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PAPER

A novel C,D-spirolactone analogue of paclitaxel: autophagy instead of apoptosis as a previously unknown mechanism of cytotoxic action for taxoids†

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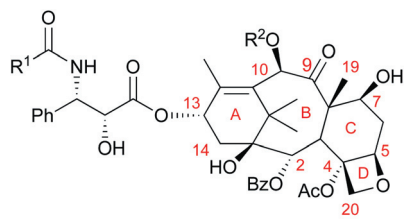
The design, synthesis and biological evaluation of a novel C,D-spirolactone analogue of paclitaxel is described. This is the first paclitaxel analogue without an oxetane D-ring that shows a significant cytotoxic effect (activity one order of magnitude lower than paclitaxel). More importantly, its cytotoxicity is a result of a different mechanism of action, involving mTOR inhibition-dependent autophagy instead of G_2/M cell cycle arrest-dependent apoptosis.

Introduction

The natural product paclitaxel **1**[1] and its semisynthetic analogue docetaxel **2**[2] (Fig. 1) are among the most efficient clinical anticancer agents, currently used for the treatment of metastatic breast cancer, advanced ovarian cancer, non-small-cell lung cancer and AIDS-related Kaposi's sarcoma.[3] Due to their importance as anticancer drugs, extensive structure–activity relationship (SAR) studies on taxoid derivatives have been conducted in order to understand which structural elements are essential for their cytotoxic activity.[2] The results of these studies should allow for the design of new paclitaxel analogues with improved pharmacological and therapeutic properties, as well as of derivatives where the pharmacophore would be contained within a less complex structure, thus simplifying their synthesis and lowering the price of the therapeutic agent, which is currently very high for the aforementioned ones. SAR studies have indicated the regions of the taxane core where considerable structural flexibility is allowed, as well as those whose strict preservation is required for the biological activity. The "northern" part of the molecule seems to be less sensitive to structural variations, and modifications of positions C-7, C-9 or C-10 have been shown to have little influence on the cytotoxicity. On the contrary, the phenylisoserine side chain at C-13 and esters at C-2 and C-4 are crucial for its biological activity.[4] On the basis of cryo-electron microscopy studies, advanced NMR studies in solution and in the solid state, computational studies and other methods, several bioactive conformations of taxoids have been proposed.[5–8] It should be pointed out that current interpretation of all SAR studies is based on the understanding that all bioactive taxoid congeners share the same mechanism of action: binding to tubulin hyperstabilizes the microtubules and arrests the cell cycle in the G_2/M phase, thus inducing apoptosis. It is also understood that the intensity of this effect is proportional to the stability of the taxoid–tubulin complex. Not only taxoids, but other compounds that have elements of a common pharmacophore with taxoids,[9] such as epothilones,[10] eleutherobin,[11] or discodermolide,[12] are also expected to act by a similar mechanism. While there is no doubt that the unique, rigid taxane skeleton is essential for biological activity,[4] the role of the oxetane D-ring (which contributes



1 R^1=Ph R^2=Ac
2 R^1=O*t*Bu R^2=H

Fig. 1 Paclitaxel **1** and docetaxel **2**.

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†Electronic supplementary information (ESI) available: Copies of ^{1}H NMR and ^{13}C NMR spectra of compounds **8–13**, **15**, **16**, **6**; data for tubulin polymerization assay; cytotoxicity of paclitaxel and compound **6** in L929 mouse fibrosarcoma cells. See DOI: 10.1039/c2ob25514f

considerably to the structural complexity of paclitaxel) is not yet fully understood, especially its importance for cytotoxicity. At present, it is unclear whether the influence of the oxetane ring is exerted through steric or electronic effects, or both. To clarify this issue, numerous D-modified and D-seco paclitaxel analogues have been designed, synthesized and submitted to the tubulin and cytotoxicity assays.

Some of them lacked the crucial C-4 ester moiety and were, as expected, inactive in both respects.[13] Several other D-seco analogues exhibited strongly reduced cytotoxic and microtubule stabilizing activity, in spite of the fact that they possessed a C-4α acetoxy group.[14] This may be the result of unfavorable steric interactions between the C-20 acetoxy group and the receptor on the β-tubulin, or the inappropriate spatial orientation of the C-4 acetyl group. To probe the electronic effect of oxygen in the oxetane ring, several analogues, with heteroatoms other than oxygen, were synthesized and tested. Activities of the azetidine[15] and thietane[16] derivatives in the tubulin test were slightly reduced, but these derivatives were practically devoid of cytotoxic activity, apparently pointing to the necessity of the presence of oxygen in ring D. However, these structural modifications brought about some conformational changes as well, and it was difficult to discern whether the lack of activity should be ascribed to these "secondary" conformational and steric effects. On the other hand, two paclitaxel analogues without a heteroatom in ring D were designed and synthesized, where the conformation of the molecule in the pharmacologically relevant regions was maintained congruent to paclitaxel, or docetaxel. In compound **3**,[17] a rigidifying role was ascribed to the cyclopropane ring, while in compound **4** steric repulsion between the C-4 and C-19 methyl groups brings the C-4 acetate into a position similar to the one it adopts in paclitaxel, and the side chains at C-2 and C-13 are presumed to adopt the appropriate orientations for the interactions with the tubulin receptor.[18] Interestingly, both compounds were highly active in the tubulin test, but again, their cytotoxicity was, by two orders of magnitude, inferior to paclitaxel, or docetaxel. Introduction of an oxygen atom at C-5 does not improve cytotoxic activity, on the contrary: compound **5** has only 1/1000 of the cytotoxicity of docetaxel (Fig. 2).[19] All these experimental results suggest that, while not crucial for productive tubulin binding, the oxetane ring might be essential for cytotoxic activity.

Besides these experimental findings, molecular modeling studies predicted that the C-4β substituent is important for the conformation of the C-ring, as well as for the appropriate orientation of the C-4 acetyl group.[14a,20] In addition, studies on the T-taxol[21] as the bioactive conformation of paclitaxel point out the importance of juxtaposition and a short distance between the C-4 acetate methyl and *ortho*-position of the phenyl moiety at C-3′.[7b,22]

Therefore, in order to better understand the role of the oxetane ring and the influence of the spatial orientation of the C-4 ester moiety in taxoids to the cytotoxic activity, we designed, synthesized and evaluated the biological activity of C,D-spirolactone **6** (Fig. 3), in which the C-4 ester group became part of the five member lactone. Introduction of the lactone ring should provide an effective rigidifying element for the taxane core and, simultaneously, conformationally fix the carboxylic structural subunit within the lactone.

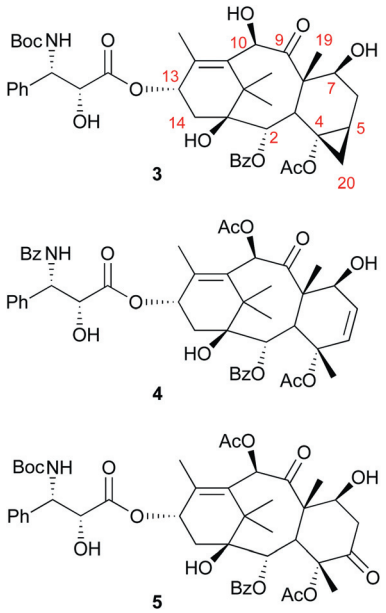


Fig. 2 Some of the D-modified and D-seco paclitaxel analogues.

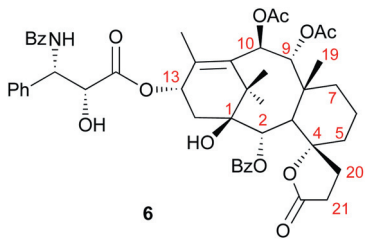
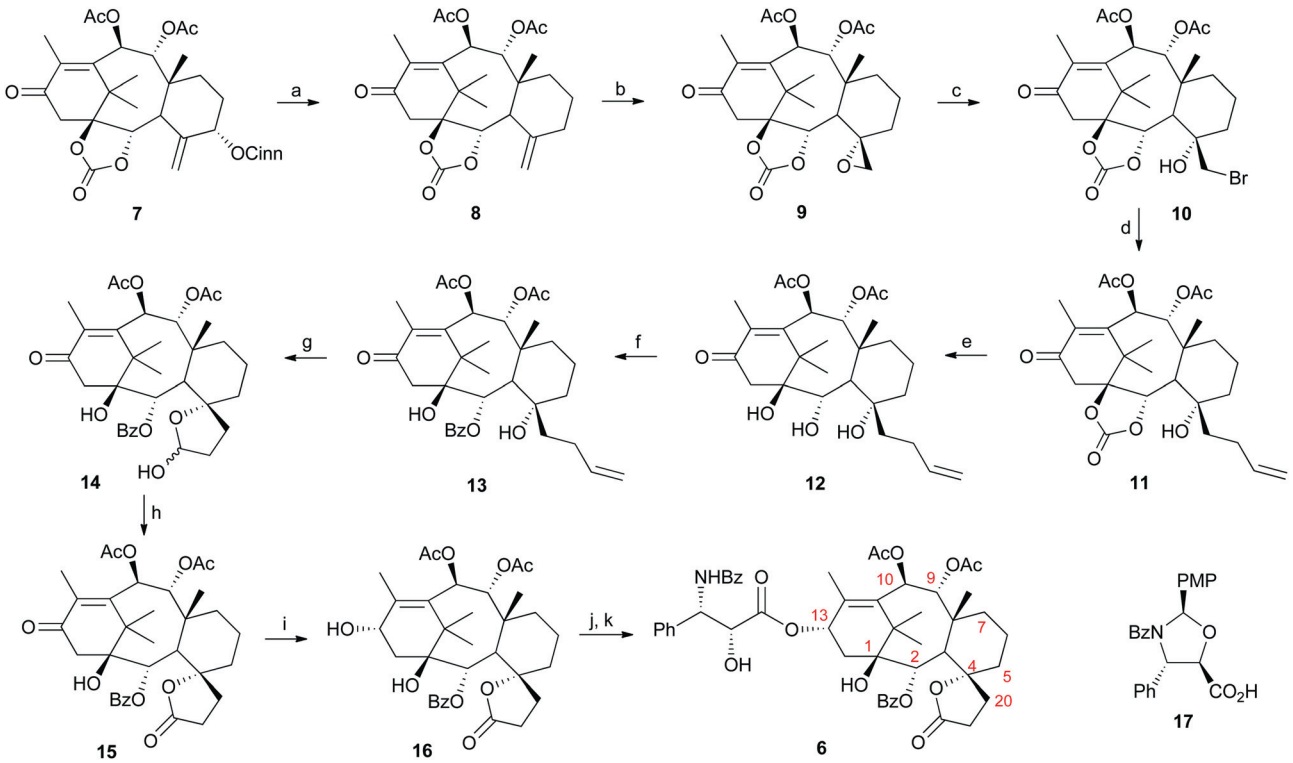


Fig. 3 C,D-Spirolactone **6**.

Results and discussion

As a starting material for the synthesis of spirolactone **6**, we chose diacetate **7** (Scheme 1), an intermediate in our semisynthesis of a C-4 carbonyl C,D-seco-paclitaxel derivative from taxine.[13c,d,23] Taxine is a mixture of alkaloids obtained by extraction from dried leaves of the European yew *Taxus baccata*.[24] Compound **7** lacks the C-7 hydroxyl group and possesses a C-9α acetoxy group instead of a carbonyl—structural modifications that were shown not to affect substantially the cytotoxic activity.[25]

The C-5 cinnamoyl side chain in diacetate **7** was selectively removed by palladium-catalyzed hydrogenolysis.[13b,26] Stereoselective epoxidation of the 4(20) double bond in **8**, followed by a Lewis acid induced nucleophilic opening of epoxide afforded bromohydrin **10**.[17,18,27] The key step of the synthetic sequence—elongation of the C-4β alkyl chain—was planned to be accomplished by radical allylation. Application of radical methodology for this transformation offered several advantages, the most important one being that there was no need for protection of the C-4 hydroxyl group (some preliminary experiments indicated that this could be a very problematic step). Thus, treatment of bromohydrin **10** with excess allyltributyltin, in the presence of AIBN, furnished compound **11** in good yield. Interestingly, the success of this reaction was highly dependent on the amount of



Scheme 1 *Reagents and conditions*: (a) HCO_2H, Et_3N, $Pd(OAc)_2$, PPh_3, THF, 45 °C, 3 d, 76%; (b) mCPBA, DCM, rt, 12 h, 83%; (c) Bu_4NBr, $BF_3{\cdot}OEt_2$, rt, 15 min, 80%; (d) allyltributyltin, AIBN, PhH, reflux, 2 h, 63%; (e) K_2CO_3, Bu_4NBr, THF, H_2O, 24 h, rt 61%; (f) benzoic acid, DCC, DMAP, PhMe, 60 °C, 12 h, 80%; (g) OsO_4 (cat.), $NaIO_4$, *t*BuOH, THF, H_2O, 1 h; (h) Jones reagent, acetone, 0 °C, 15 min, 79% from **13**; (i) $NaBH_4$, THF, MeOH, rt, 15 min, 54%; (j) **17**, DCC, DMAP, PhMe, 75 °C, 1 h; (k) 5% *p*-TsOH in MeOH, 30 min, 61% from **16**.

the radical initiator added (at least 6 equiv.) and the purity of the starting compound **10**. At this stage of the synthesis, we planned to install the C-2 benzoate group by opening of the cyclic carbonate in **11** with phenyllithium (followed by reacetylation of the hydroxyl groups at C-10 and C-9), which is a well known protocol for the introduction of the C-2 benzoate moiety in taxoids.[13c,28] Surprisingly, the cyclic carbonate in **11** proved unreactive toward a large excess of phenyllithium, or phenylmagnesium bromide, even after 24 hours at 0 °C. To overcome this problem, we decided to attempt a selective hydrolysis of the carbonate, in the presence of acetate units at the C-9 and the C-10 positions. Our hypothesis was that the free C-4 hydroxyl group could activate the carbonate toward hydrolysis by intramolecular hydrogen bonding. After considerable experimentation, we found that selective removal of the cyclic carbonate could be accomplished with potassium carbonate, under phase-transfer conditions, to give triol **12**. Esterification of the C-2 hydroxyl group in **12** under standard reaction conditions afforded benzoate **13**. Oxidative cleavage of the terminal alkene in **13**, which was effected by treatment with OsO_4 and $NaIO_4$, gave hemiacetal **14**, as a mixture of two diastereoisomers. Oxidation of the diastereomeric mixture **14** with Jones reagent, followed by stereoselective reduction of the C-13 carbonyl group[14b] in **15** furnished compound **16**. Finally, the coupling of the protected side chain **17** with alcohol **16** was carried out according to the previously described protocol.[29] Removal of the *p*-methoxybenzylidene protecting group with *p*-toluenesulfonic acid gave C,D-spirolactone **6**.

The activity of spirolactone **6** was first evaluated in an *in vitro* tubulin polymerization assay,[30] where **6** was found to stabilize microtubules, being 13 times less active than paclitaxel. The IC_{50} value for **6** was 9 μM, while paclitaxel, which was used as a positive control, showed an IC_{50} value of 0.7 μM.[31] More importantly, **6** was found to possess cytotoxic activity: all three assays for cell number/viability (crystal violet, MTT and acid phosphatase) demonstrated that **6** has cytotoxic activity towards U251 human glioma cells, which was approximately one order of magnitude lower with respect to paclitaxel (the IC_{50} values of 0.25–0.5 μM for paclitaxel and 4–8 μM for **6**; Fig. 4A). Similar results were obtained in the L929 mouse fibrosarcoma cell line (see ESI,† Fig. 3A). While a number of taxane derivatives are known that, without the oxetane ring, still show activity in the tubulin test, to the best of our knowledge compound **6** is the first such analogue that shows a significant cytotoxic activity, being at least one order of magnitude more potent than any other member of the class.

A light microscopy analysis of cell morphology revealed that paclitaxel, but not **6**, caused cell enlargement and change to a spindle-like shape at high (0.5 μM for taxol; 4 μM for **6**) and low concentrations (0.125 μM for taxol; 1 μM for **6**), respectively (Fig. 4B), indicating different cytotoxic mechanisms of the two taxoids. This was further confirmed by flow cytometric analysis, which demonstrated that, at equipotent concentrations, only paclitaxel was able to increase cell size (FSC) and granularity (SSC) (Fig. 5A), induce G_2/M cell cycle arrest (Fig. 5B) and activate caspases (Fig. 5C), the key enzymes involved in

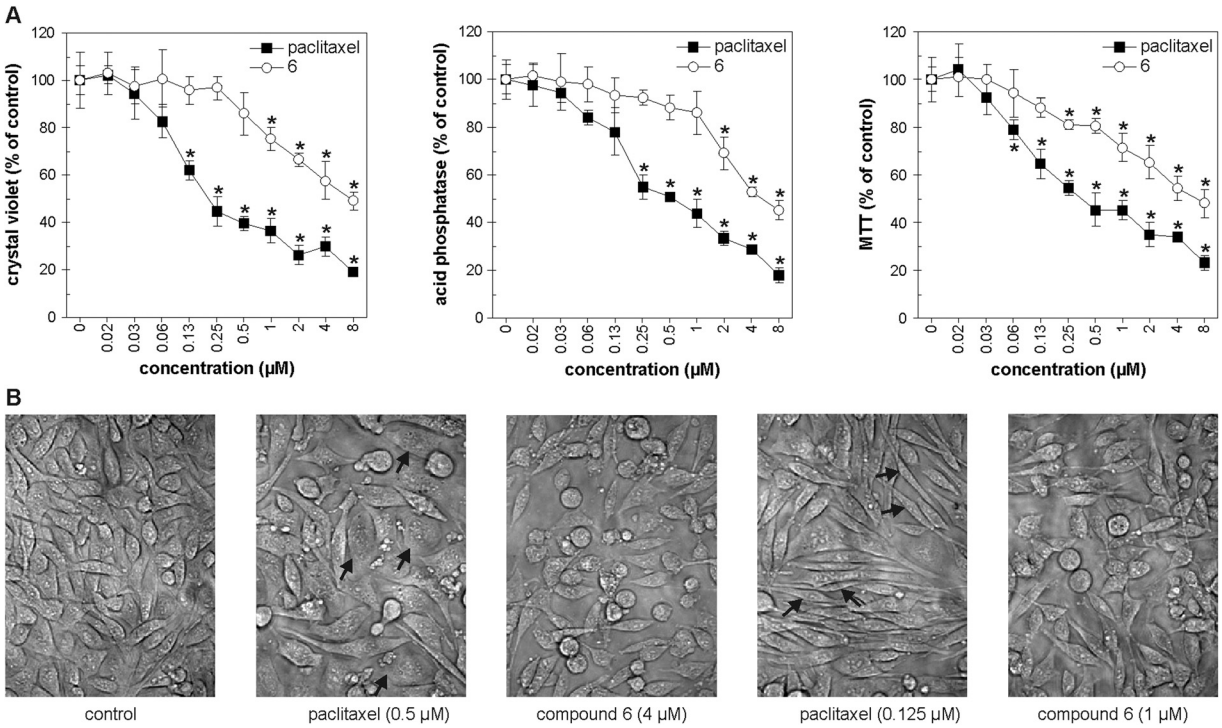


Fig. 4 *In vitro* anticancer activity of paclitaxel and compound **6**. (A) U251 glioma cells were incubated with various concentrations of paclitaxel or compound **6** for 24 h and cell viability was determined using crystal violet, acid phosphatase or MTT assay (the data are mean ± SD values of triplicate measurements from a representative of four independent experiments; $*p < 0.05$). (B) The representative light photomicrographs of U251 cells treated for 24 h with the indicated concentrations of paclitaxel or compound **6**. The arrows indicate enlarged and spindle-shaped cells in cell cultures exposed to 0.5 and 0.125 μM of paclitaxel, respectively.

apoptosis execution.[32] Similar results were obtained in L929 cells (see ESI,† Fig. 3B, C).

Very recently, paclitaxel has been found to induce autophagy,[33] a process in which cytoplasmic content is sequestered in double-membrane vesicles called autophagosomes, and targeted for degradation following their fusion with lysosomes.[34] Depending on the cytotoxic stimulus, autophagy can cause cell death or act as a survival mechanism against metabolic stress or chemotherapy.[35] The latter was apparently true for paclitaxel, as autophagy inhibition sensitized various cancer cell lines to its cytotoxic action.[33c] However, the role of autophagy in cancer cell death induced by other taxoids, to the best of our knowledge, has not been investigated thus far. Therefore, we next investigated the role of autophagy in U251 glioma cell death induced by paclitaxel and compound **6**. Both agents induced intracellular acidification consistent with the autophagy induction,[36] as revealed by staining with the pH-sensitive dye acridine orange (Fig. 6A). Accordingly, both paclitaxel and compound **6** readily triggered a conversion of microtubule-associated protein 1 light-chain 3 (LC3)-I isoform to autophagosome-associated LC3-II (Fig. 6B) and reduced the levels of the selective autophagic target p62,[37] thus confirming the increase in autophagic proteolysis (Fig. 6B). The autophagic response triggered by compound **6**, unlike that induced by paclitaxel, was associated with a decrease in the phosphorylation of p70S6 kinase (S6K) (Fig. 6B), which is a direct downstream target of the main negative autophagy regulator mammalian target of rapamycin (mTOR).[38] Similar results were obtained in L929 cells (see ESI,† Fig. 3D). Finally, downregulation of autophagy by short hairpin (sh) RNA-mediated silencing of autophagy-essential LC3b increased U251 glioma cell death induced by paclitaxel (Fig. 6C), which is consistent with the protective role of autophagy in the paclitaxel-induced death of A549 adenocarcinoma, U87 glioma, PC-3 prostate cancer and HT-29 colon cancer cell lines.[33c] Somewhat surprisingly, LC3b knockdown significantly reduced the cytotoxicity of compound **6** (Fig. 6D). It therefore appears that selective induction of autophagy in the absence of apoptosis might contribute to the anticancer effect of compound **6**, in contrast to a concomitant activation of both the apoptotic and autophagic response by paclitaxel, where the latter acts as a survival mechanism.

Conclusions

Several important conclusions can be drawn: introduction of the lactone ring into the taxane core reduces the *in vitro* anticancer activity of paclitaxel by one order of magnitude. Moreover, the obtained spirolactone analogue displays mechanistically distinct cytotoxic action in comparison with paclitaxel, involving mTOR inhibition-dependent autophagy instead of G_2/M cell cycle block-associated apoptosis. These results suggest that the oxetane D-ring is important, not only for cytotoxic efficiency, but also for the mechanism of reaction, *i.e.* the ability of taxoids to induce apoptotic death of cancer cells. It also indicates that care should be taken when performing SAR studies of taxane analogues, as quantitative differences in activities of structurally

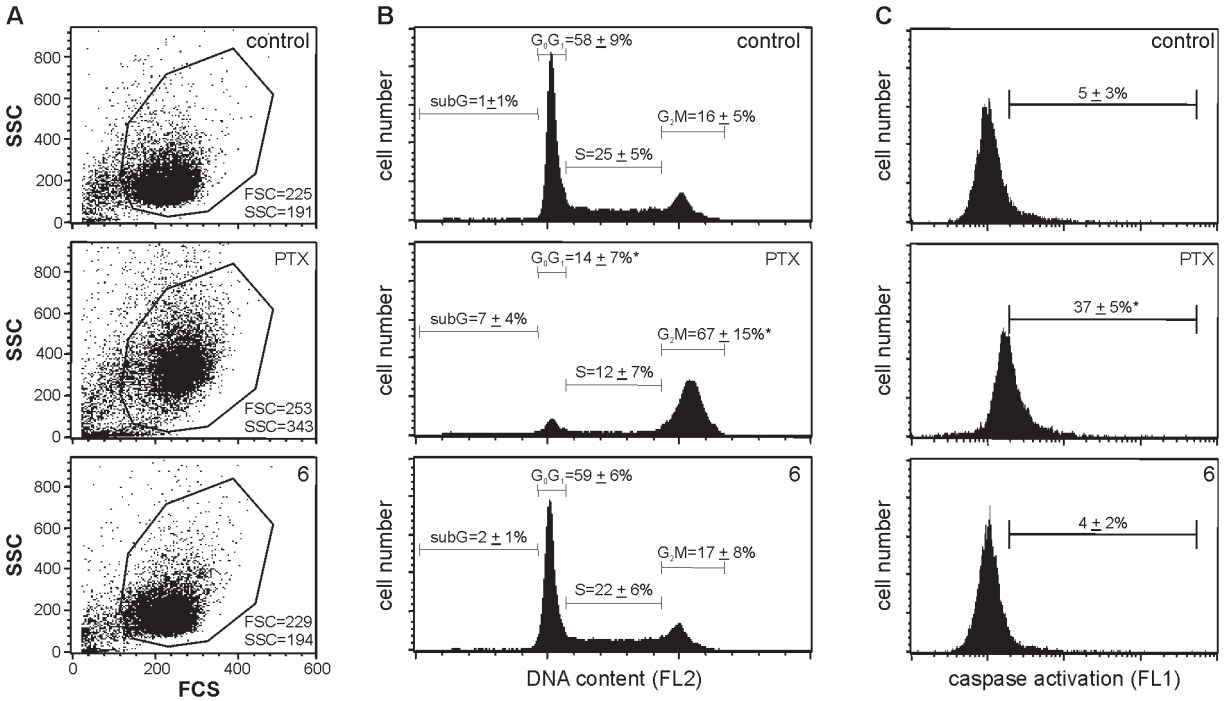


Fig. 5 The effects of paclitaxel and compound **6** on cell size/granularity, cell cycle and caspase activation. U251 cells were incubated with paclitaxel (PTX; 0.5 μM) or compound **6** (4 μM) for 24 h. The cell size (FSC) and granularity (SSC) (A), cell cycle distribution (B), and caspase activation (C) were examined by flow cytometry. The dot plots and histograms from a representative of three independent experiments are presented. The data in (B) and (C) are mean ± SD values (*$p < 0.05$).

close derivatives may reflect qualitative differences in the mechanism of action. The yew tree is an old organism which has developed its secondary metabolites early on in the evolutionary scale; therefore, the taxane core appears to have the properties of a privileged structure, where small structural changes can lead to significant changes of mechanism of action within a large scope of possible biological activities. Having in mind the fact that the acquired resistance to apoptosis limits the clinical success of the taxane anticancer agents, the present study provides a conceptual framework for development of novel taxane analogues able to kill cancer cells through induction of autophagy.

Experimental section

General experimental

All chromatographic separations[39] were performed on silica, 10–18, 60A, ICN Biomedicals. Standard techniques were used for the purification of reagents and solvents.[40] NMR spectra were recorded on a Bruker Avance III 500. Chemical shifts are expressed in ppm (δ) using tetramethylsilane as internal standard. IR spectra were recorded on a Nicolet 6700 FT instrument, and are expressed in cm^{-1}. Mass spectra were obtained on an Agilent technologies 6210 TOF LC/MS instrument (LC: series 1200). Microanalyses were performed at the Vario EL III instrument CHNOS Elementar Analyzer, Elementar Analysensysteme GmbH, Hanau, Germany. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. The purity of compound **6** that was synthesized and tested for cytotoxic activity was ≥95%, as determined by HPLC analysis. The purity of the compound was determined by Agilent 1200 Series HPLC with UV detection at wavelengths 230, 248 and 274 nm (method A: Zorbax SB-C18, 100 mm × 2.1 mm; 1.8 μm, with 60–100% acetonitrile–water and 0.35 mL min^{-1} elution at 40 °C; method B: Nucleosil C18, 150 mm × 4.0 mm; 5 μm, with 60–100% acetonitrile–water and 1.0 mL min^{-1} elution at 25 °C).

Compound 8. Palladium acetate (29.6 mg, 0.13 mmol) and triphenylphosphine (207 mg, 0.79 mmol) were dissolved in THF (6 mL) and stirred for 10 min under an argon atmosphere. After cooling the yellow solution to 0 °C, triethylamine (4.27 g, 42.2 mmol), formic acid (1.94 g, 42.2 mmol) and the solution of **7** (800 mg, 1.32 mmol) in THF (6 mL) were added successively. The reaction mixture was then allowed to warm to rt and, after 1 h at rt, was stirred for 72 h at 45 °C. The dark brown solution was cooled to rt, diluted with CH_2Cl_2, washed with 1.5 M HCl, sat. aq. $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by two dry-flash chromatographies (1. eluent: petrol ether–ethyl acetate = 7 : 3 and 2. eluent: benzene–ethyl acetate = 9 : 1) to give compound **8** (460 mg, 76%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.03 (1H, d, J = 10.5 Hz, H-10), 5.73 (1H, d, J = 10.5 Hz, H-9), 5.35 (1H, bs, H-20), 5.06 (1H, bs, H-20), 4.90 (1H, d, J = 6.0 Hz, H-2), 2.92 (1H, d, J = 19.5 Hz, H-14), 2.88 (1H, d, J = 19.5 Hz, H-14), 2.66 (1H, d, J = 5.5 Hz, H-3), 2.27–2.24 (1H, m, H-5), 2.20 (3H, s, H-18), 2.09 (3H, s, Ac), 2.06 (3H, s, Ac), 1.96–1.92 (1H, m, H-7), 1.75–1.63 (2H, m, H-5 and H-6), 1.69 (3H, s, H-16 or H-17), 1.57–1.50 (1H, m, H-6), 1.36 (1H, dd, J = 4.0 and 13.5 Hz, H-7), 1.32 (3H, s, H-16 or H-17), 0.98 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$)

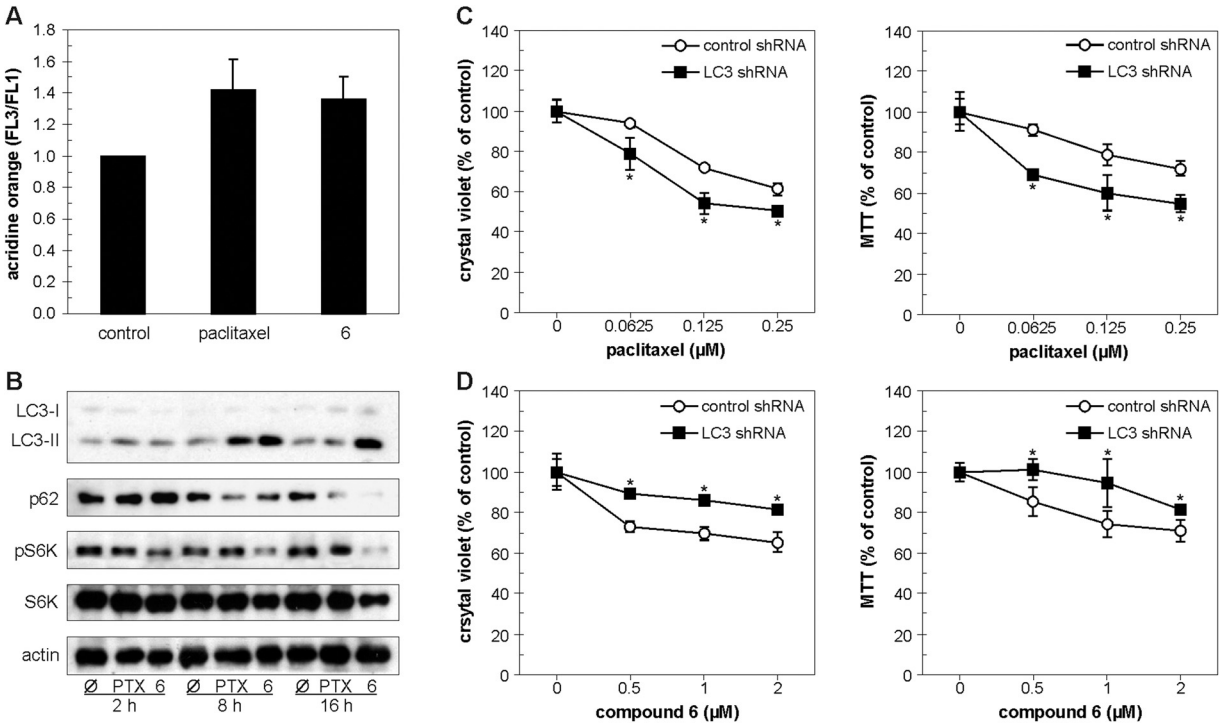


Fig. 6 The role of autophagy in the anticancer activity of paclitaxel and compound **6**. (A, B) U251 cells were incubated with paclitaxel (PTX; 0.5 μM) or compound **6** (4 μM). Intracellular acidification, as a marker of autophagy, was assessed by flow cytometric analysis of acridine orange-stained cells after 24 h (A), while LC3 conversion, p62 and phospho-S6K (pS6K) levels were determined by immunoblotting (B). (C, D) U251 cells stably transfected with control, or LC3b shRNA, were incubated with the indicated concentrations of paclitaxel (C) or compound **6** (D) for 24 h and cell viability was assessed by crystal violet or MTT assay. The data are mean + SD values from three independent experiments (A), or mean + SD values of triplicates from a representative of three separate experiments (C, D).

δ: 196.4 (C, C-13), 169.9 (C, Ac), 169.5 (C, Ac), 152.5 (C, CO), 149.9 (C, C-11), 141.9 (C, C-12), 141.4 (C, C-4), 114.9 (CH_2, C-20), 88.5 (C, C-1), 79.9 (CH, C-2), 75.4 (CH, C-9), 72.5 (CH, C-10), 49.1 (CH, C-3), 45.3 (C, C-8), 41.0 (C, C-15), 40.9 (CH_2, C-14), 40.4 (CH_2, C-5), 32.7 (CH_3, C-16 or C-17), 32.0 (CH_2, C-7), 23.2 (CH_2, C-6), 20.8 (CH_3, Ac), 20.6 (CH_3, Ac), 20.1 (CH_3, C-16 or C-17), 18.2 (CH_3, C-19), 14.7 (CH_3, C-18). IR (film) ν_{max}: 2995, 2940, 2869, 1815, 1745, 1689, 1374, 1270, 1235, 1030. HRMS (ESI-TOF high acc) calcd for $C_{25}H_{33}O_8$ (MH^+): 461.2170, found: 461.2180. $[\alpha]_D^{20}$ +160° (*c* 0.2, EtOAc).

Compound 9. A solution of **8** (1.7 g, 3.69 mmol) and mCPBA (1.27 g, 7.36 mmol) in CH_2Cl_2 (120 mL) was stirred overnight at rt. The reaction mixture was diluted with CH_2Cl_2, washed successively with sat. aq. $NaHCO_3$ and water, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by two dry-flash chromatographies (1. eluent: petrol ether–ethyl acetate = 6 : 4; 2. eluent: benzene–ethyl acetate = 8 : 2) gave compound **9** (1.45 g, 83%) as a colorless film. ^1H NMR (500 MHz, $CDCl_3$) δ: 6.02 (1H, d, *J* = 10.5 Hz, H-10), 5.72 (1H, d, *J* = 10.5 Hz, H-9), 4.72 (1H, d, *J* = 5.0 Hz, H-2), 3.29 (1H, dd, *J* = 1.5 and 4.5 Hz, H-20), 3.22 (1H, d, *J* = 19.5 Hz, H-14), 2.91 (1H, d, *J* = 19.5 Hz, H-14), 2.56 (1H, d, *J* = 5.0 Hz, H-3), 2.53 (1H, d, *J* = 4.5 Hz, H-20), 2.19 (3H, s, H-18), 2.11 (3H, s, Ac), 2.06 (3H, s, Ac), 2.03–1.97 (1H, m, H-7), 1.81–1.76 (1H, m, H-6), 1.71–1.58 (2H, m, H-5 and H-6), 1.63 (3H, s, H-16 or H-17), 1.31 (3H, s, H-16 or H-17), 1.29–1.24 (2H, m, H-5 and H-7), 1.08 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 196.7 (C, C-13), 170.0 (C, Ac), 169.4 (C, Ac), 152.4 (C, CO), 149.4 (C, C-11), 142.8 (C, C-12), 87.6 (C, C-1), 78.8 (CH, C-2), 75.1 (CH, C-9), 72.2 (CH, C-10), 58.8 (C, C-4), 53.0 (CH_2, C-20), 45.2 (CH, C-3), 45.1 (C, C-8), 41.5 (C, C-15), 41.1 (CH_2, C-14), 38.0 (CH_2, C-5), 32.4 (CH_3, C-16 or C-17), 31.1 (CH_2, C-7), 20.7 (CH_3, Ac), 20.5 (CH_3, Ac), 20.0 (CH_3, C-16 or C-17), 19.9 (CH_2, C-6), 18.1 (CH_3, C-19), 14.6 (CH_3, C-18). IR (film) ν_{max}: 3061, 2995, 2945, 2873, 1809, 1744, 1689, 1374, 1270, 1235, 1033. HRMS (ESI-TOF high acc) calcd for $C_{25}H_{32}O_9Na$ (MNa^+): 499.1938, found: 499.1928. $[\alpha]_D^{20}$ +214° (*c* 0.1, MeOH).

Compound 10. $BF_3{\cdot}Et_2O$ (475 mg, 3.35 mmol) was added dropwise to a solution of **9** (1.45 g, 3.04 mmol) and Bu_4NBr (3.92 g, 12.16 mmol) in CH_2Cl_2 (50 mL), at rt, under an argon atmosphere. The reaction mixture was stirred for 15 min, then diluted with CH_2Cl_2, washed successively with water and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by dry-flash chromatography (eluent: benzene–ethyl acetate = 6 : 4) gave compound **10** (1.35 g, 80%) as white powder, mp = 126–129 °C. ^1H NMR (500 MHz, $CDCl_3$) δ: 6.01 (1H, d, *J* = 10.5 Hz, H-10), 5.66 (1H, d, *J* = 10.5 Hz, H-9), 4.80 (1H, d, *J* = 4.5 Hz, H-2), 4.16 (1H, dd, *J* = 2.0 and 10.0 Hz, H-20), 4.03 (1H, d, *J* = 19.5 Hz, H-14), 3.83 (1H, d, *J* = 10.5 Hz, H-20), 2.82 (1H, d, *J* = 19.5 Hz, H-14), 2.67

(1H, d, J = 5.0 Hz, H-3), 2.66 (1H, s, OH-4), 2.32–2.27 (1H, m, H-5), 2.15 (3H, s, H-18), 2.10 (3H, s, Ac), 2.04 (3H, s, Ac), 1.92–1.87 (1H, m, H-7), 1.73–1.67 (1H, m, H-6), 1.67 (3H, s, H-16 or H-17), 1.45–1.36 (1H, m, H-6), 1.33 (3H, s, H-16 or H-17), 1.29–1.23 (1H, m, H-7), 1.02–0.95 (1H, m, H-5), 0.99 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 197.6 (C, C-13), 170.2 (C, Ac), 169.3 (C, Ac), 152.4 (C, CO), 149.0 (C, C-11), 142.5 (C, C-12), 88.6 (C, C-1), 80.0 (CH, C-2), 75.3 (CH, C-9), 72.7 (C, C-4), 72.2 (CH, C-10), 49.7 (CH, C-3), 43.9 (C, C-8), 41.6 (C, C-15), 41.1 (CH_2, C-14), 40.6 (CH_2, C-20), 39.7 (CH_2, C-5), 32.6 (CH_3, C-16 or C-17), 31.4 (CH_2, C-7), 20.7 (CH_3, Ac), 20.6 (CH_3, Ac), 20.2 (CH_3, C-16 or C-17), 19.5 (CH_2, C-6), 18.7 (CH_3, C-19), 14.1 (CH_3, C-18). IR (film) ν_{max}: 3495, 2995, 2937, 2867, 1810, 1744, 1689, 1372, 1269, 1233, 1033. HRMS (ESI-TOF high acc) calcd for $C_{25}H_{33}O_9BrK$ (MK^+): 595.0939, found: 595.0942. Elemental analysis (%) calcd for $C_{25}H_{33}O_9Br$: C 53.87, H 5.97, found: C 53.54, H 5.88. $[\alpha]_D^{20}$ +166° (c 0.2, MeOH).

Compound 11. A deaerated solution of **10** (259 mg, 0.46 mmol), allyltributyltin (1.85 g, 5.59 mmol) and AIBN (229 mg, 1.39 mmol) in benzene (35 mL) was stirred at 80 °C under an argon atmosphere. After 1 h, a second batch of AIBN (229 mg, 1.39 mmol) was added and the reaction mixture was continued for 1 h. After removal of the solvent under reduced pressure, the residue was purified by dry-flash chromatography (eluent: benzene–ethyl acetate = 8 : 2) to give compound **11** (152 mg, 63%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.00 (1H, d, J = 10.5 Hz, H-10), 5.89–5.81 (1H, m, H-22), 5.63 (1H, d, J = 10.5 Hz, H-9), 5.08 (1H, broad dd, J = 1.5 and 17.0 Hz, H-23), 5.00 (1H, dd, J = 1.5 and 10.0 Hz, H-23), 4.85 (1H, d, J = 5.0 Hz, H-2), 3.90 (1H, d, J = 19.5 Hz, H-14), 2.81 (1H, d, J = 19.0 Hz, H-14), 2.24 (1H, d, J = 5.5 Hz, H-3), 2.22 (1H, s, OH-4), 2.21–2.16 (1H, m, H-21), 2.14 (3H, s, H-18), 2.13–2.08 (2H, m, H-20 and H-21), 2.09 (3H, s, Ac), 2.04 (3H, s, Ac), 1.98–1.94 (1H, m, H-5), 1.88–1.83 (1H, m, H-7), 1.80–1.74 (1H, m, H-20), 1.72–1.66 (1H, m, H-6), 1.68 (3H, s, H-16 or H-17), 1.52–1.45 (1H, m, H-6), 1.32 (3H, s, H-16 or H-17), 1.26–1.19 (1H, m, H-7), 1.02 (3H, s, H-19), 1.01–0.95 (1H, m, H-5). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 197.4 (C, C-13), 170.2 (C, Ac), 169.4 (C, Ac), 152.6 (C, CO), 148.7 (C, C-11), 142.2 (C, C-12), 138.7 (CH, C-22), 115.5 (CH_2, C-23), 89.0 (C, C-1), 81.1 (CH, C-2), 75.6 (CH, C-9), 75.3 (C, C-4), 72.4 (CH, C-10), 52.5 (CH, C-3), 44.1 (C, C-8), 41.3 (C, C-15), 41.2 (CH_2, C-14), 39.4 (CH_2, C-5), 35.0 (CH_2, C-20), 32.6 (CH_3, C-16 or C-17), 31.6 (CH_2, C-7), 28.4 (CH_2, C-21), 20.8 (CH_3, Ac), 20.7 (CH_3, Ac), 20.2 (CH_3, C-16 or C-17), 19.3 (CH_3, C-19), 18.9 (CH_2, C-6), 14.1 (CH_3, C-18). IR (film) ν_{max}: 3523, 3362, 2976, 2934, 2871, 1811, 1744, 1689, 1372, 1267, 1235, 1031. HRMS (ESI-TOF high acc) calcd for $C_{28}H_{38}O_9Na$ (MNa^+): 541.2408, found: 541.2393. $[\alpha]_D^{20}$ +170° (c 0.2, MeOH).

Compound 12. Bu_4NBr (4.6 mg, 0.014 mmol) was added to a solution of K_2CO_3 (49 mg, 0.35 mmol) and **11** (18.4 mg, 0.035 mmol) in water (1.25 mL) and THF (2.5 mL). The reaction mixture was stirred for 24 hours at rt, diluted with CH_2Cl_2 and washed with aq. NH_4Cl. The aqueous phase was extracted with CH_2Cl_2, the combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (eluent: benzene–ethyl acetate = 6 : 4) gave compound **12** (10.7 mg, 61%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.01 (1H, d, J = 10.5 Hz, H-10), 5.87–5.79 (1H, m, H-22), 5.80 (1H, d, J = 10.5 Hz, H-9), 5.05 (1H, ddd, J = 1.5, 3.0 and 17.0 Hz, H-23), 4.98 (1H, broad dd, J = 1.5 and 10.0 Hz, H-23), 4.43 (1H, d, J = 9.5 Hz, OH-2), 4.04 (1H, dd, J = 9.0 and 5.5 Hz, H-2), 3.85 (1H, s, OH-1), 3.24 (1H, d, J = 19.5 Hz, H-14), 2.72 (1H, s, OH-4), 2.62 (1H, dd, J = 1.0 and 19.5 Hz, H-14), 2.47–2.40 (1H, m, H-20), 2.27 (1H, d, J = 5.0 Hz, H-3), 2.21–2.15 (1H, m, H-21), 2.09 (3H, s, H-18), 2.08 (3H, s, Ac), 2.05–2.03 (1H, m, H-21), 2.04 (3H, s, Ac), 1.91–1.82 (2H, m, H-5 and H-7), 1.72–1.65 (2H, m, H-6 and H-20), 1.63 (3H, s, H-16 or H-17), 1.52–1.44 (1H, m, H-6), 1.30–1.23 (1H, m, H-7), 1.27 (3H, s, H-16 or H-17), 1.02–0.95 (1H, m, H-5), 0.99 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 201.2 (C, C-13), 170.4 (C, Ac), 169.6 (C, Ac), 152.9 (C, C-11), 139.6 (C, C-12), 138.9 (CH, C-22), 115.2 (CH_2, C-23), 77.6 (C, C-1), 77.1 (C, C-4), 75.4 (CH, C-9), 74.4 (CH, C-2), 72.5 (CH, C-10), 54.2 (CH, C-3), 44.0 (CH_2, C-14), 43.2 (C, C-8), 42.6 (C, C-15), 40.6 (CH_2, C-5), 35.5 (CH_2, C-20), 34.3 (CH_3, C-16 or C-17), 31.9 (CH_2, C-7), 28.8 (CH_2, C-21), 20.9 (CH_3, Ac), 20.7 (CH_3, Ac), 20.4 (CH_3, C-16 or C-17), 19.0 (CH_3, C-19), 18.9 (CH_2, C-6), 13.7 (CH_3, C-18). IR (film) ν_{max}: 3416, 2936, 2869, 1745, 1677, 1450, 1372, 1228, 1022. HRMS (ESI-TOF high acc) calcd for $C_{27}H_{40}O_8Na$ (MNH_4^+): 510.3061, found: 510.3065. $[\alpha]_D^{20}$ +113° (c 0.2, EtOAc).

Compound 13. A solution of **12** (24 mg, 0.049 mmol), benzoic acid (41 mg, 0.335 mmol), DCC (70 mg, 0.339 mmol) and DMAP (6 mg, 0.049 mmol) in toluene (1.5 mL) was stirred overnight at 60 °C under an argon atmosphere. The reaction mixture was diluted with CH_2Cl_2, washed successively with sat. aq. $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by dry-flash chromatography (eluent: benzene–ethyl acetate = 8 : 2) gave compound **13** (23.2 mg, 80%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.02 (2H, dd, J = 1.0 and 8.5 Hz, Ar), 7.64–7.60 (1H, m, Ar), 7.51–7.48 (2H, m, Ar), 6.08 (1H, d, J = 10.5 Hz, H-10), 5.95 (1H, bd, J = 5.5 Hz, H-2), 5.91 (1H, d, J = 10.5 Hz, H-9), 5.29–5.21 (1H, m H-22), 4.58–4.55 (1H, m, H-23), 4.33 (1H, ddd, J = 1.0, 3.0 and 17.0 Hz, H-23), 3.67 (1H, d, J = 19.5 Hz, H-14), 2.68 (1H, d, J = 19.0 Hz, H-14), 2.61 (1H, d, J = 5.5 Hz, H-3), 2.50 (1H, s, OH-4), 2.15 (3H, s, H-18), 2.10 (3H, s, Ac), 2.06 (3H, s, Ac), 1.86 (1H, s, OH-1), 1.87–1.77 (3H, m, H-5, H-7 and H-21), 1.75 (3H, s, H-16 or H-17), 1.74–1.61 (2H, m, H-6 and H-21), 1.56–1.40 (3H, m, H-6 and 2 × H-20), 1.35–1.30 (1H, m, H-7), 1.25 (3H, s, H-16 or H-17), 1.03–0.98 (1H, m, H-5), 1.00 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 199.6 (C, C-13), 170.2 (C, Ac), 169.5 (C, Ac), 166.9 (C, Bz), 151.5 (C, C-11), 140.2 (C, C-12), 138.0 (CH, C-22), 133.8 (CH, Ar), 129.8 (CH, Ar), 129.3 (C, Ar), 128.8 (CH, Ar), 114.5 (CH_2, C-23), 78.7 (C, C-1), 75.3 (2 × CH, C-2 and C-9), 75.1 (C, C-4), 72.3 (CH, C-10), 55.3 (CH, C-3), 43.8 (CH_2, C-14), 43.3 (C, C-8), 43.0 (C, C-15), 39.9 (CH_2, C-5), 36.0 (CH_2, C-20), 34.2 (CH_3, C-16 or C-17), 31.9 (CH_2, C-7), 28.7 (CH_2, C-21), 20.9 (CH_3, Ac), 20.7 (CH_3, Ac), 20.1 (CH_3, C-16 or C-17), 18.9 (CH_3, C-19), 18.7 (CH_2, C-6),

13.8 (CH_3, C-18). IR (film) ν_{max}: 3498, 2933, 2865, 1745, 1677, 1451, 1373, 1153, 1024. HRMS (ESI-TOF high acc) calcd for $C_{34}H_{44}O_9Na$ (MNa^+): 619.2877, found: 619.2893. $[\alpha]_D^{20}$ +56° (*c* 0.2, EtOAc).

Compound 15. OsO_4 (0.1 mL of a 2.5% solution in *t*-BuOH) was added to a solution of **13** (98 mg, 0.164 mmol) in a mixture of THF (5.8 mL) and water (2.9 mL), followed by the addition of $NaIO_4$ (422 mg, 1.97 mmol). The reaction mixture was stirred for 1 h at rt, then sodium dithionite (500 mg) was added and stirring was continued for 30 min. The reaction mixture was diluted with CH_2Cl_2, washed with aq. $Na_2S_2O_3$ and water, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude product was roughly purified by dry-flash chromatography (eluent: petrol ether–ethyl acetate = 6 : 4) affording a mixture of hemiacetals **14** (79 mg) that was used in the next step.

Jones reagent (160 μL, 0.53 mmol) was added to a solution of **14** (79 mg, 0.132 mmol) in acetone (14.2 mL) at 0 °C, and the resulting mixture was stirred for 15 min, when isopropanol (2.5 mL) was added. The reaction mixture was diluted with EtOAc, washed with brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by dry-flash chromatography (eluent: benzene–ethyl acetate = 1 : 1) gave compound **15** (62 mg, 79%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.04–8.01 (2H, m, Ar), 7.64–7.58 (1H, m, Ar), 7.52–7.47 (2H, m, Ar), 6.09 (1H, d, J = 10.5 Hz, H-10), 6.01 (1H, bd, J = 5.5 Hz, H-2), 5.99 (1H, d, J = 11.0 Hz, H-9), 3.73 (1H, d, J = 19.5 Hz, H-14), 2.93 (1H, d, J = 5.0 Hz, H-3), 2.71 (1H, dd, J = 1.0 and 19.5 Hz, H-14), 2.41 (1H, m, H-21), 2.15 (3H, s, H-18), 2.11 (3H, s, Ac), 2.07 (3H, s, Ac), 2.05–2.00 (1H, m, H-20), 1.94–1.89 (1H, m, H-7), 1.86–1.75 (4H, m, H-5, H-6, H-20 and H-21), 1.73 (3H, s, H-16 or H-17), 1.53–1.45 (1H, m, H-6), 1.43–1.30 (2H, m, H-5 and H-7), 1.24 (3H, s, H-16 or H-17), 1.04 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 199.1 (C, C-13), 175.4 (CH, C-22), 170.0 (C, Ac), 169.5 (C, Ac), 167.4 (C, Bz), 151.0 (C, C-11), 140.5 (C, C-12), 134.0 (CH, Ar), 130.0 (CH, Ar), 128.9 (CH, Ar), 128.5 (C, Ar), 88.0 (C, C-4), 77.9 (C, C-1), 74.6 (CH, C-2 or C-9), 74.5 (CH, C-2 or C-9), 72.1 (CH, C-10), 50.1 (CH, C-3), 44.1 (CH_2, C-14), 43.9 (C, C-8), 43.0 (C, C-15), 41.3 (CH_2, C-5), 33.9 (CH_3, C-16 or C-17), 31.1 (CH_2, C-7), 29.4 (CH_2, C-20), 28.7 (CH_2, C-21), 20.9 (CH_3, Ac), 20.7 (CH_3, Ac), 20.1 (CH_3, C-16 or C-17), 18.3 (CH_3, C-19), 18.1 (CH_2, C-6), 13.9 (CH_3, C-18). IR (film) ν_{max}: 3428, 3440, 2926, 2858, 1769, 1746, 1713, 1677, 1272, 1221, 1024. HRMS (ESI-TOF high acc) calcd for $C_{33}H_{40}O_{10}K$ (MK^+): 635.2253, found: 635.2256. $[\alpha]_D^{20}$ +52° (*c* 0.2, EtOAc).

Compound 16. A mixture of compound **15** (22 mg, 0.037), $NaBH_4$ (21 mg, 0.555 mmol), THF (1.1 mL) and MeOH (0.19 mL), was stirred for 15 min at rt. The reaction mixture was diluted with EtOAc, washed with 1.5 M HCl, water and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (eluent: benzene–ethyl acetate = 1 : 2) gave compound **16** (12 mg, 54%) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.04–8.01 (2H, m, Ar), 7.62–7.58 (1H, m, Ar), 7.50–7.46 (2H, m, Ar), 6.07 (1H, d, J = 10.5 Hz, H-10), 5.90–5.86 (2H, m, H-2 and H-9), 4.63 (1H, d, J = 9.0 Hz, H-13), 3.27 (1H, d, J = 5.0 Hz, H-3), 2.74 (1H, dd, J = 3.0 and 15.5 Hz, H-14), 2.60–2.53 (1H, m, H-14), 2.43–2.30 (2H, m, H-21 and OH-1), 2.19 (3H, d, J = 1.0 Hz, H-18), 2.08 (3H, s, Ac), 2.08–2.05 (1H, m, H-20), 2.03 (3H, s, Ac), 1.90–1.74 (5H, m, H-5, H-6, H-7, H-20 and H-21), 1.65 (3H, s, H-16 or H-17), 1.58 (1H, s, OH-13), 1.56–1.42 (3H, m, H-5, H-6 and H-7), 1.04 (3H, s, H-16 or H-17), 1.01 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.4 (C, C-22), 170.1 (C, Ac), 169.7 (C, Ac), 167.5 (C, Bz), 144.7 (C, C-11), 133.8 (CH, Ar), 133.7 (C, C-12), 129.9 (CH, Ar), 128.9 (C, Ar), 128.8 (CH, Ar), 89.3 (C, C-4), 77.6 (C, C-1), 75.3 (2 × CH, C-2 and C-9), 71.9 (CH, C-10), 69.0 (CH, C-13), 49.8 (CH, C-3), 43.8 (C, C-8), 41.7 (C, C-15), 41.4 (CH_2, C-5), 39.6 (CH_2, C-14), 30.9 (CH_2, C-7), 29.8 (CH_3, C-16 or C-17), 29.3 (CH_2, C-20), 28.6 (CH_2, C-21), 21.0 (CH_3, Ac), 20.7 (CH_3, Ac), 20.5 (CH_3, C-16 or C-17), 18.2 (CH_3, C-19), 18.1 (CH_2, C-6), 16.7 (CH_3, C-18). IR (film) ν_{max}: 3486, 3061, 2934, 2869, 1743, 1719, 1451, 1373, 1272, 1227. HRMS (ESI-TOF high acc) calcd for $C_{33}H_{42}O_{10}Na$ (MNa^+): 621.2670, found: 621.2666. $[\alpha]_D^{20}$ +6.9° (*c* 0.3, $CHCl_3$).

Compound 6. A solution of DCC (16 mg, 0.077 mmol) in toluene (1.3 mL) was added to a solution of **16** (9.8 mg, 0.0164 mmol), carboxylic acid **17** (26 mg, 0.064 mmol) and DMAP (9.4 mg, 0.077 mmol) in toluene (3.3 mL), at rt, under an argon atmosphere. The reaction mixture was slowly (over 30 min) heated up to 75 °C, and was stirred at that temperature for an additional 30 min. Then, a batch of solid carboxylic acid **17** (6 mg, 0.015 mmol) and DCC (4 mg, 0.019 mmol) was added to the reaction mixture and stirring was continued at 75 °C for 30 min. After removal of the solvent, the reaction mixture was roughly purified by column chromatography (eluent: benzene–ethyl acetate = 7 : 3) to give the crude protected ester as a colorless film. The protected ester was stirred with 5% methanolic *p*-TsOH (3.7 mL) at rt for 30 min. The reaction mixture was diluted with CH_2Cl_2, washed successively with aq. $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification by two column chromatographies (1. eluent: benzene–ethyl acetate = 6 : 4; 2. eluent: hexane–acetone = 6 : 4) gave compound **6** (8.6 mg, 61% over two steps) as a colorless film. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.71 (1H, d, J = 7.0 Hz, NH), 8.03–8.01 (2H, m, Ar), 7.98–7.96 (2H, m, Ar), 7.63–7.60 (1H, m, Ar), 7.58–7.54 (1H, m, Ar), 7.50–7.42 (6H, m, Ar), 7.35–7.32 (2H, m, Ar), 7.29–7.25 (1H, m, Ar), 6.46 (1H, d, J = 9.0 Hz, OH'), 6.05–6.01 (2H, m + d, J = 10.5 Hz, H-13 and H-10), 5.89 (1H, d, J = 4.0 Hz, H-2), 5.88 (1H, d, J = 10.0 Hz, H-9), 5.39 (1H, dd, J = 3.5 and 7.0 Hz, H-3′), 4.91 (1H, dd, J = 4.0 and 9.0 Hz, H-2′), 3.16 (1H, d, J = 5.5 Hz, H-3), 2.81 (1H, dd, J = 4.0 and 15.0 Hz, H-14), 2.45 (1H, dd, J = 10.5 and 15.0 Hz, H-14), 2.41–2.35 (1H, m, H-21), 2.14–2.07 (1H, m, H-20), 2.09 (3H, s, Ac), 2.03 (3H, s, Ac), 1.98 (3H, d, J = 1.0 Hz, H-18), 1.93–1.78 (5H, m, H-5, H-6, H-7, H-20 and H-21), 1.66 (3H, s, H-16 or H-17), 1.64–1.51 (2H, m, H-5 and H-6), 1.55 (1H, s, OH-1), 1.42–1.36 (1H, m, H-7), 1.09 (3H, s, H-16 or H-17), 1.02 (3H, s, H-19). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 177.6 (C, C-22), 171.2 (C, C-1′), 170.0 (C, Ac), 169.7 (C, Ac), 169.2 (C, Bz′), 167.4 (C, Bz), 139.7 (C, C-12), 138.2 (C, Ar), 136.1 (C, C-11), 133.9 (C, Ar), 133.9 (CH, Ar), 131.9 (CH, Ar), 129.8 (CH, Ar), 128.8 (C, Ar), 128.7 (CH, Ar), 128.6 (CH, Ar), 128.5 (CH, Ar),

127.6 (CH, Ar), 127.5 (CH, Ar), 127.1 (CH, Ar), 90.0 (C, C-4), 77.5 (C, C-1), 75.3 (CH, C-2), 75.1 (CH, C-9), 74.0 (CH, C-2′), 71.5 (CH, C-10), 71.1 (CH, C-13), 58.3 (CH, C-3′), 50.1 (CH, C-3), 43.9 (C, C-8), 42.1 (C, C-15), 41.5 (CH_2, C-5), 36.3 (CH_2, C-14), 30.8 (CH_2, C-7), 29.6 (CH_2, C-20), 29.3 (CH_3, C-16 or C-17), 28.8 (CH_2, C-21), 20.9 (CH_3, Ac), 20.7 (CH_3, Ac), 20.5 (CH_3, C-16 or C-17), 18.3 (CH_3, C-19), 18.2 (CH_2, C-6), 16.3 (CH_3, C-18). IR (film) ν_{max}: 3429, 3332, 2931, 2864, 1743, 1652, 1525, 1486, 1270, 1230. HRMS (ESI-TOF high acc) calcd for $C_{49}H_{56}O_{13}$ (MH^+): 866.3746, found: 866.3741. $[\alpha]_D^{20}$ +71° (*c* 0.2, $CHCl_3$).

Cells and cell culture

The human glioma cell line U251 was kindly donated by Dr Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The mouse L929 fibrosarcoma cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The tumor cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in a HEPES-buffered RPMI 1640 cell culture medium supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine and 1% antibiotic–antimycotic mixture (all from PAA Laboratories, Pasching, Austria). The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA and incubated in 96-well flat-bottom plates (1×10^4 cells per well) for the cell viability assessment, 24-well plates (5×10^4 cells per well) for the flow cytometric analysis, or 60 mm cell culture Petri dishes (1×10^6 cells) for the immunoblotting. Cells were rested for 24 h and then treated with compound **6** or paclitaxel as described in Results and the Figure legends.

Cell viability determination

The cell number, mitochondrial dehydrogenase activity and intracellular acid phosphatase activity, as markers of cell viability, were determined as previously described, using crystal violet, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and acid phosphatase assay.[41] The crystal violet, MTT and *p*-nitrophenol absorbance was measured in an automated microplate reader at 570 nm. The results were presented as a % of the control value obtained in untreated cells.

Flow cytometry

A FACSCalibur flow cytometer (BD, Heidelberg, Germany) and the Cell Quest Pro software (BD) were used for the flow cytometric analysis of the cell/size granularity, cell cycle phases, caspase activation and intracellular acidification. The extent of forward-scattering (FSC) and side-scattering (SSC) of the laser beam was used as a measure of cell size and granularity, respectively. Cell cycle was analyzed by measuring the amount of propidium iodide-labeled DNA in fixed cells, as previously described,[41] using a peak fluorescence gate to exclude cell aggregates. Activation of caspases was measured after labeling the cells with a cell-permeable, fluorescein isothiocyanate (FITC)-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The increase in intracellular acidification was determined as an increase in red/green fluorescence ratio (FL3/FL1) of a redox-sensitive dye acridine orange, as previously described.[36]

Immunoblotting

Western blotting followed by antibody-mediated detection of specific proteins was used to monitor autophagy. Cells were lysed in lysis buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail; all from Sigma-Aldrich, St. Louis, MO) on ice for 30 min, centrifuged at 14 000*g* for 15 min at 4 °C, and the supernatants were collected. Equal amounts of protein from each sample were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Marnes-la-Coquette, France). Following incubation with antibodies against microtubule-associated protein 1 light-chain 3 (LC3), p62, phospho-S6K, S6K or actin (Cell Signaling Technology, Beverly, MA) as primary antibodies and peroxidase-conjugated goat anti-rabbit IgG (Jackson IP Laboratories, West Grove, PA) as a secondary antibody, specific protein bands were visualized using enhanced chemiluminescence reagents for Western blot analysis (Amersham Pharmacia Biotech, Piscataway, NJ).

Transfection with shRNA

The shRNA targeting human LC3b gene, as well as scrambled control shRNA, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Subconfluent U251 cells were transfected with LC3b or control shRNA according to the manufacturer's protocol, using a shRNA plasmid transfection reagent and medium (Santa Cruz Biotechnology). The stably transfected cells were selected as recommended by the manufacturer and maintained in a selection medium containing puromycin (10 μg mL^{-1}).

Statistical analysis

The statistical significance of the differences was analyzed by *t*-test. A *p* value of less than 0.05 was considered statistically significant.

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