptosis in U-937 cells

A series of assays were performed in order to determine whether the decrease in U-937 cell viability evoked by toddaculin (6) at cytotoxic micromolar concentrations resulted from induction of apoptosis. Both after 24-h and 48-h (Figure 3.A and B, respectively), toddaculin (6) at 250 µM significantly increased the percentage of U-937 cells in the sub G0/G1 phase of the cell cycle. Also, treatment of U-937 cells with 250 µM toddaculin (6) for 24 h induced morphological changes typically associated with the apoptotic process, such as cell shrinkage, chromatin condensation (pyknosis) and nuclear segmentation (Figure 3.C).

Apoptotic and necrotic cells were detected by studying chromatin condensation with HO test and nuclear staining with PI. Figure 3.D shows that apoptosis was the prevailing form of cell death induced by toddaculin (6) at lower concentrations than 500 µM, whereas necrosis was negligible. However, when cells were exposed to higher concentrations of toddaculin (6), the percentage of
necrotic cells increased significantly, suggesting that toddaculin (6) is capable of inducing apoptosis or necrosis in U-937 cells depending on the concentration.

In subsequent experiments, the effect of toddaculin (6) treatment on the activation of caspase-3, a key enzyme involved in the programmed cell death process, was evaluated. Toddaculin (6) was able to induce caspase-3 cleavage in U-937 cells, in a time and concentration dependent manner (Figure 4.A and B, respectively). Caspase-3-mediated proteolytic cleavage of the 116 kDa Poly(ADPribose)-polymerase protein (PARP) results in the separation of the N-terminal binding domain (24 kDa) from its C-terminal catalytic domain (89 kDa). It can be seen in figure 4.C that cleaved PARP (89 kDa) levels increased significantly after treatment of U-937 cells with toddaculin (6) at concentrations higher than 100 µM. These results were further confirmed by caspase-3 enzymatic activity measurements (Figure 4.D), indicating that toddaculin (6)-induced apoptosis in U-937 cells occurs through activation of caspase-3. Interestingly, none of the remaining natural coumarins herein evaluated (1 – 5) nor the synthetic one (7) displayed pro-apoptotic activity in the same range of concentrations as toddaculin (6, data not shown).

The pro-apoptotic activity of toddaculin (6) in U-937 cells entails down-regulation of p-ERK and p-Akt but not p-JNK or p-p38.

The treatment of U-937 cells with toddaculin (6; 250 µM) for up to 24 h had no effect on JNK or p38 apoptosis-associated signaling pathways (Figure 5.A and B, respectively). Nevertheless, a short exposure (< 1 h) to 250 µM toddaculin (6) induced p-ERK 1/2 and p-Akt downregulation (Figures 5.C and D, respectively). Collectively, these findings indicate that the pro-apoptotic activity of toddaculin (6) in the U-937 cell line implies down-regulation of kinases involved in cell survival, with no effect on MAPKs stress/apoptosis-related pathways.

Toddaculin (6) induces partial differentiation in U-937 leukemic cells
Figures 6.A, D and E show the inducing effect of a 48-h treatment with 50 µM toddaculin (6) on CD11b expression, NBT reduction capacity and functional CD88 expression in U-937 cells, respectively. However, toddaculin (6) induced U-937 cells to undergo partial differentiation since CD14 up-regulation or enhanced chemotactic response in U-937 cells were not observed in treated U-937 cells. The rest of the coumarins listed in Table 1 were unable to induce the expression of the differentiation markers herein evaluated in U-937 cells (data not shown). Furthermore, Figure 7 shows that the partial differentiating activity of toddaculin (6) in U-937 cells did not involve modulation of MAPKs or Akt signaling pathways (Figure 7.A, B, C and D, respectively).

Discussion

In an effort to offer novel compounds with well-established structure for the development of new chemotherapeutic agents, over the last years we have focused our research predominantly on coumarins. In the present work, we found toddaculin (6) to be the lead molecule among a series of natural prenylated coumarins isolated from *Toddalia asiatica*, owing to its highest capacity to induce anti-proliferative and cytotoxic effects in a concentration-dependent manner. Importantly, these effects remained after exposure to the coumarin, restricting the capacity of U-937 cells to form colonies in soft agar. Further studies revealed the involvement of caspase-3 activation in the pro-apoptotic activity of toddaculin in (6) U-937 cells, suggesting it may serve as a starting structure for the development of potential cytotoxic anti-leukemic agents.

The pro-apoptotic activity of toddaculin (6) involved a rapid down regulation of both p-Akt and p-ERK 1/2 levels. The Ras/Raf/MEK/ERK pathway is over-expressed in different cancer types and it has been proposed that this might influence resistance of some cancers to chemotherapeutic agents (McCubrey, 2007. Ozaki, 2010). The serine/threonine kinase Akt is the most studied effector of the phosphoinositide 3-kinase (PI3K). Emerging studies have revealed a key role of the PI3K/Akt pathway in carcinogenesis and chemoresistance in several tumor cells (Falasca, 2010. Martelli, 2006. Yuan, 2003). In the particular case of acute myeloid leukemia, several authors have pointed
out the existence of a correlation between elevated PI3K/Akt signaling and poor prognosis of these patients (Gallay and Kornblau, 2009). The cited chemoresistance has been linked to the fact that the PI3K/Akt pathway controls the expression of the multidrug resistance-associated protein 1 (MDR1), which mediates the extrusion of several chemotherapeutic drugs from leukemic cells and is associated with a lower survival rate (Schaich, 2005; Tazzari, 2007). Hence, the down-regulation of both the Ras/Raf/MEK/ERK and PI3K/Akt pathways is nowadays considered an important strategy for the treatment of cancer (Falasca, 2010; Martelli, 2006; Yuan, 2003). In this regard, toddaculin (6) makes an attractive lead compound for further development of promising anti-leukemic agents.

Another reason why toddaculin (6) should be viewed as an interesting compound for novel anti-leukemic drug design is that it induces apoptosis in a p53-independent manner, this argument is based on the fact that U-937 cells are p53-deficient. Often, cancer cells develop resistance to a given chemotherapeutic agent when it exerts pro-apoptotic effects through the p53 pathway. Thus, the development of new drugs which act in a p53-independent form would constitute a novel therapeutic strategy for overcoming multidrug-resistance in leukemia.

Our group and others have described the differentiating activity of certain coumarins in different malignant cell lines, but none is structurally related to toddaculin (6) (Finn, 2004; Kawai, 2000; Riveiro, 2004 and 2009; Zhang, 2003). Likewise, in the literature it is well reported that various cytotoxic agents also trigger leukemic cell differentiation when they are used at low concentrations (Cai, 2000; Lemarie, 2006; Pérez, 1994). Therefore, the possibility that low concentrations of toddaculin might induce differentiation in U-937 cells was also evaluated. In this regard, the present report demonstrates for the first time the capacity of toddaculin (6) to induce both CD11b and functional C5a receptor (CD88) expression in U-937 cells, as well as the metabolic changes leading to NBT reduction. In contrast, Toddaculin (6) did not induce CD14 expression or chemotaxis of U-937 cells in response to C5a. Of interest, this differentiating activity of toddaculin (6) did not involve modulation of ERK 1/2 and Akt phosphorylation levels as the pro-apoptotic effect did. Certainly, understanding the precise mechanisms of action of toddaculin (6) in U-937 cells represents an interesting topic for further investigation. Moreover, in accordance with other authors, we are of the
opinion that compounds with dual pharmacological behavior, such as toddaculin (6), might serve as a useful criterion for identification and prioritization of candidate compounds with potential application in the development of novel anti-leukemic drugs (Vizirianakis, 2010).

Our results also provide evidence of a correlation between some structural properties of toddaculin (6) and its anti-leukemic activities in vitro. Findings obtained for coumarin 7 during the present research point out that the methoxyl groups at positions 5 and 7 would not be involved in the pro-apoptotic or differentiating effects of toddaculin (6) in U-937 cells. On the other hand, our data illustrate the key role of the prenyl residue at position 6 for both activities. Of note, aeculeatin (1), coumarin 2, toddanol (4) and toddalactone (5) also have a prenyl residue at position 6, and the substituent at C-6 of coumarin 2 is very similar to that of toddaculin (6). However, the latter was the unique compound of the series with pro-apoptotic activity. These observations emphasize the direct relationship between the cited residue and the pharmacological activities of toddaculin (6). Thus, future structure-activity relationship studies of toddaculin (6) should be based on this portion of the molecule.

In conclusion, this is the first report describing the dual pharmacological profile of toddacullin (6) in U-937 cells, as well as the reasons that justify the ongoing study of this molecule in order to move toward clinical development. Further research will confirm whether toddaculin (6)-induced apoptosis and partial differentiation represent two different suitable approaches for the management of leukemia.

Conflict of Interest Statement

No potential conflicts of interest were disclosed.

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Figure 1. Concentration and time-dependent decrease of U-937 cells viability due to toddaculin (6) treatment. U-937 cells in exponential growth were treated with increasing concentrations of toddaculin (6) (5 µM, 25 µM, 50 µM, 100 µM and 250 µM) or 0.1% (v/v) DMSO (vehicle control group, not shown), and the effects on cell proliferation (graphic A) and viability (graphic B) were evaluated at different times (24 h, 48 h and 72 h) employing a Coulter Z-1 cell counter and performing the trypan blue exclusion assay, respectively. In both graphics, one-way ANOVA test (p < 0.01) was performed followed by SNK a posteriori test (**p < 0.01; ***p < 0.001). Significant differences were evaluated respect to vehicle control group (data not shown) at each time, in order to determine the effect of the treatment concentration on the biological effects. Each bar and vertical line represents the mean ± SEM (n ≥ 3).
Figure 2. Irreversible effects of toddaculin (6) on U-937 cells viability. U-937 cells in exponential growth were exposed to increasing concentrations of toddaculin (6) (5 µM, 25 µM, 50 µM, 100 µM and 250 µM) or 0.1% (v/v) DMSO (vehicle control group). After 24 h of treatment, cells from each treatment were seeded in soft agar and the number of colonies generated in these conditions was determined 2 to 3 weeks later. One-way ANOVA test (p < 0.01) was performed followed by SNK a posteriori test (**p < 0.01 vs. vehicle control group). Each bar and vertical line represents the mean ± SEM (n = 3).
**Figure 3. Pro-apoptotic effects of toddaculin (6) in U-937 cells.** Control cells, 2% (v/v) DMSO-treated cells (pro-apoptotic positive control group) or U-937 cells treated with increasing concentrations of toddaculin (6) (25 µM, 50 µM, 100 µM and 250 µM) were analyzed in order to detect cell cycle variations after a 24-h or a 48-h treatment (graphic A and B, respectively). Graphic C shows the typical morphological changes associated with apoptosis in U-937 cells after a 24-h treatment with 250 µM toddaculin (6), as visualized by double staining with HO and PI. Arrows represent condensed chromatin, fragment nuclei and apoptotic bodies. Graphic D shows the nuclear morphology changes of U-937 cells treated with increasing concentrations of toddaculin (6) for 24 h. Necrosis was assessed by the staining of nuclei with PI, whereas apoptosis was visualized by the appropriate changes of nuclei stained with HO. In graphics A, B and D, each bar and vertical line represents the mean ± SEM (n ≥ 3). In these cases one-way ANOVA test (p < 0.01) was performed followed by SNK *a posteriori* test (**p < 0.01; *p < 0.05). In graphic C, each picture is representative of three independent experiments.
Figure 4. Toddaculin (6)-mediated caspase-3 activation in U-937 cells. U-937 cells were treated with different concentrations of toddaculin (6) (50 µM, 100 µM, 250 µM and 500 µM) or 2% DMSO (positive control of apoptosis) and harvested after 24 h. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with an anti-caspase-3 antibody detecting the procaspase-3 (Pro-C3) and the cleavage fragments (C3 20 kDa, C3 17 kDa and C3 11kDa, respectively. Graphic A). Graphic B shows the kinetics of caspase-3 activation in U-937 cells, as detected by Western blot after treatment with 250 µM toddaculin (6). Graphic C shows the toddaculin (6) concentration-dependent cleavage of PARP in U-937 cells after a 24-h treatment. The enzymatic activity of caspase-3 was measured in control cells, 0.1% (v/v) DMSO (vehicle control group), 2% (v/v) DMSO (positive control group) or 50 µM, 100 µM and 250 µM toddaculin (6)-treated U-937 cells after 24 h (Graphic D). Data in this graphic were log-transformed before performing a one-way ANOVA test (p < 0.01) followed by SNK a posteriori test (**p < 0.01 vs. vehicle control group) to evaluate significant variations in caspase-3 activity. Each bar and vertical line represents the mean ± SEM (n = 3). Pictures A, B and C are representative of at least three independent experiments.
Figure 5. Toddaculin (6) regulates Akt and ERK 1/2 signaling pathways at pro-apoptotic concentrations in U-937 cells. Cells in exponential growth were treated with 250 µM toddaculin (6) and harvested at different times during a period of 24 h. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti-p-JNK and JNK antibodies (A), anti-p-p38 and p38 antibodies (B), anti-p-ERK 1/2 and ERK 1/2 antibodies (C) and anti-p-Akt and Akt antibodies (D). Pictures A – D are representative of at least three independent experiments.
Figure 6. U-937 cell expression of differentiation markers induced by low concentrations of toddaculin (6). Expression of CD11b (A) and CD14 (B), chemotactic ability (C), NBT reduction (D) and intracellular Ca\(^{2+}\) mobilization induced by rhC5a due to CD88 expression (E) were evaluated after a 48-h (A, D and E) or a 72-h (B and C) treatment of U-937 cells with increasing concentrations of toddaculin (6) (5 µM, 25 µM, 50 µM) and 0.1% (v/v) DMSO (vehicle control). In graphs A – D, differentiation markers are expressed as relative to control cells. Logarithmic transformation of the data before performing one-way ANOVA test (p < 0.01) followed by SNK a posteriori test (**p < 0.01; *p < 0.05) was applied when necessary. Each bar and vertical line represents the mean ± SEM (n ≥ 3). db-cAMP (400 µM) was employed as positive control of CD11b and CD14 expression and chemotaxis in response to rhC5a (A, B and C, respectively), whereas 1 µM ATRA was the positive control of NBT reduction. In graphic E, arrows indicate the addition of rhC5a (C5a) or ATP, as positive control of intracellular Ca\(^{2+}\) mobilization. Cells treated with 400 µM db-cAMP served as positive control of CD88 expression. Similar results were obtained in at least three independent experiments.
Figure 7. Evaluation of cell signaling pathways in U-937 cells under differentiating concentrations of toddaculin (6). U-937 cells in exponential growth were treated with 50 µM Toddaculin (6) and harvested at different times during a period of 72 h. Equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blot with anti-p-JNK and JNK antibodies (A), anti-p-p38 and p38 antibodies (B), anti-p-ERK 1/2 and ERK 1/2 antibodies (C) and anti-p-Akt and Akt antibodies (D). Pictures A – D are representative of three independent experiments.