Bioorganic & Medicinal Chemistry 20 (2012) 5774–5786

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of novel ursolic acid heterocyclic derivatives with improved abilities of antiproliferation and induction of p53, p21^{waf1} and NOXA in pancreatic cancer cells

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ARTICLE INFO

Article history:
Received 5 June 2012
Revised 18 July 2012
Accepted 8 August 2012
Available online 16 August 2012

Keywords:
Ursolic acid
Heterocyclic derivatives
Antiproliferative
p53
NOXA

ABSTRACT

A series of new heterocyclic derivatives of ursolic acid **1** were synthesized and evaluated for their antiproliferative activity against AsPC-1 pancreatic cancer cells. Compounds **24–32**, with an α,β unsaturated ketone in conjugation with an heterocyclic ring in ring A have improved antiproliferative activities. Compound **32** is the most active compound with an IC_{50} of 1.9 μM which is sevenfold more active than ursolic acid **1**. Compound **32** arrests cell cycle in G1 phase and induces apoptosis in AsPC-1 cells with upregulation of p53, p21^{waf1} and NOXA protein levels.

1. Introduction

Ursolic acid **1** is a pentacyclic triterpenoid presenting in medicinal plants, vegetables and fruits.[1,2] Ursolic acid **1** has been reported to have antiproliferative effects,[3–8] to induce apoptosis,[9–11] and/or to arrest cell cycle in cancer cells.[12,13] However, the antitumor activity of ursolic acid **1** is poor. With the intent to improve the antitumor activity of ursolic acid **1**, chemical modifications have been made. The modifications performed so far are mostly on the alcohol group at position C_3 and on the carboxylic acid group at position C_{28}. Amides and esters are the most common semisynthetic derivatives of ursolic acid **1** and some derivatives present a better antiproliferative profile than ursolic acid **1**.[14–20]

Introduction of a heterocyclic ring(s) into triterpenoids has been performed in betulinic acid and betulin, affording several derivatives with better antiproliferative activities than parental compounds.[21–24] We introduced imidazole, methyl-imidazole or triazole as heterocyclic rings on several positions of ursolic acid **1** and synthesized a group of new *N*-alkylimidazoles and *N*-acylimidazoles. The antiproliferative effects of these new compounds were tested in AsPC-1, a pancreatic cancer cell line, and the structure–activity relationship (SAR) was analyzed. More active compounds were further tested for their antiproliferative activities in other cancer cell lines and the most active, compound **32**, was selected to explore the mechanism of action in AsPC-1 cancer cells.

2. Results and discussion

2.1. Chemistry

Within this work we successfully synthesized *N*-acylimidazoles **12–23** (Schemes 1 and 2), and *N*-alkylimidazoles **24–32** (Schemes 2 and 3). These compounds were prepared by the reaction of ursolic acid **1** or derivatives with the appropriate heterocyclic reagent, 1,1′-carbonyldiimidazole (CDI), 1,1′-carbonylbis(2′-methylimidazole) (CBMI) or 1,1′-carbonyl-di(1,2,4-triazole) (CDT), in reflux of THF under inert atmosphere, the best found reaction conditions. The synthesis of *N*-acylimidazoles and *N*-alkylimidazoles was dependent on the nature of the reacting group, alcohol type or acid, and the reaction conditions, which in this work were maintained for several derivatives.[25–28]

The biological activity of triterpenoid compounds could benefit by the presence of a carbonyl group in ring C. In ursolic acid **1** the presence of a methyl group at position C_{19} increases the steric hindrance around position C_{12} and renders the functionalization of

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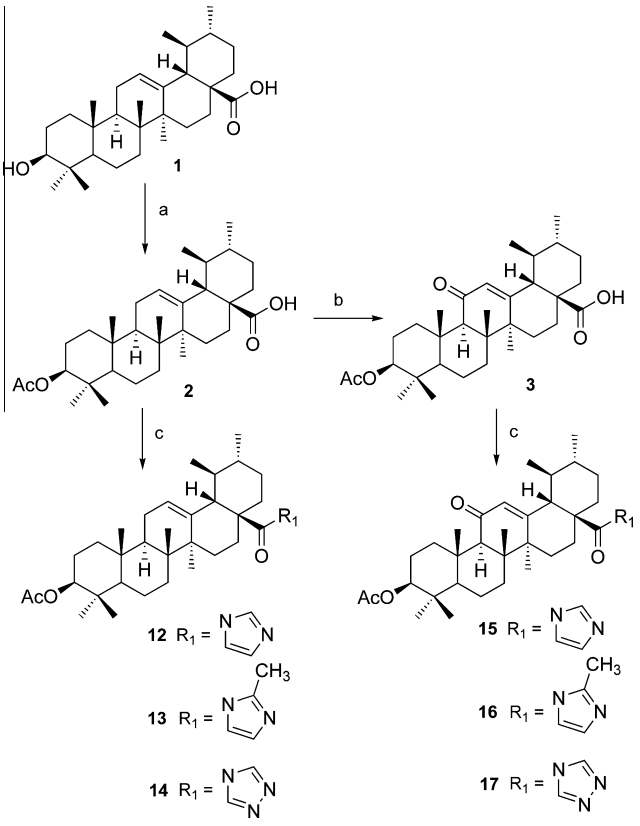
http://dx.doi.org/10.1016/j.bmc.2012.08.010



Scheme 1. Reagents: (a) Acetic anhydride, DMAP, THF, rt; (b) $KMnO_4$, $Fe_2(SO_4)_3{\cdot}nH_2O$, *t*-BuOH, H_2O, CH_2Cl_2; (c) CDI, CBMI or CDT, THF, 70 °C, N_2.

Δ^{12} moiety in ring C more difficult.[29] The reaction of compounds **2**, **4** and **8** with a mixture of potassium permanganate and iron sulfate ($Fe_2(SO_4)_3{\cdot}nH_2O$) affords compounds **3**, **5** and **9**, respectively, with a carbonyl group at C_{11} (Schemes 1–3). This oxidative mixture was previously reported for the formation of epoxides in unsaturated steroids,[30] although in ursane triterpenoids the reaction affords an α,β-unsaturated ketone in ring C, with yields higher than 80%. The presence of the α,β unsaturated carbonyl group in ring C is confirmed by the presence of a band around 1700 cm^{-1} on the IR spectra characteristic of the carbonyl group at the α,β unsaturated ketone moiety, by the presence of the δ for proton H_{12}, approximately at 5.6 ppm in ^1H NMR spectrum, slightly higher than in ursane compounds without the carbonyl group at C_{11}, and through the presence of an δ around 199 ppm for a carbonyl group in the ^{13}C NMR spectrum. In compounds with the presence of a carbonyl group at C_{11} the δ signals for carbons C_{12} and C_{13} in ^{13}C NMR spectrum are higher than in compounds without the carbonyl group at C_{11}.

The reaction of ursolic acid derivatives **2**–**5** with CDI, CMBI or CDT gives amides **12–23** (Schemes 1 and 2). The presence of the amide functional group at C_{28} is confirmed by the presence of a band at 1680 cm^{-1} in IR spectrum correspondent to the carbonyl group, different from the usual value for free acids, 1710 cm^{-1}, and through the presence of a δ signal around 178 ppm in the ^{13}C NMR spectrum, a lower value than the usually registered for a free acid, δ of 183 ppm.

N-Alkylimidazoles derivatives **24–32** (Schemes 2 and 3), were prepared by reaction of the vinyl alcohol at C_2, previously introduced in compounds **6**, **10** and **11**, in which the hydroxyl group was replaced by an heterocyclic ring. The introduction of the vinyl alcohol at position C_2 was previously described (Schemes 2 and 3).[31] Compounds **24–32** were prepared with the intent of exploring the influence of the heterocyclic ring on the biological activity of compounds bearing the α,β unsaturated ketone moiety.

The successful preparation of *N*-alkylimidazoles **24–32**, was confirmed by the presence of a δ signal around 7.8–7.7 ppm correspondent to the proton of the exocyclic double bond at C_2 in the ^1H NMR spectrum. On ^{13}C NMR spectrum the presence of this functionality is confirmed by the δ signal around 131 ppm, the δ signal for carbon C_2 shows around 123 ppm.

Full structural elucidation of the new semisynthetic ursane derivatives was made by means of infra-red spectroscopy (IR), 1D and 2D nuclear magnetic resonance (NMR), and mass spectrometry (MS). The presence of the heterocyclic ring can be detected by the presence of the extra δ signals in ^1H and ^{13}C NMR spectra. The imidazole group has three specific protons that appear on the ^1H NMR spectrum at δ values higher than 7 ppm, the methyl-imidazole group has only two protons on the ^1H NMR spectrum ranging from δ 6.7 to 7.4 ppm, and the triazole group has two proton signals with δ higher than 8 ppm on the ^1H NMR spectrum. In the ^{13}C NMR spectrum the δ signal of the carbons for the heterocyclic ring are present for values higher than 130 ppm, varying in accordance with the different heterocyclic rings.

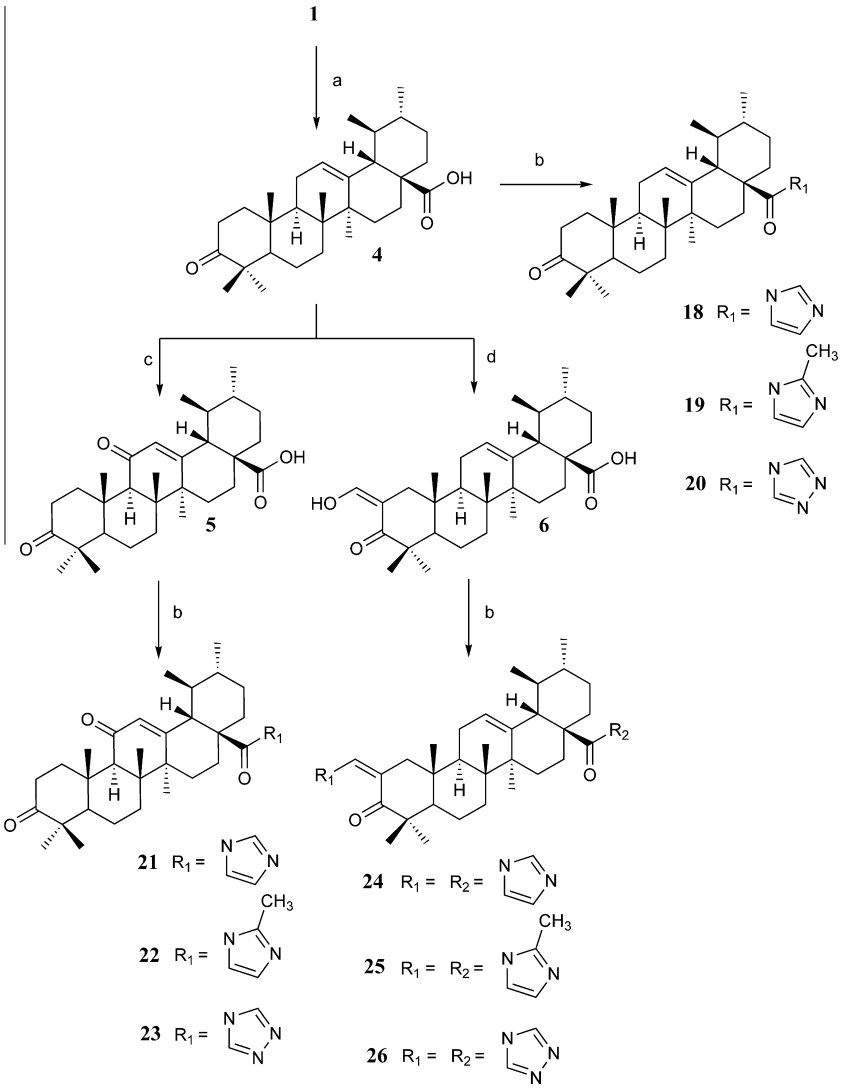
For compound **31** the correlations in HMBC allowed the attribution of several signals in the ^1H and ^{13}C NMR spectra (Fig. 1). Carbon C_1 correlates with the proton of the exocyclic double bond, and this carbon with proton H_5' of the methyl-imidazole ring. Proton of the H_4' of the heterocyclic ring correlates with the C_2' of the same ring, allowing the identification of all the signals in the methyl-imidazole ring. Proton H_{18} of the ursane structure correlates with carbon C_{19}, allowing the identification of C_{19} and of the methyl group attached (Fig. 1). This same rational was used for other compounds whose results are expressed in the experimental section.

2.2. Biological activity

The antiproliferative activities of these new semisynthetic compounds were determined using the MTT assay and evaluated based on the values of IC_{50} (μM) against AsPC-1 cells, a pancreatic cancer cell line (Table 1). Overall these new compounds have better antiproliferative activities than the intermediates and ursolic acid **1** in AsPC-1 cells. Compounds **24–32** with a heterocyclic ring conjugated with an α,β unsaturated ketone in ring A except **25** shown better antiproliferative activities than compounds **12–23** with a heterocyclic ring at C28 and a ketone or an acetyl ester in ring A except **22** (Table 1).

Based on the antiproliferative activities against AsPC-1 cells SAR was established for ursolic acid **1** and compounds **6**, **10**, **11** and **24–32** with an α,β unsaturated ketone in ring A (Fig. 2). Compounds **27**, **28**, **31** and **32** have the best antiproliferative activities against AsPC-1 cells. The heterocyclic ring present at C_2 position influences the antiproliferative activities, depending on the initial scaffold. Triazole substituent with an α,β unsaturated ketone in ring A appears to generate the most active compounds. Compound **32** is the most active compound. It is sevenfold more potent than ursolic acid **1**, and fourfold more potent than compound **11** (Fig. 2).

Compounds **27–32** were further studied in other pancreatic (PANC-1 and MIA PaCa-2), hepatic (Hep G2), breast (MCF7), lung (A549) and prostate (PC-3) cancer cell lines (Table 2). *N*-Alkyltriazole **32** presented consistent better antiproliferative activity in all tested cell lines, with IC_{50} values ranging from 1.9 to 4.9 μM. The presence of the *N*-alkyltriazole in ring A conjugated with an α,β unsaturated ketone affords a better Michael acceptor in compound **32** and allows better interaction with the potential target proteins.[32] This could explain the observed differences in the antiproliferative values of compounds with different substituent at C_2.

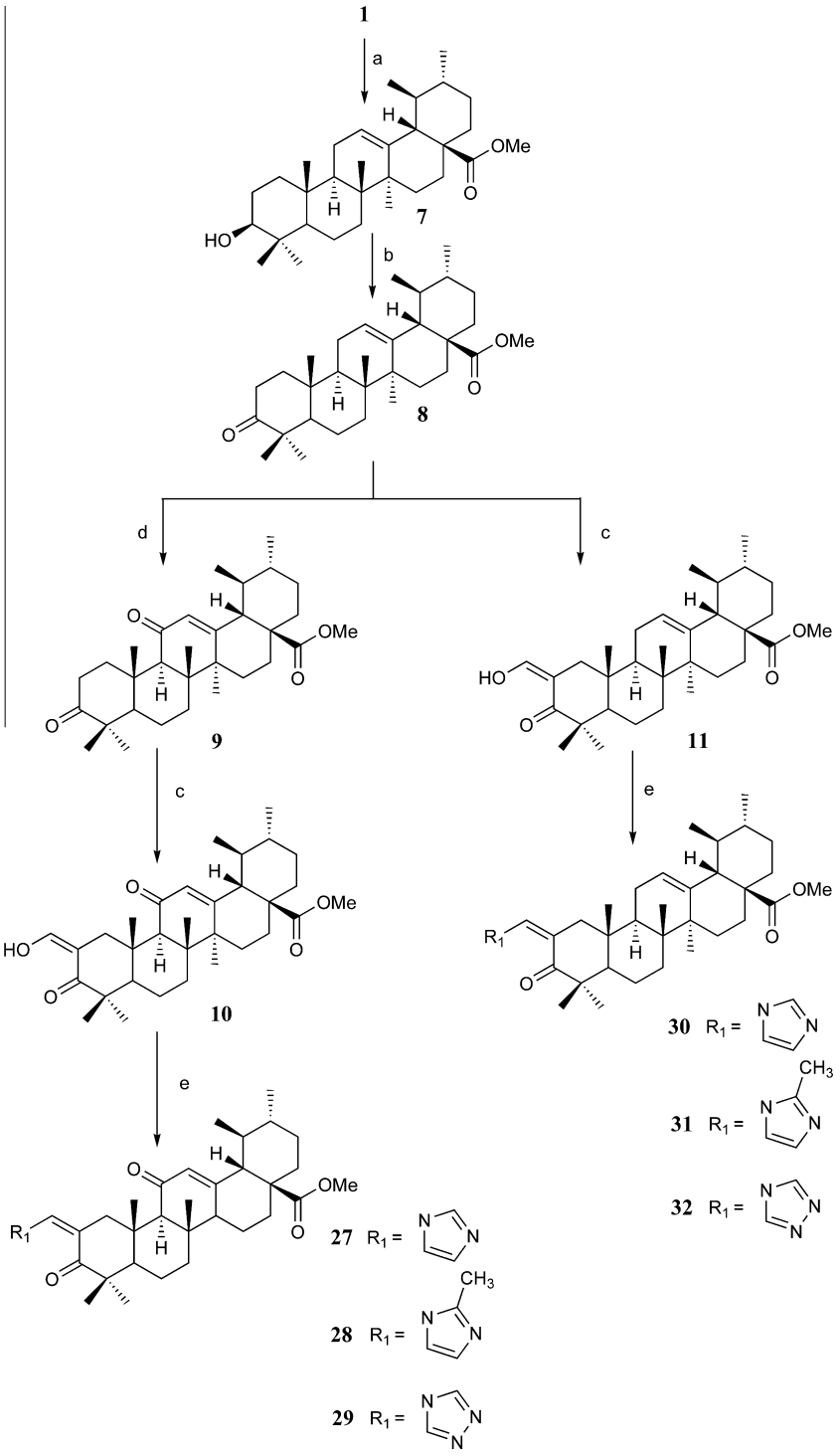


Scheme 2. Reagents: (a) Jones reagent, acetone, ice; (b) CDI, CBMI or CDT, THF, 70 °C, N_2; (c) $KMnO_4$, $Fe_2(SO_4)_3{\cdot}nH_2O$, *t*-BuOH, H_2O, CH_2Cl_2; (d) Ethyl formate, benzene, NaOMe, rt.

Pancreatic cancer AsPC-1 cells are the most susceptible to these compounds and compound **32** is the most active one. AsPC-1 cells were used to study the underlying antiproliferative mechanisms of compound **32**. AsPC-1 cells were treated with 2–10 μM of compound **32** for 24 h. Compound **32** induced cell cycle arrest at G1 phase at the concentration of 6 μM and the population of cells at sub-G1 phase was increased after treatment with compound **32** at higher concentrations (Fig. 3A and B), implying that the antiproliferative effects of this compound are due to cell cycle arrest and induction of apoptosis. The levels of a few of cell cycle and apoptosis related proteins were determined (Fig. 4). $p21^{waf1}$ is a cell cycle regulator of G1 phase which is not expressed in pancreatic cancer cells.[33] NOXA is a BH3 protein responsible for the regulation of antiapoptotic protein Mcl-1. The levels of $p21^{waf1}$ and NOXA were increased significantly in AsPC-1 cells treated with compound **32** at concentrations higher than 6 μM. Unlike other triterpenoid derivatives which decreased the levels of Mcl-1,[34] compound **32** did not decrease the levels of Mcl-1. This result suggests that this compound has a new mechanism of action. The apoptosis induction may rely on the induction of NOXA which antagonize the function of Mcl-1 and then lead to apoptosis via activation of caspases 9 and 3. NOXA and $p21^{waf1}$ are regulated by p53 and compound **32** increased the levels of p53 protein. The role of p53 in compound **32**-mediated cell cycle arrest and apoptosis induction as well as the mechanism of compound **32** to increase p53 level need to be studied further.

3. Conclusions

In summary, a series of new *N*-acylimidazoles and *N*-alkylimidazoles ursolic acid derivatives with the introduction of heterocyclic rings of imidazole, methyl-imidazole or triazole in different positions of the ursane backbone were synthesized. These new semisynthetic derivatives have improved antiproliferative activities over ursolic acid **1** in AsPC-1 cells. Compounds **27**, **28, 31** and **32** with the introduction of a heterocyclic ring in conjugation with an α,β unsaturated ketone in ring A of ursane skeleton were more active in inhibiting AsPC-1 cell growth. Compound **32** is the most active one with induction of p53, $p21^{waf1}$ and NOXA which leads to cell cycle arrest and apoptosis of AsPC-1 pancreatic cancer cells. Compounds with an α,β unsaturated ketone conjugated with an heterocyclic ring in ring A represent a new group of ursane derivatives as good starting points for drug discovery in cancer.



Scheme 3. Reagents: (a) CH_3I, K_2CO_3, DMF, rt, N_2; (b) Jones reagent, acetone, ice; (c) Ethyl formate, benzene, NaOMe, rt; (d) $KMnO_4$, $Fe_2(SO_4)_3{\cdot}nH_2O$, *t*-BuOH, H_2O, CH_2Cl_2; (e) CDI, CBMI or CDT, THF, 70 °C, N_2.

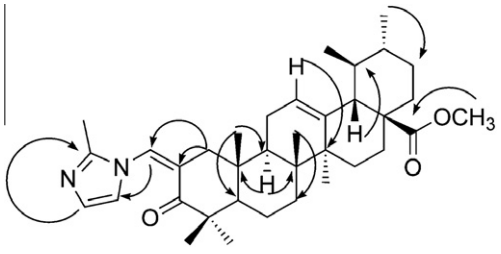


Figure 1. Selected HMBC correlations for compound **31**.

4. Experimental

4.1. Chemistry

IR spectra were recorded in JASCO FT/IR-420. ^1H, ^{13}C, 135 DEPT, HMQC, HMBC, COESY and NOESY spectra were recorded in a Bruker Avance III 400 MHz spectrometer. The chemical shifts were recorded in δ (ppm) using the δ 7.26 of $CHCl_3$ (^1H NMR) and the δ 77.00 (^{13}C NMR) as internal standards. Chemical shifts measures

Table 1
The IC_{50} (μM) of ursolic acid heterocyclic derivatives to inhibit AsPC-1 cell growth.

Compd	AsPC-1[a]	Compd	AsPC-1[a]
1	12.6 ± 0.03	**18**	15.2 ± 0.1
2	71.0 ± 0.09	**19**	19.6 ± 0.02
3	33.5 ± 0.05	**20**	19.2 ± 0.04
4	24.5 ± 0.1	**21**	11.7 ± 0.05
5	54.9 ± 0.03	**22**	5.1 ± 0.03
6	13.4 ± 0.03	**23**	9.9 ± 0.03
7	17.7 ± 0.06	**24**	5.1 ± 0.02
10	9.0 ± 0.04	**25**	11.3 ± 0.02
11	7.2 ± 0.02	**26**	3.9 ± 0.04
12	15.2 ± 0.03	**27**	2.3 ± 0.02
13	20.2 ± 0.06	**28**	2.1 ± 0.01
14	23.3 ± 0.05	**29**	8.1 ± 0.02
15	13.9 ± 0.04	**30**	5.8 ± 0.05
16	15.3 ± 0.06	**31**	2.1 ± 0.02
17	10.2 ± 0.02	**32**	1.9 ± 0.02

[a] AsPC-1 cells were treated with the indicated compounds with a variety of concentrations for 72 h. The antiproliferative effects were determined by MTT assay and the IC_{50} was calculated. The results shown are of means ± SE of three independent experiments.

were given in ppm and coupling constants (*J*) in hertz (Hz). Low resolution mass spectrometry was obtained in a Finnigan Polaris QGC/MS Benchtop Ion Trap spectrometer with a direct insertion probe. Melting points were determined using a BUCHI melting point B-540 apparatus and were uncorrected. For thin layer chromatography (TLC) analysis Kiesel gel 60HF254/kiesel gel 60G was used and FCC was performed using Kieselgel 60 (230–400 mesh, Merck). Ursolic acid **1**, dimethylaminopyridine (DMAP), acetic anhydride, ethyl formate, sodium methoxide, THF, dimethylformamide (DMF), benzene, CDI, CBMI and CDT were purchased from Sigma Aldrich Co. The solvents used in the work ups were purchase from VWR Portugal, and were of analytical grade. Potassium bicarbonate, sodium chloride, sodium bicarbonate, sodium sulfite were purchased from Merck Co. All the solvents used in the reactions were previously purified and dried according to the literature procedures.

The purities of all compounds were determinate to be great than 95% by NMR and elemental analysis.

4.1.1. 3β-Acetoxy-urs-12-en-28-oic acid (2)

Preparation of **2** was made according to previously described method,[19] providing a solid (92%). Mp 287.4–290.8 °C. IR (film $CHCl_3$): 2926.5, 1735.6, 1691.3 1460.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.21 (1H t *J* = 6.54), 4.47 (1H dd *J* = 15.99), 2.16 (1H d *J* = 11.51), 2.02 (3H s), 1.05 (3H s), 0.94 (3H s), 0.84 (6H s), 0.83 (3H s), 0.74 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 183.84 (C28), 171.03 ($OCOCH_3$), 137.92 (C13), 125.71 (C12), 80.92 (C3). EI-MS *m*/*z*: 497.82 (1) M^+, 437.89 (23), 93.76 (37), 91.78 (50), 89.79 (100), 81.87 (22), 79.88 (83), 77.9 (73), 75.89 (34), 67.87 (23), 65.86 (62). Anal. Calcd for $C_{32}H_{50}O_4$: C, 77.06; H 10.10. Found: C, 77.46; H 10.05.

4.1.2. 3β-Acetoxy-11-oxours-12-en-28-oic acid (3)

To a stirred solution of **2** (500 mg 1 mmol) in dichloromethane (15 mL) at room temperature was added a mixture of $KMnO_4$ (2 g) and $Fe_2(SO_4)_3{\cdot}nH_2O$ (1 g), previous reduced to fine powder, water (0.1 mL) and *t*-butanol (0.46 mL). After 23 h the reaction mixture was diluted with ether (60 mL) and mixed for 30 min. The resulting mixture is filtered through a celite plate, which is washed with ether (100 ml). The resulting organic phase was washed with $NaHCO_3$ (2 × 100 mL) and water (2 × 100 mL), dried over Na_2SO_4, filtered and evaporated to the dryness, to afford a white powder (83%). Mp 315.7–318.1 °C. IR (film $CHCl_3$): 2941.9, 2873.4, 1725.0, 1690.3, 1661.4, 1459.9, 1367.3, 1318.1, 1252.5, 1214.0, 1167.7, 1141.7 cm^{-1}. EI-MS *m*/*z*: 513.30 (15) M^+, 303.10 (73), 262.09 (63), 257.17 (68), 134.19 (80), 189.27 (100), 175.30 (63), 174.33 (63), 162.29 (46), 161.28 (99), 120.08 (47), 119.21 (87), 105.28 (53), 91.17 (45), 79.27 (45).

4.1.3. 3-Oxours-12-en-28-oic acid (4)

Compound **4** was prepared according to the literature,[35] from **1** to give a solid (99%). Mp 281.5–286.8 °C. IR (film $CHCl_3$): 2972.7, 2929.3, 2872.4, 1690.3, 1457.9, 1385.6, 1316.2, 1276.7, 1255.4, 1235.2, 1214.0 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.26 (1H t), 1.08 (3H s), 1.08 (3H s), 1.05 (3H s), 1.02 (3H s), 0.95 (3H s), 0.87 (3H s), 0.82 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 217.89 (C3), 183.92 (C28), 137.98 (C13), 125.53 (C12). EI-MS *m*/*z*: 455.3 (2) M^{+1}, 249.3 (20), 248.3 (100), 219.4 (22), 205.5 (20), 204.5 (22), 203.5 (55), 134.3 (22), 133.3 (44). Anal. Calcd for $C_{30}H_{46}O_3$ C, 79.25; H, 10.20. Found C, 78.98; H 10.34.

4.1.4. 3,11-Dioxours-12-en-28-oic acid (5)

Compound **5** was prepared using the same method as for the preparation of **3**, starting with **4** (71%). Mp 285.8–300.4 °C. IR (film $CHCl_3$): 2945.7, 2873.4, 1700.9, 1691.3, 1661.4, 1460.8, 1385.6, 1312.3, 1276.7, 1255.4, 1231.3, 1212.0 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.62 (1H s), 1.30 (3H s), 1.24 (3H s), 1.08 (3H s), 1.02 (3H s), 0.97 (3H d *J* = 6.14), 0.94 (3H s), 0.86 (3H d *J* = 6.25). 13C NMR (100 MHz $CDCl_3$): δ 217.11 (C3), 199.26 (C11), 182.65 (C28), 163.12 (C13), 130.66 (C12), 60.67, 55.31, 52.49, 47.66, 47.48, 44.50, 43.79, 39.70, 38.55, 38.52, 36.72, 35.97, 34.14, 32.34, 30.21, 28.42, 26.44, 23.59, 21.32, 20.99, 20.91, 18.92, 18.65, 16.99, 15.57. EI-MS *m*/*z*: 469.13 (14) M^+, 303.04 (49), 262.08 (37), 257.13 (66), 134.04 (56), 190.09 (31), 189.06 (84), 175.14 (33), 161.13 (100), 119.19 (79), 105.14 (43), 95.19 (34), 91.04 (45), 79.11 (40), 67.07 (34), 55.04 (32).

4.1.5. 2-Hydroxymethylene-3-oxours-12-en-28-oic acid (6)

Preparation of **6** was made according to previously described method,[36,37] providing a solid (96%). Mp 148.0–150.4 °C. IR (film $CHCl_3$): 3440.4, 2925.5, 2872.5, 1694.2, 1640.2, 1580.4, 1456.0, 1379.8, 1314.3, 1234.2 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 14.89 (1H s), 8.58 (1H s), 5.29 (1H s), 1.18 (3H s), 1.09 (3H s), 1.08 (3H s), 0.95 (3H d *J* = 5.93), 0.91 (3H), 0.86 (3H t *J* = 12.40), 0.82 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 190.60 (C3), 188.43, 184.01 (C28), 137.94 (C13), 125.56 (C12), 105.72 (C2). EI-MS *m*/*z*: 482.57 (7) M^+, 202.50 (25), 172.57 (12), 146.63 (13), 144.66 (13), 133.72 (12), 132.72 (100), 130.76 (13), 120.70 (13), 118.75 (29), 116.72 (11), 106.69 (17), 104.71 (28), 94.65 (14), 92.62 (28). Anal. Calcd for $C_{31}H_{46}O_4.0.25H_2O$ C, 76.42; H, 9.62. Found: C, 76.18; H, 10.02.

4.1.6. Methyl 3β-hydroxy-urs-12-en-28-oate (7)

Compound **7** was prepared according to the literature[22,38] from **1** to give a solid (93%). Mp 170.7–172.0 °C. IR (film $CHCl_3$): 3434.6, 2926.5, 1718.3, 1645.9, 1457.0 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.23 (1H t *J* = 7.32 H12), 3.59 (3H s $COOCH_3$), 3.20 (1H dd *J* = 15.83 H3), 1.07 (3H s), 0.98 (3H s), 0.93 (3H s), 0.91 (3H s), 0.85 (3H d *J* = 6.48), 0.77 (3H s), 0.73 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 178.05 (C28), 138.12 (C13), 125.55 (C12), 79.03 (C3). EI-MS *m*/*z*: 471.04 (7) M^{+1}, 261.99 (53), 207.08 (21), 203.07 (89), 202.15 (20), 189.19 (28), 134.11 (38), 133.21 (100), 119.13 (26), 91.22 (21). Anal. Calcd for $C_{31}H_{50}O_3$: C, 79.30; H, 10.95.0.25 hexane. Found: C, 79.68; H, 11.31.

4.1.7. Methyl 3-oxours-12-en-28-oate (8)

Compound **8** was prepared according to the literature,[35] from **7** to give a solid (93%). Mp 196.3–197.2 °C. IR (film $CHCl_3$): 2946.7, 2872.5, 1725.0, 1704.8, 1456.0, 1384.6, 1309.4, 1271.8, 1230.4, 1198.5, 1143.6, 1111.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.26 (1H s), 3.61 (3H s), 1.08 (6H s), 1.04 (6H s), 0.94 (3H d *J* = 5.73), 0.86 (3H d *J* = 6.33), 0.79 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ

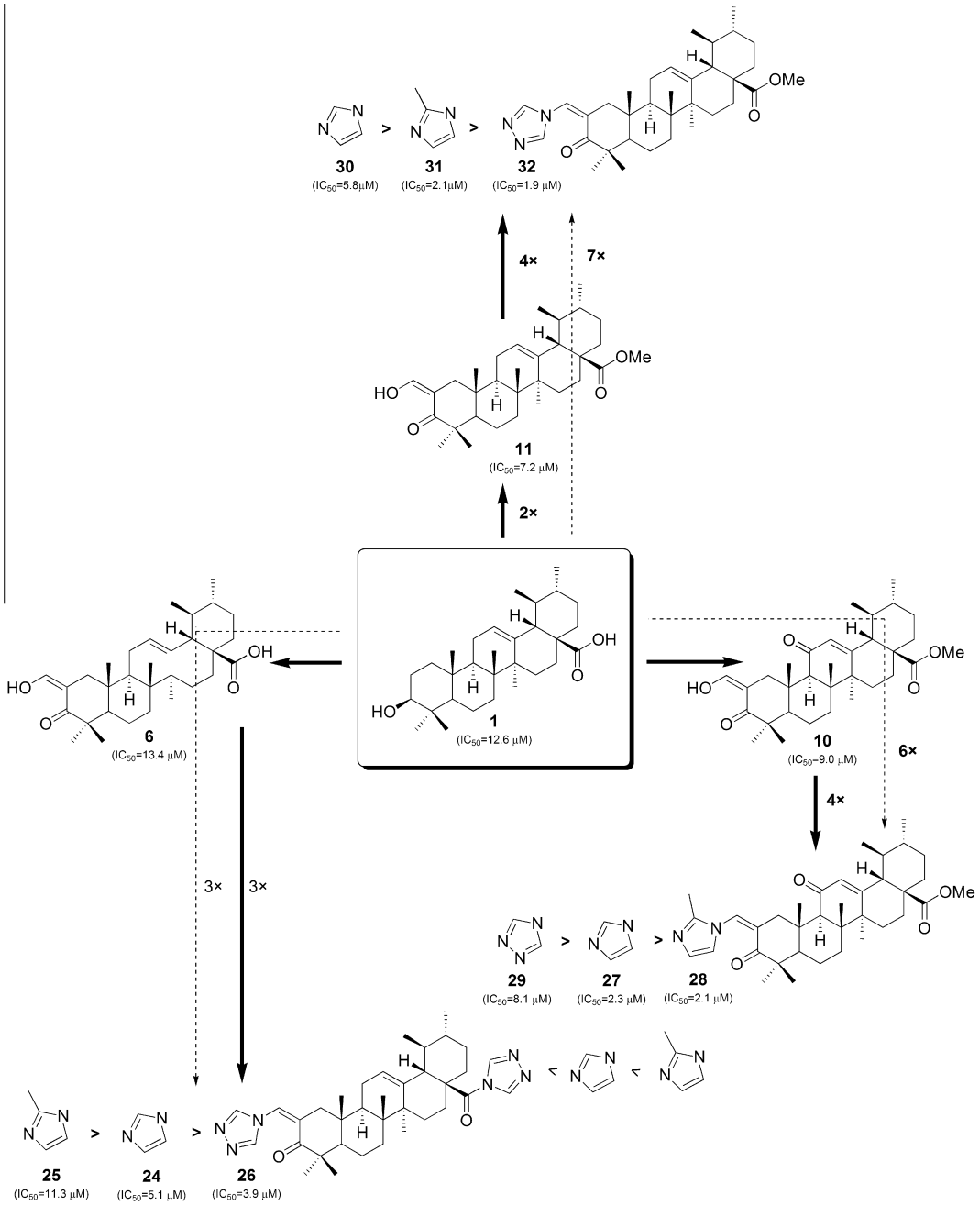


Figure 2. Graphical presentation of the SAR relationship among ursolic acid **1** and novel derivatives with a modification at C_2 of the ursane structure. Comparisons were based on the IC_{50} of each compound to inhibit AsPC-1 cell growth. Compound **32** is the most active one with fourfold more active than compound **11** and sevenfold more active than ursolic acid **1**. Compound **28** is fourfold more active than compound **10** and sixfold more active than ursolic acid **1**. Compound **26** is threefold more active than compound **6** and ursolic acid **1**.

Table 2
The IC_{50} (μM) of ursolic acid heterocyclic derivatives to inhibit growth of pancreatic (PANC-1 and MIA PaCa-2), breast (MCF7), prostate (PC-3), hepatic (Hep G2) and lung (A549) cancer cell lines.

Compd	PANC-1[a]	MIA PaCa-2[a]	Hep G2[a]	MCF7[a]	A549[a]	PC-3[a]
1	14.9 ± 0.04	10.4 ± 1.0	15.0 ± 0.09	12.3 ± 0.02	11.4 ± 0.03	20.8 ± 0.01
27	18.2 ± 0.06	24.3 ± 0.03	27.6 ± 0.05	9.4 ± 0.05	12.2 ± 0.08	12.9 ± 0.05
28	14.4 ± 0.04	8.3 ± 0.02	8.5 ± 0.06	3.5 ± 0.05	18.7 ± 0.03	5.7 ± 0.02
29	12.1 ± 0.2	7.8 ± 0.03	7.9 ± 0.03	12.5 ± 0.06	12.9 ± 0.02	9.5 ± 0.03
30	5.1 ± 0.04	7.3 ± 0.04	2.0 ± 0.03	5.3 ± 0.02	5.6 ± 0.05	6.8 ± 0.02
31	5.6 ± 0.04	5.6 ± 0.01	4.0 ± 0.01	2.2 ± 0.03	5.3 ± 0.02	3.5 ± 0.02
32	3.5 ± 0.03	4.0 ± 0.05	3.2 ± 0.03	2.3 ± 0.02	4.9 ± 0.01	3.0 ± 0.01

[a] PANC-1, MIA PaCa 2, MCF7, PC-3, Hep G2 and A549 cells were treated with the indicated compounds with a variety of concentrations for 72 h. The antiproliferative effects were determined by MTT assay and the IC_{50} was calculated. The results shown are means ± SE of three independent experiments.

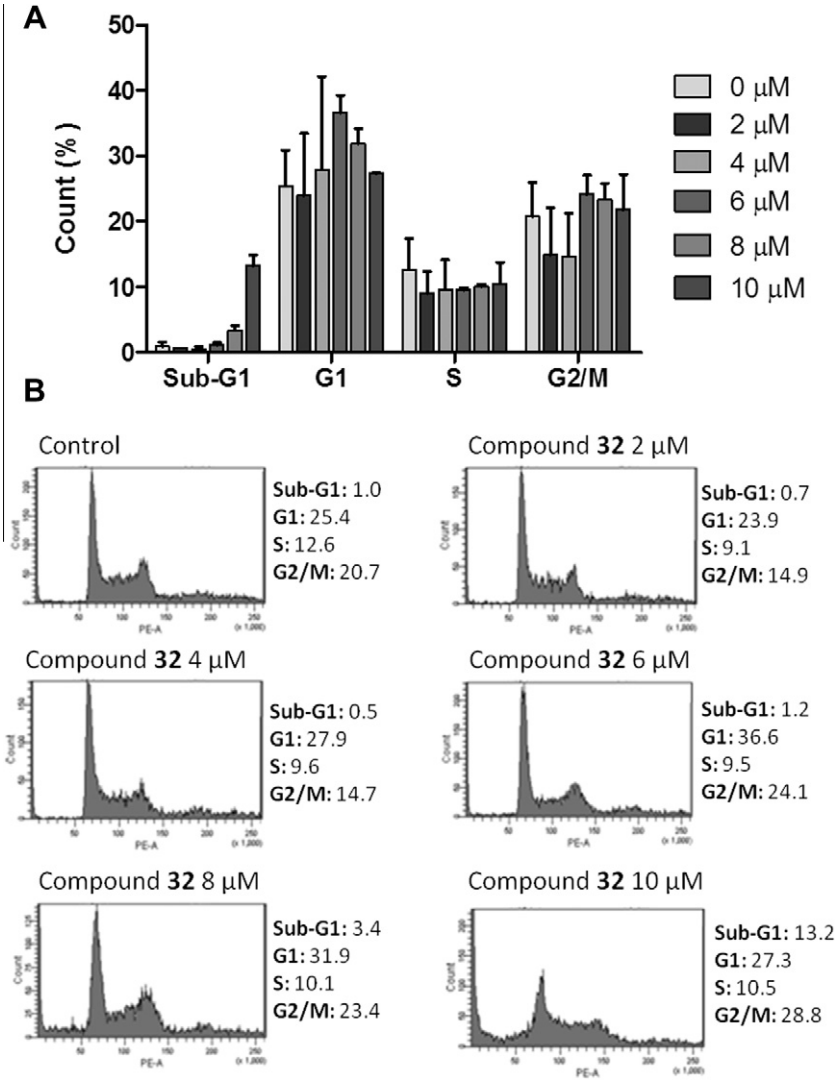


Figure 3. Cell cycle arrest and apoptosis induction of compound **32** in AsPC-1 cells. (A) Cell cycle analysis of AsPC-1 cells treated with compound **32**. The results shown are means ± SE of three independent experiments. (B) Representative FACS analyses of cell cycle of AsPC-1 cells treated with compound **32** at the indicated concentrations for 24 h. The sub-G1 phase (apoptotic cells) of AsPC-1 cells were detected only in cells treated with compound **32** at 10 μM.

217.70 (C3), 177.99 (C28), 138.29 (C13), 125.33 (C12). EI-MS *m*/*z*: 470.48 (27) M^+, 469.47 (94), 264.09 (51), 262.48 (100), 248.92 (16), 205.74 (18), 204.63 (19), 203.81 (25), 203.20 (16), 202.52 (26), 201.52 (23), 200.44 (18), 189.08 (16), 134.34 (25), 133.12 (64), 131.30 (16).

4.1.8. Methyl 3,11-dioxours-12-en-28-oate (9)

Compound **9** was prepared using the same method as for the preparation of **5**, with the obtention of a solid (98%). Mp 128.5–130.7 °C. IR (film $CHCl_3$): 2948.6, 2872.5, 1725.0, 1704.8, 1660.4, 1457.0, 1386.6, 1273.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 5.63 (1H s), 3.60 (3H s), 1.29 (3H s), 1.24 (3H s), 1.08 (3H s), 1.04 (3H s), 0.96 (3H d J = 6.53), 0.93 (3H s), 0.86 (3H d J = 5.82). 13C NMR (100 MHz $CDCl_3$): δ 217.12 (C3), 199.06 (C11), 177.13 (C28), 163.26 (C13), 130.55 (C12). EI-MS *m*/*z*: 483.38 (28) M^+, 482.35 (28), 467.36 (37), 454.38 (27), 317.32 (99), 276.37 (34), 258.45 (31), 257.4 (100), 248.49 (60), 233.5 (38), 190.47 (34), 189.42 (78), 162.45 (41), 161.36 (58), 119.33 (46).

4.1.9. Methyl 2-hydroxymethylene-3,11-dioxours-12-en-28-oate (10)

Compound **10** was prepared using the same method as for the preparation of **6**,[36] starting with **9** (95%). Mp 134.7–138.1 °C. IR (film $CHCl_3$): 3436.5, 2949.6, 2870.5, 1726.0, 1656.6, 1588.1, 1457.0, 1387.5, 1320.0, 1272.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 14.86 (1H s), 8.62 (1H s), 5.67 (1H s), 3.62 (3H s), 3.45 (1H d J = 14.76), 2.45 (1H d J = 11.19), 1.31 (3H s), 1.19 (3H s), 1.13 (3H s), 1.11 (3H s), 0.97 (6H d J = 8.98), 0.88 (3H d J = 6.40). 13C NMR (100 MHz $CDCl_3$): δ 199.20 (C3), 189.57 (C11), 188.82, 177.15 (C28), 163.48 (C13), 130.68 (C12), 105.79 (C2). EI-MS *m*/*z*: 510.28 (11) M^+, 495.32 (18), 317.15 (52), 258.17 (20), 257.14 (100), 189.16 (22), 187.19 (22), 175.18 (16), 173.19 (18), 161.18 (96), 135.20 (25), 119.20 (25), 105.18 (18), 91.15 (21). Anal. Calcd for $C_{32}H_{46}O_5.0.5H_2O$ C, 73.95; H, 9.12. Found: C, 74.1; H, 9.52.

4.1.10. Methyl 2-hydroxymethylene-3-oxours-12-en-28-oate (11)

Compound **11** was prepared using the same method as for the preparation of **6**, with the obtention of a solid (97%). Mp 127.9–131.7 °C. IR (film $CHCl_3$): 3441.3, 2924.5, 2872.5, 1725.0, 1634.4, 1586.2, 1456.0, 1377.9, 1307.5, 1271.8, 1229.4, 1200.5, 1143.6 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 14.90 (1H s), 8.57 (1H s), 5.30 (1H s), 3.61 (3H s), 1.19 (3H s), 1.11 (3H s), 1.09 (3H s), 0.94 (3H d J = 5.98), 0.91 (3H s), 0.87 (3H d J = 6.39), 0.81 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 190.84 (C3), 188.17, 178.00 (C28), 138.23 (C13), 125.35 (C12), 105.79 (C2). EI-MS *m*/*z*: 497.40 (30) M^+,

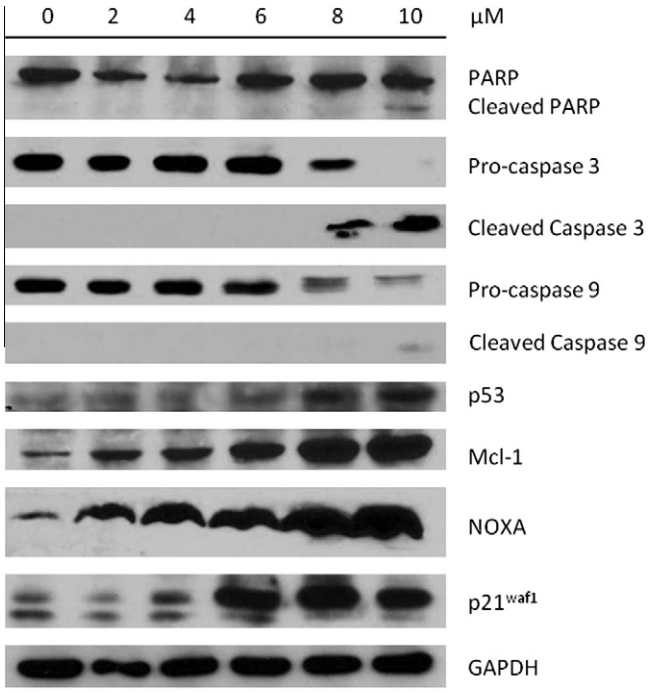


Figure 4. Compound **32** increased the levels of $p21^{waf1}$, NOXA and p53 protein in AsPC-1 cells. AsPC-1 cells were treated with compound **32** at the indicated concentrations for 24 h. The relative levels of indicated proteins were determined by Western blot analysis. GAPDH was used as a loading control.

263.63 (40), 262.47 (99), 233.44 (52), 204.71 (31), 203.41 (95), 189.41 (45), 187.33 (25), 134.35 (48), 133.23 (100), 119.23 (27). Anal. Calcd for $C_{32}H_{48}O_4.0.25H_2O$ C, 76.68; H, 9.75. Found: C, 76.63; H, 10.20.

4.1.11. 3β-Acetoxy-urs-12-en-28-yl-1*H*-imidazole-1-carboxylate (12)

To a stirred solution of **2** (300 mg 0.60 mmol) in THF (4 mL), under N_2 atmosphere at 70 °C, was added CDI (194.58 mg 1.2 mmol). After 10 h the reaction mixture was diluted with water (60 mL), the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The resulting organic phases were washed with NaCl 10% (3 × 50 mL), dried over Na_2SO_4, filtered and evaporated to the dryness, to afford a yellow solid. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (75:25) to (70:30)], to afford **12** (88%). Mp 204.0–207.5 °C. IR (film $CHCl_3$): 3151.1, 2927.4, 2873.4, 1725.0, 1466.6, 1368.3, 1275.7, 1246.8, 1205.3, 1101.2 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.22 (1H s), 7.51 (1H s), 7.01 (1H s), 5.20 (1H s), 4.46 (1H t J = 15.70), 2.43 (1H d J = 11.03), 2.02 (3H s), 1.07 (3H s), 0.98 (3H d J = 6.17), 0.90 (6H s), 0.82 (3H s), 0.81 (3H s), 0.65 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 174.67, 170.88, 137.02, 136.92, 129.57, 126.48, 117.41, 80.74, 55.22, 54.10, 50.86, 47.33, 42.03, 39.39, 39.19, 38.64, 38.23, 37.57, 36.75, 35.54, 32.68, 30.33, 27.97, 27.68, 24.98, 23.52, 23.44, 23.24, 21.22, 20.98, 18.01, 17.06, 16.69, 16.63, 15.44. EI-MS m/z: 548.91 (14) M^+, 248.95 (30), 203.01 (45), 189.06 (55), 187.09 (22), 147.1 (23), 133.11 (36), 119.12 (32), 107.12 (34), 105.15 (34), 95.16 (29), 91.15 (33), 79.14 (32), 69.15 (100). Anal. Calcd for $C_{35}H_{52}N_2O_3$ C, 76.60; H, 9.55; N, 5.10. Found: C, 76.38; H, 9.74; N, 5.09.

4.1.12. 3β-Acetoxy-urs-12-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (13)

To a stirred solution of **2** (300 mg 0.60 mmol) in THF (4 mL), under N_2 atmosphere at 70 °C, was added CBMI (228.25 mg 1.2 mmol). After 88 h the reaction mixture was diluted with water (60 mL), the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The resulting organic phases were washed with NaCl 10% (3 × 50 mL), dried over Na_2SO_4, filtered and evaporated to the dryness, to afford a yellow solid. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (75:25) to (65:35)], to afford **13** (66%). Mp 216.1–219.7 °C. IR (film $CHCl_3$): 3180.0, 3116.4, 2926.5, 2872.5, 1720.2, 1509.0, 1457.9, 1366.3, 1245.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.53 (1H s), 6.86 (1H s), 5.22 (1H s), 4.47 (1H t J = 14.86), 2.55 (3H s), 2.03 (3H s), 1.09 (3H s), 0.99 (3H d J = 6.00), 0.92 (6H s), 0.84 (3H s), 0.83 (3H s), 0.71 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 176.65, 170.96, 149.35, 137.13, 127.24, 126.39, 117.35, 80.79, 55.25, 54.04, 51.83, 47.39, 42.19, 39.55, 39.49, 38.64, 38.29, 37.64, 36.80, 35.55, 32.70, 30.45, 28.01, 27.75, 24.88, 23.49, 23.41, 23.26. 21.27, 21.09, 18.06, 17.84, 17.25, 17.18, 16.67, 15.51. EI-MS m/z: 562.94 (20) M^+, 248.94 (61), 203.06 (47), 191.08 (25), 189.07 (100), 187.13 (28), 119.14 (38), 107.14 (36), 105.16 (35), 95.19 (41), 91.18 (35), 81.15 (31), 79.14 (29). Anal. Calcd for $C_{36}H_{54}N_2O_3$ C, 76.82; H, 9.67; N, 4.98. Found: C, 77.04; H, 9.34; N, 5.03.

4.1.13. 3β-Acetoxy-urs-12-en-28-yl-4*H*-triazole-1-carboxylate (14)

To a stirred solution of **2** (300 mg 0.60 mmol) in THF (4 mL), under N_2 atmosphere at 70 °C, was added CDT (295.43 mg 1.8 mmol). After 2 h the reaction mixture was diluted with water (60 mL), the aqueous phase was extracted with ethyl acetate (3 × 50 mL). The resulting organic phases were washed with NaCl 10% (3 × 50 mL), dried over Na_2SO_4, filtered and evaporated to the dryness, to afford a yellow solid. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (85:15) to (80:20)], to afford **14** (94%). Mp 124.0–128.8 °C. IR (film $CHCl_3$): 3139.5, 2950.6, 2924.5, 2872.5, 1732.7, 1511.9, 1456.0, 1348.0, 1275.7, 1246.7, 1183.1 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.81 (1H s), 7.98 (1H s), 5.23 (1H s), 4.48 (1H t J = 15.77), 2.04 (3H s), 1.10 (3H s), 0.98 (3H d J = 6.29), 0.92 (6H s), 0.85 (3H s), 0.84 (3H s), 0.66 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ174.87, 170.99, 152.20, 145.30, 137.54, 126.09, 80.84, 55.28, 52.96, 51.30, 47.43, 42.14, 39.47, 39.11, 38.54, 38.29, 37.66, 36.82, 34.44, 32.76, 30.45, 28.15, 28.03, 23.56, 23.51 (2C), 23.29, 21.29, 21.06, 18.09, 17.05, 16.74, 16.69, 15.51. EI-MS m/z: 548.86 (1) M^+, 452.08 (23), 202.96 (32), 201.92 (100), 201.03 (25), 190.04 (85), 189 (53), 188.05 (26), 145.02 (21), 133.05 (31), 131.93 (97), 119.02 (39), 117.04 (53), 104.98 (28), 91.00 (30), 78.96 (22). Anal. Calcd for $C_{34}H_{51}N_3O_3$ C, 74.28; H, 9.35; N, 7.64. Found: C, 74.56; H, 9.21; N, 8.00.

4.1.14. 3β-Acetoxy-11-oxours-12-en-28-yl-1*H*-imidazole-1-carboxylate (15)

Compound **15** was prepared using the same method as for **12**, using **3** (290 mg 0.57 mmol) as a starting material and CDI (184.85 mg 1.14 mmol). The workup was performed after 2.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (55:45)], to afford **15** (56%). Mp 145.7–152.4 °C. IR (film $CHCl_3$): 3139.5, 2969.8, 1872.5, 1721.2, 1656.6, 1465.6, 1365.4, 1245.8, 1208.2 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.28 (1H s Himidazole), 7.52 (1H s Himidazole), 7.05 (1H s Himidazole), 5.64 (1H s H12), 4.48 (1H dd J = 15.98 H3), 2.77 (1H d J = 13.55), 2.60 (1H d J = 11.11), 2.02 (3H s OCOCH_3), 1.30 (3H s), 1.12 (3H s), 1.01 (3H d J = 6.17), 0.92 (3H d J = 6.24), 0.84 (9H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 199.15, 173.78, 170.94, 161.02, 136.87, 131.24, 130.04, 117.27, 80.48, 61.28, 55.00, 53.99, 50.60, 44.61, 43.71, 38.78, 38.69, 38.34, 37.94, 36.94, 34.84, 32.83, 30.08, 28.17, 27.98, 24.47, 23.47, 21.24, 20.84, 20.80, 18.44, 17.19 (2C), 16.62, 16.33. EI-MS m/z: 563.00 (14) M^+, 217.02 (73), 189.04 (100), 175.05 (78), 161.05 (33), 147.06 (74), 133.07 (34), 119.07 (52), 107.06 (33), 105.06 (53), 95.06 (54),

91.06 (49), 79.03 (32), 68.99 (78). Anal. Calcd for $C_{35}H_{50}N_2O_4$ C, 74.70; H, 8.95; N, 4.98. Found: C, 74.30; H, 9.35; N, 4.58.

4.1.15. 3β-Acetoxy-11-oxours-12-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (16)

Compound **16** was prepared using the same method as for **13**, using **3** (290 mg 0.57 mmol) as a starting material and CBMI (216.84 mg 1.14 mmol). The workup was performed after 7 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (60:40) to (50:50)], to afford **16** (39%). Mp 170.0–171.0 °C. IR (film $CHCl_3$): 2950.6, 2872.5, 1722.1, 1656.6, 1458.9, 1365.4, 1247.7 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.52 (1H s Himidazole), 6.89 (1H s Himidazole), 5.65 (1H s H12), 4.48 (1H dd J = 15.82 H3), 2.77 (1H d J = 13.47), 2.65 (1H d J = 10.98), 2.53 (3H s), 2.03 (3H s), 1.31 (3H s), 1.13 (3H s), 1.00 (3H d J = 6.19), 0.92 (3H d J = 6.28), 0.88 (3H s), 0.84 (6H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 199.26, 175.43, 170.97, 161.22, 149.70, 131.19, 127.72, 116.99, 80.50, 61.28, 55.01, 54.18, 51.56, 44.68, 43.85, 38.91, 38.80, 38.34, 37.96, 36.95, 34.69, 32.84, 30.11, 28.21, 28.00, 24.13, 23.48, 21.26, 20.87, 20.74, 18.86, 18.15, 17.30, 17.19, 16.64, 16.36. EI-MS m/z: 577.02 (5) M^+, 216.94 (31), 199.02 (12), 189.99 (17), 188.97 (100), 176.05 (11), 175.04 (40), 173.07 (11), 161.06 (10), 147.07 (34), 133.07 (10), 119.05 (13), 105.07 (12), 95.07 (17), 91.06 (10), 83.04 (20), 80.99 (14). Anal. Calcd for $C_{36}H_{52}N_2O_4$ C, 74.96; H, 9.09; N, 4.86. Found: C, 74.7; H, 9.48; N, 4.48.

4.1.16. 3β-Acetoxy-11-oxours-12-en-28-yl-4*H*-triazole-4-carboxylate (17)

Compound **17** was prepared using the same method as for **14**, using **3** (290 mg 0.57 mmol) as a starting material and CDT (280.66 mg 1.71 mmol). The workup was performed after 2 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (80:20) to (75:25)], to afford **17** (46%). Mp 151.8–156.5 °C. IR (film $CHCl_3$): 3133.8, 2951.5, 2872.5, 1730.8, 1661.4, 1459.9, 1349.9, 1246.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.83 (1H s Htriazole), 8.00 (1H s Htriazole), 5.64 (1H s H12), 4.49 (1H dd J = 16.28 H3), 2.79 (3H d J = 2.77), 2.48 (1H d J = 12.60), 2.31 (1H s H9), 2.03 (3H s CH_3), 1.32 (3H s C27), 1.12 (3H s C25), 1.00 (3H d J = 6.29 C30), 0.93 (3H d J = 6.36 C29), 0.84 (6H s C23 or C24), 0.83 (3H s C26), 0.75 (1H d J = 11.55 H5). ^{13}C NMR (100 MHz $CDCl_3$): δ 199.33 (C11), 174.12 (C28), 170.97 ($OCOCH_3$), 161.72 (C13), 152.61 (Ctriazole), 145.36 (Ctriazole), 131.05 (C12), 80.53 (C3), 61.34 (C9), 55.02 (C5), 53.10 (C18), 50.99 (C17), 44.65 (C8), 43.81 (C14), 38.80 (C1), 38.65 (C19 or C20), 38.32 (C19 or C20), 37.97 (C4), 36.96 (C10), 33.73, 32.86 (C7), 30.13 (C21), 28.60 (C15), 28.01 (C23 or C24), 23.51, 22.96, 21.27 ($OCOCH_3$), 20.89 (C27), 20.87 (C30), 18.53 (C26), 17.22, 17.14 (C29), 16.66 (C23 or C24), 16.33 (C25). EI-MS m/z: 563.78 (4) M^+, 465.94 (16), 353.84 (21), 257.99 (23), 256.95 (100), 217.01 (60), 216.01 (49), 176.08 (19), 175.08 (20), 174.06 (18), 161.08 (28), 159.10 (17), 147.05 (16), 146.03 (20), 91.12 (17). Anal. Calcd for $C_{34}H_{49}N_3O_4$ C, 72.43; H, 8.76; N, 7.45. Found: C, 72.2; H, 9.16; N, 7.05.

4.1.17. 3-Oxours-12-en-28-yl-1*H*-imidazole-1-carboxylate (18)

Compound **18** was prepared using the same method as for **12**, using **4** (300 mg 0.6 mmol) as a starting material and CDI (194.58 mg 1.2 mmol). The workup was performed after 18.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (70:30) to (60:40)], to afford **18** (84%). Mp 141.0–147.3 °C. IR (film $CHCl_3$): 3128.0, 2927.4, 1703.8, 1459.0, 1381.8, 1362.5, 1277.6, 1225.5, 1204.3 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.46 (1H s), 7.56 (1H s), 7.11 (1H s), 5.25 (1H t J = 7.32 H12), 1.10 (3H s C27), 1.06 (3H s C23 or C24), 1.03 (3H s C23 or C24), 1.02 (3H s C25), 1.00 (3H d J = 6.32 C30), 0.91 (3H d J = 6.46 C29), 0.72 (3H s C26). ^{13}C NMR (100 MHz $CDCl_3$): δ 217.61 (C3), 174.26 (C28), 136.88 (C13), 136.69 (Cimidazole), 127.97 (Cimidazole), 126.55 (C12), 117.79 (Cimidazole), 55.23 (C5), 54.22 (C18), 51.16 (C17), 47.38 (C4), 46.62 (C9), 42.20 (C14), 39.38 (C8), 39.26, 39.22 (C19), 38.62 (C20), 36.60 (C10), 35.48, 34.11, 32.29 (C7), 30.30 (C21), 27.72 (C15), 26.41 (C23 or C24), 24.95, 23.50 (C27), 23.41, 21.45, 20.97, 19.33, 17.04 (C29), 16.73 (C26), 15.17 (C25). EI-MS m/z: 504.99 (11) M^+, 205 (44), 201.01 (77), 175.06 (40), 133.04 (54), 119.04 (48), 107.03 (52), 105.04 (51), 95.04 (51), 91.03 (51), 79 (43), 68.97 (100). Anal. Calcd for $C_{33}H_{48}N_2O_2$ C, 78.53; H, 9.59; N, 5.55. Found. C, 78.93; H, 9.89; N, 5.67.

4.1.18. 3-Oxours-12-en-28-yl-2′-methyl-1*H*-imidazole-1-carboxylate (19)

Compound **19** was prepared using the same method as for **13**, using **4** (300 mg 0.66 mmol) as a starting material and CBMI (251.1 mg 1.32 mmol). The workup was performed after 24 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (50:50)], to afford **19** (36%). Mp 196.0–199.7 °C. IR (film $CHCl_3$): 3735.4, 2927.4, 2872.5, 1703.8, 1508.1, 1457.9, 1385.6, 1268.9, 1247.7 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.53 (1H s Himidazole), 6.87 (1H s Himidazole), 5.24 (1H s H12), 2.56 (3H s CH_3imidazole), 1.10 (3H s), 1.06 (3H s), 1.03 (3H s), 1.01 (3H s), 0.98 (3H d J = 6.25), 0.90 (3H d J = 6.32), 0.76 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 217.53, 176.55, 149.34, 137.20, 127.04, 126.25, 117.37, 55.24, 54.08, 51.86, 47.37, 46.66, 42.30, 39.50 (2C), 39.28, 38.61, 36.60, 35.48, 34.10, 32.27, 30.42, 27.74, 26.42, 24.83, 23.39, 23.36, 21.44, 21.06, 19.41, 17.76, 17.20, 17.13, 15.18. EI-MS m/z: 518.91 (13) M^+, 408.98 (96), 244.96 (44), 204.95 (73), 203.00 (100), 89.02 (50), 175.06 (58), 121.07 (44), 119.07 (42), 107.08 (42), 105.10 (40), 95.11 (48). Anal. Calcd for $C_{34}H_{50}N_2O_2$ C, 78.72, H, 9.71; N, 5.40. Found: C, 78.96; H, 9.91; N, 5.56.

4.1.19. 3-Oxours-12-en-28-yl-4*H*-triazole-4-carboxylate (20)

Compound **20** was prepared using the same method as for **14**, using **4** (200 mg 0.44 mmol) as a starting material and CDT (216.65 mg 1.32 mmol). The workup was performed after 5 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (75:25) to (70:30)], to afford **20** (98%). Mp 129.4–133.9 °C. IR (film $CHCl_3$): 3133.8, 2925.5, 2870.5, 1733.7, 1703.8, 1510.0, 1457.0, 1384.6, 1348.0, 1275.7, 1181.1 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.82 (1H s Htriazole), 7.98 (1H s Htriazole), 5.24 (1H s H12), 1.10 (3H s), 1.06 (3H s), 1.02 (3H s), 1.01 (3H s), 0.97 (3H d J = 6.31), 0.90 (3H d J = 6.37), 0.71 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 217.58, 174.80, 152.20, 145.28, 137.60, 125.92, 55.24, 53.0, 51.30, 47.36, 46.69, 42.25, 39.41, 39.27, 39.10, 38.49, 36.60, 34.37, 34.11, 32.33, 30.42, 28.12, 26.44, 23.49, 23.44, 23.40, 21.44, 21.02, 19.44, 16.99, 16.69, 15.17. EI-MS m/z: 505.84 (1) M^+, 409.02 (38), 408.01 (100), 202.98 (18), 201.94 (47), 201.01 (28), 190.00 (58), 189.02 (42), 145.09 (24), 133.04 (26), 132.03 (62), 131.05 (23), 119.08 (32), 117.10 (48), 105.10 (23), 91.12 (24). Anal. Calcd for $C_{32}H_{47}N_3O_2$ C, 76.00; H, 9.37; N, 8.31. Found: C, 75.66; H, 9.45; N, 8.70.

4.1.20. 3,11-Dioxours-12-en-28-yl-1*H*-imidazole-1-carboxylate (21)

Compound **21** was prepared using the same method as for **12**, using **5** (300 mg 0.64 mmol) as a starting material and CDI (207.55 mg 1.28 mmol). The workup was performed after 4.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (70:30) to (60:40)], to afford **21** (52%). Mp 211.9–218.8 °C. IR (film $CHCl_3$): 3133.8, 2927.4, 2871.5, 1703.8, 1658.5, 1460.8, 1386.6, 1364.4, 1278.6, 1224.6, 1208.2 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.27 (1H s Himidazole),

7.53 (1H s Himidazole), 7.06 (1H s Himidazole), 5.68 (1H s H12), 1.32 (3H s C27), 1.24 (3H s C25), 1.06 (3H s C23 or C24), 1.02 (6H d J = 7.81 C23 or C24 or C30), 0.93 (3H d J = 6.20 C29), 0.89 (3H s C26). ^{13}C NMR (100 MHz $CDCl_3$): δ 216.91 (C3), 198.55 (C11), 173.82 (C28), 161.40 (C13), 136.89 (Cimidazole), 131.21 (C12), 130.14 (Cimidazole), 117.28 (Cimidazole), 60.68 (C9), 55.44 (C5), 54.06 (C18), 50.65 (C17), 47.69 (C4), 44.47 (C8), 43.86 (C14), 39.76 (C1), 38.78 (C20), 38.37 (C21), 36.73 (C10), 34.86, 34.14, 32.33 (C7), 30.11, 28.29 (C15), 26.34 (C23 or C24), 24.50, 21.36 (C23 or C24 or C30), 20.87 (C23 or C24 or C30), 20.80 (C27), 18.61, 18.36 (C26), 17.18 (C29), 15.57 (C25). EI-MS m/z: 519.00 (5) M^+, 216.98 (29), 188.98 (100), 147.03 (62), 133.05 (22), 119.03 (33), 107.03 (23), 105.02 (32), 95.02 (43), 91.01 (37), 81.01 (30), 79.00 (25), 68.96 (55), 66.97 (33), 54.95 (26). Anal. Calcd for $C_{33}H_{46}N_2O_3$.0.5EtOAc C, 74.70; H, 8.95; N, 4.98. Found: C, 74.76; H, 9.35; N 4.84.

4.1.21. 3,11-Dioxours-12-en-28-yl-2′methyl-1*H*-imidazole-1-carboxylate (22)

Compound **22** was prepared using the same method as for **13** (300 mg 0.64 mmol), using **5** as a starting material and CBMI (243.47 mg 1.28 mmol). The workup was performed after 6 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (50:50)], to afford **22** (34%). Mp 279.4–285.3 °C. IR (film $CHCl_3$): 3182.9, 3120.3, 2927.4, 1871.5, 1704.8, 1659.5, 1549.5, 1511.9, 1460.8, 1384.6, 1365.4, 1270.9, 1248.7, 1232.3, 1215.9 cm^{-1}. ^{1}H NMR (400 MHz $CDCl_3$): δ 7.53 (1H s), 6.91 (1H s), 5.69 (1H s), 2.55 (3H s), 1.33 (3H s), 1.25 (3H s), 1.07 (3H s), 1.03 (3H s), 1.01 (3H d J = 6.18), 0.92 (6H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 216.94, 198.65, 175.42, 161.60, 149.73, 131.14, 127.69, 116.99, 60.65, 55.43, 54.24, 51.60, 47.71, 44.52, 43.98, 39.75, 38.98, 38.35, 36.72, 34.67, 34.15, 32.32, 30.11, 28.32, 26.33, 24.13, 21.38, 20.87, 20.76, 18.76, 18.61, 18.13, 17.28, 15.61. EI-MS m/z: 532.95 (7) M^+, 423.01 (12), 216.89 (18), 190.96 (16), 189.96 (16), 188.96 (100), 173.09 (12), 147.10 (38), 119.09 (14), 105.10 (12), 95.15 (23), 91.12 (13), 83.14 (24), 81.10 (14). Anal. Calcd for $C_{34}H_{48}N_2O_3$ C, 76.65; H, 9.08; N, 5.26. Found: C, 76.26; H, 9.42; N, 4.86.

4.1.22. 3,11-Dioxours-12-en-28-yl-4*H*-triazole-4-carboxylate (23)

Compound **23** was prepared using the same method as for **14**, using **5** (300 mg 0.64 mmol) as a starting material and CDT (315.13 mg 1.92 mmol). The workup was performed after 4 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (80:20) to (70:30)], to afford **23** (43%). Mp 169.3–172.7 °C. IR (film $CHCl_3$): 3128.0, 2955.4, 2872.5, 1732.7, 1702.8, 1659.5, 1513.9, 1458.9, 1385.6, 1349.9, 1275.7 cm^{-1}. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.84 (1H s), 8.01 (1H s), 5.67 (1H s), 1.33 (3H s), 1.23 (3H s), 1.06 (3H s), 1.02 (3H s), 1.00 (3H d J = 6.34), 0.92 (3H d J = 6.38), 0.87 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 216.98, 198.71, 174.14, 162.11, 152.65, 145.37, 130.97, 60.70, 55.41, 53.13, 51.00, 47.69, 44.46, 43.92, 39.73, 38.69, 38.30, 36.70, 34.15, 33.71, 32.31, 30.12, 28.67, 26.33, 22.94, 21.37, 20.89, 20.85, 18.62, 18.41, 17.10, 15.56. EI-MS m/z: 519.84 (8) M^+, 421.97 (46), 407.03 (43), 256.99 (100), 217.04 (73), 216.04 (71), 161.11 (45), 147.07 (32), 146.05 (42), 105.14 (31), 91.2 (39). Anal. Calcd for $C_{32}H_{45}N_3O_3$.0.5H_2O C, 72.69; H, 8.77; N, 7.95. Found: C, 73.08; H, 8.85; N, 7.61.

4.1.23. 28-(1*H*-Imidazol-1-yl)-3,28-dioxours-12-en-2-(1*H*-imidazol-1-yl)-methylene (24)

Compound **24** was prepared using the same method as for **12**, using **6** (323 mg 0.67 mmol) as a starting material and CDI (326 mg 2.06 mmol). The workup was performed after 5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (45:55) to (20:80)], to afford **24** (46%). Mp 281.7–285.0 °C. IR (film $CHCl_3$): 3116.4, 2967.0, 2924.5, 2870.5, 1719.2, 1685.5, 1606.4, 1486.9, 1381.8, 1303.6, 1224.6 cm^{-1}. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.22 (1H s), 7.78 (1H s), 7.68 (1H s), 7.51 (1H s), 7.21 (1H s), 7.14 (3H s), 7.02 (1H s), 5.27 (1H s), 2.86 (1H d J = 15.90), 2.48 (1H d J = 11.04), 1.13 (6H s), 1.09 (3H s), 0.99 (3H d J = 5.78), 0.95 (3H d J = 5.85), 0.86 (3H s), 0.71 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.40, 174.59, 138.37, 137.15, 136.95, 130.76, 130.42, 129.61, 125.94, 122.67, 119.36, 117.37, 54.21, 52.42, 50.89, 45.09, 45.05, 43.06, 42.25, 39.26, 39.20, 38.57, 35.87, 35.42, 31.73, 30.32, 29.67, 27.61, 24.88, 23.51, 23.35, 22.33, 20.94, 20.03, 17.08, 16.36, 15.55. EI-MS m/z: 582.97 (7) M^+, 488.19 (35), 487.18 (100), 486.13 (27), 297.10 (31), 145.10 (30), 133.09 (39), 131.11 (32), 119.10 (46), 117.11 (32), 107.11 (41), 105.11 (56), 95.11 (33), 91.14 (64), 79.10 (48), 69.08 (73). Anal. Calcd for $C_{37}H_{50}N_4O_2$.0.5EtOAc C, 74.72; H, 8.68; N, 8.94. Found: C, 74.93; H, 9.08; N, 9.16.

4.1.24. 28-(2′-Methyl-1*H*-imidazol-1-yl)-3,28-dioxours-12-en-2-(2′-methyl-1*H*-imidazol-1-yl)-methylene (25)

Compound **25** was prepared using the same method as for **13**, using **6** (300 mg 0.62 mmol) as a starting material and CBMI (353.79 mg 1.86 mmol). The workup was performed after 24.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (40:60) to (10:90)], to afford **25** (36%). Mp 283.3–286.1 °C. IR (film $CHCl_3$): 3116.4, 2952.5, 2870.5, 1717.3, 1684.5, 1603.5, 1540.9, 1411.6, 1383.7, 1247.7 cm^{-1}. ^{1}H NMR (400 MHz $CDCl_3$): δ 7.67 (1H s C2C*H*), 7.53 (1H s H4″or H5″), 7.16 (1H s H5′), 7.00 (1H s H4′), 6.87 (1H s H4″ or H5″), 5.28 (1H s H12), 2.86 (1H d J = 15.80 H1), 2.54 (4H s CH_3 C2″ and H18), 2.47 (3H s CH_3 C2′), 1.15 (6H s C27 or C23 or C24), 1.12 (3H s C23 or C24), 1.00 (3H d J = 6.13 C30), 0.95 (3H d J = 6.13 C29), 0.87 (3H s C25), 0.77 (3H s C26). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.50 (C3), 176.51 (C28), 149.35 (C2″), 147.39 (C2′), 137.37 (C13), 130.50 (C2*C*H), 128.68 (C4′), 127.37, 125.87 (C12), 123.07 (C2), 118.11 (C5′), 117.31, 54.16 (C18), 52.64 (C5), 51.84 (C17), 45.20 (C4), 45.15 (C9), 42.40 (C14), 42.36 (C1), 39.55 (C19), 39.39 (C8), 38.59 (C20), 36.03 (C10), 35.43 (C11), 31.78 (C7), 30.42 (C21), 29.66 (C3 or C24), 27.72 (C15), 24.75, 23.49, 23.28 (C27), 22.52 (C23 or C24), 21.07 (C30), 20.08 (C6), 17.93 (CH_3 C2″), 17.24 (C29), 16.90 (C26), 15.51 (C25), 13.75 (CH_3 C2′). EI-MS m/z: 611.12 (5) M^+, 502.17 (38), 501.20 (100), 311.14 (14), 197.16 (25), 119.11 (10), 105.13 (11), 91.17 (12), 83.17 (14), 81.10 (9), 79.10 (8). Anal. Calcd for $C_{39}H_{54}N_4O_2$ C, 76.68; H, 8.91; N, 9.17. Found C, 76.28; H, 9.31; N, 8.89.

4.1.25. 28-(4*H*-Triazol-4-yl)-3,28-dioxours-12-en-2-(4*H*-triazol-4-yl)-methylene (26)

Compound **26** was prepared using the same method as for **14**, using **6** (300 mg 0.62 mmol) as a starting material and CDT (407 mg 2.48 mmol). The workup was performed after 7.5 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (80:20) to (70:30)], to afford **26** (21%). Mp 245.5–251.1 °C. IR (film $CHCl_3$): 3134.1, 2953.5, 2924.5, 2869.6, 1733.7, 1689.3, 1616.1, 1510.0, 1457.0, 1382.7, 1349.0, 1276.7, 1222.7, 1184.1 cm^{-1}. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.82 (1H s), 8.37 (1H s), 8.10 (1H s), 7.99 (1H s), 7.80 (1H s), 5.31 (1H s), 3.41 (1H d J = 17.27), 1.25 (2H), 1.16 (3H s), 1.15 (3H s), 1.11 (3H s), 0.99 (3H d J = 6.26), 0.96 (3H d J = 6.38), 0.88 (3H s), 0.72 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.95, 174.81, 152.96, 152.24, 145.30, 137.60, 137.03, 128.37, 125.92, 125.29, 52.75, 51.39, 45.28 (2C), 44.99, 43.18, 42.40, 39.30, 39.21, 38.53, 35.74, 34.39, 31.87, 30.46, 29.66, 28.13, 23.59, 23.42 (2C), 22.48, 21.02, 20.16, 17.08, 16.43, 15.68. EI-MS m/z: 584.94 (4) M^+, 488.01 (31), 487.00 (70), 202.01 (42), 190.07 (52), 189.09 (100), 187.13 (38), 133.03 (31), 132.03 (51), 119.05 (45), 117.08 (48). Anal. Calcd for

$C_{35}H_{48}N_6O_2$.0.5hexane.0.5EtOAc C, 71.5; H, 8.85; N, 12.51. Found: C, 71.26; H, 9.03; N, 12.42.

4.1.26. Methyl 2-(1*H*-imidazol-1-yl)-methylene-3,11-dioxours-12-en-28-oate (27)

Compound **27** was prepared using the same method as for **13**, using **10** (300 mg 0.59 mmol) as a starting material and CDI (191.34 mg 1.18 mmol). The workup was performed after 5.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (50:50)], to afford **27** (56%). Mp 137.3–141.0 °C. IR (film $CHCl_3$): 3116.4, 2949.6, 1866.7, 1725.0, 1686.4, 1652.7, 1613.2, 1517.7, 1486.9, 1383.7, 1302.7, 1272.8, 1212.0 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.84 (1H s), 7.65 (1H s), 7.35 (1H s), 7.14 (1H s), 5.68 (1H s), 4.16 (1H d J = 16.47), 3.61 (3H s), 2.46 (2H s), 2.25 (1H d J = 16.45), 1.33 (3H s), 1.18 (3H s), 1.12 (6H s), 0.97 (3H d J = 6.23), 0.94 (3H s), 0.90 (3H d J = 6.33). 13C NMR (100 MHz $CDCl_3$): δ 206.25, 198.67, 177.11, 164.05, 138.85, 130.51, 130.26, 130.11, 122.70, 119.21, 58.91, 52.85, 52.79, 51.86, 47.63, 45.28, 44.15, 43.85, 43.17, 38.72, 38.58, 35.97, 35.88, 31.59, 30.24, 29.71, 28.45, 23.81, 22.34, 20.97, 20.93, 19.45, 18.19, 17.17, 15.44. EI-MS m/z: 561.20 (24) M^+, 229.01 (30), 216.99 (32), 215.98 (100), 201.08 (48), 188.10 (42), 174.13 (42), 105.14 (21), 91.17 (27), 69.13 (36). Anal. Calcd for $C_{35}H_{48}N_2O_4$.0.25H_2O C 74.37, H, 8.65; N, 4.96. Found: C, 74.29; H, 9.05; N, 4.83.

4.1.27. Methyl 2-(2̂-methyl-1*H*-imidazol-1-yl)-methylene-3,11-dioxours-12-en-28-oate (28)

Compound **28** was prepared using the same method as for **13**, using **10** (300 mg 0.59 mmol) as a starting material and CBMI (224.45 mg 1.18 mmol). The workup was performed after 5.5 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (50:50)], to afford **28** (39%). Mp 263.4–266.1 °C. IR (film $CHCl_3$): 3119.3, 2949.6, 2867.6, 1726.0, 1684.5, 1654.6, 1604.5, 1540.8, 1504.2, 1456.0, 1410.7, 1382.7, 1320.0, 1273.8, 1244.8 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.62 (1H s H32), 7.32 (1H s H4′), 6.99 (1H s H5′), 5.67 (1H s H12), 4.14 (1H d J = 16.36 H1), 3.61 (3H s COOMe), 2.49 (3H s CH_3 imidazole), 2.45 (2H H18 and H9), 2.18 (1H d J = 16.46 H1), 1.33 (3H s C27), 1.18 (3H s C23 or C24), 1.13 (3H s C23 or C24), 1.12 (3H s C25), 0.97 (3H d J = 6.28 C30), 0.94 (3H s C26), 0.89 (3H d J = 6.22 C29). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.36 (C3), 198.71 (C11), 177.11 (C28), 163.99 (C13), 147.23 (C2′), 130.53 (C12), 129.69 (C32), 128.38 (C4′), 122.98 (C2), 118.24 (C5′), 58.90 (C9), 53.02 (C5), 52.80 (C18), 51.87 (COOMe), 47.64 (C17), 45.39 (C4), 44.19 (C8), 43.86 (C14), 42.51 (C1), 38.72 (C20), 38.59 (C19), 36.07 (C10), 35.90, 31.65 (C7), 30.25, 29.67 (C23 or C24), 28.47 (C15), 23.82, 22.47 (C23 or C24), 21.00 (C27), 20.94 (C30), 19.44, 18.26 (C26), 17.19 (C29), 15.39 (C25), 13.66 (CH_3 imidazole). EI-MS m/z: 575.20 (17) M^+, 243.01 (44), 231.00 (23), 229.98 (100), 229.07 (19), 215.1 (54), 202.11 (36), 189.12 (18), 188.14 (50), 83.14 (25). Anal. Calcd for $C_{36}H_{50}N_2O_4$ C, 75.22; H, 8.77; N, 4.87. Found: C, 74.88; H, 9.12; N, 4.86.

4.1.28. Methyl 2-(4*H*-triazol-4-yl)-methylene-3,11-dioxours-12-en-28-oate (29)

Compound **29** was prepared using the same method as for **14**, using **10** (280 mg 0.55 mmol) as a starting material and CDT (180.54 mg 1.1 mmol). The workup was performed after 23.5 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (65:35) to (55:45)], to afford **29** (46%). Mp 250.2–251.2 °C. IR (film $CHCl_3$): 3122.2, 2949.6, 2869.6, 1725.0, 1688.4, 1654.6, 1617.0, 1508.1, 1457.0, 1383.7, 1275.7, 1202.4 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.46 (1H s), 8.08 (1H s), 7.79 (1H s), 5.68 (1H s), 4.39 (1H d J = 17.42), 3.61 (3H s), 1.33 (3H s), 1.19 (3H s), 1.12 (6H s), 0.97 (3H d J = 6.150), 0.94 (3H s), 0.91 (3H d J = 6.26). 13C NMR (100 MHz $CDCl_3$): δ 206.32, 198.51, 177.13, 163.66, 152.49, 145.23, 130.61, 128.77, 125.27, 58.71, 53.01, 52.80, 51.87, 47.66, 45.38, 44.19, 43.86, 43.56, 38.74, 38.61, 35.93, 35.81, 31.64, 30.27, 29.73, 28.48, 23.85, 22.34, 20.97, 20.95, 19.50, 18.21, 17.19, 15.45. EI-MS m/z: 562.08 (12) M^+, 317.91 (24), 316.93 (86), 285.03 (25), 258.03 (23), 256.97 (100), 217.01 (37), 216.04 (28), 202.03 (14), 189.05 (19), 187.10 (23), 173.12 (21), 162.07 (20), 161.16 (91), 135.18 (22), 119.16 (26). Anal. Calcd for $C_{34}H_{47}N_3O_4$.0.25EtOAc C, 72.01; H, 8.46; N, 7.20. Found: C, 71.72; H, 8.85; N, 6.88.

4.1.29. Methyl 2-(1*H*-imidazol-1-yl)-methylene-3-oxours-12-en-28-oate

Compound **30** was prepared using the same method as for **12**, using **11** (300 mg 0.60 mmol) as a starting material and CDI (194.58 mg 1.2 mmol). The workup was performed after 4 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (80:20) to (60:40)], to afford **30** (63%). Mp 134.0–137.5 °C. IR (film $CHCl_3$): 3116.4, 2948.6, 2872.5, 1774.2, 1722.1, 1685.5, 1607.3, 1519.6, 1457.0, 1380.8, 1306.5, 1225.5 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.81 (1H s), 7.70 (1H s), 7.23 (1H s), 7.16 (1H s), 5.30 (1H s), 3.60 (3H s), 2.89 (1H d J = 15.97), 1.17 (3H s), 1.12 (6H s), 0.95 (3H d J = 5.90), 0.89 (6H s), 0.80 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.58, 177.93, 138.46 (2C), 130.70, 130.41, 124.94, 123.08, 119.41, 53.03, 52.62, 51.45, 48.16, 45.31, 45.18, 43.14, 42.23, 39.36, 39.15, 38.84, 36.55, 36.01, 31.98, 30.65, 29.76, 27.96, 24.18, 23.59, 23.41, 22.42, 21.11, 20.25, 17.07, 16.57, 15.59. EI-MS m/z: 546.13 (35) M^+, 285.07 (48), 202.97 (56), 187.04 (22), 133.02 (100), 119.03 (33), 105.02 (29), 91.00 (31), 78.97 (21), 68.94 (36). Anal. Calcd for $C_{35}H_{50}N_2O_3$.0.5H_2O.0.5EtOAc C, 74.09; H, 9.24; N, 4.67. Found: C, 74.36; H, 9.54; N, 4.63.

4.1.30. Methyl 2-(2′-methyl-1*H*-imidazol-1-yl)-methylene-3-oxours-12-en-28-oate (31)

Compound **31** was prepared using the same method as for **13**, using **11** (300 mg 0.60 mmol) as a starting material and CBMI (228.25 mg 1.2 mmol). The workup was performed after 7 h. The solid was subjected to flash column chromatography [hexanes–ethyl acetate from (75:25) to (60:40)], to afford **31** (55%). Mp 229.5–231.7 °C. IR (film $CHCl_3$): 2948.6, 2872.5, 1724.1, 1685.5, 1603.5, 1540.9, 1500.4, 1456.0, 1411.6, 1274.7, 1242.9 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 7.66 (1H s C2C*H*), 7.16 (1H s C5′), 7.01 (1H s C4′), 5.29 (1H s C12), 3.60 (3H s COOMe), 2.89 (1H d J = 15.84 C1), 2.47 (3H s CH_3 imidazole), 2.26 (1H d J = 11.27 C18), 2.17 (1H d J = 15.49 C1), 1.16 (3H s C23 or C24), 1.14 (3H s C23 or C24), 1.12 (3H s C27), 0.95 (3H d J = 5.85 C30), 0.89 (6H s C29 and C25), 0.79 (3H s C26). ^{13}C NMR (100 MHz $CDCl_3$): δ 206.63 (C3), 177.92 (C28), 147.31 (C2′), 138.45 (C13), 130.38 (C2CH), 128.57 (C4′ imidazole), 124.98 (C12), 123.60 (C2), 118.19 (C5′ imidazole), 53.01 (C18), 52.78 (C5), 51.45 (COOMe), 48.14 (C17), 45.31 (C4), 45.28 (C9), 42.37 (C1), 42.21 (C14), 39.38 (C8), 39.12 (C20), 38.83 (C19), 36.55, 36.12 (C10), 32.01 (C7), 30.63 (C21), 29.67 (C23 or C24), 27.97 (C15), 24.17, 23.54, 23.43 (C27), 22.57 (C23 or C24), 21.11 (C30), 20.23, 17.07 (C29), 16.62 (C26), 15.46 (C25), 13.68 (CH_3 imidazole). EI-MS m/z: 561.17 (12) M^+, 300.07 (17), 299.06 (75), 297.08 (18), 243.06 (16), 203.03 (47), 134.01 (26), 133.15 (100), 131.1 (16), 119.07 (26), 117.09 (16), 107.05 (16), 105.09 (23), 91.13 (29), 83.18 (39), 79.08 (18). Anal. Calcd for $C_{36}H_{52}N_2O_3$ C, 77.10; H, 9.35; N, 5.00. Found: C, 76.70; H, 9.56; N, 5.02.

4.1.31. Methyl 2-(4*H*-triazol-4-yl)-methylene-3-oxours-12-en-28-oate (32)

Compound **32** was prepared using the same method as for **14**, using **11** (300 mg 0.60 mmol) as a starting material and CDT

(295.43 mg 1.8 mmol). The workup was performed after 23 h. The residue was subjected to flash column chromatography [hexanes–ethyl acetate from (80:20) to (70:30)], to afford **32** (40%). Mp 202.1–206.3 °C. IR (film $CHCl_3$): 3116.4, 2947.7, 1721.2, 1688.4, 1618.0, 1509.0, 1454.1, 1381.8, 1281.5, 1226.5 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$): δ 8.37 (1H s), 8.10 (1H s), 7.80 (1H s), 5.32 (1H s), 3.60 (3H s), 3.40 (1H d J = 17.43), 1.18 (3H s), 1.13 (3H s), 1.12 (3H s), 0.95 (3H d J = 5.92), 0.90 (6H s), 0.80 (3H s). ^{13}C NMR (100 MHz $CDCl_3$): δ 207.06, 177.97, 152.82, 145.84, 138.26, 128.35, 125.53, 125.26, 53.05, 52.80, 51.45, 48.17, 45.31, 45.07, 43.20, 42.22, 39.33, 39.13, 38.86, 36.58, 35.79, 32.00, 30.65, 29.68, 27.98, 24.20, 23.59, 23.42, 22.49, 21.13, 20.29, 17.08, 16.60, 15.67. EI-MS m/z: 584.05 (8) M^+, 298.95 (25), 261.96 (44), 204.04 (17), 203.05 (83), 202.08 (22), 189.12 (26), 187.13 (15), 134.05 (40), 133.13 (100), 119.06 (24), 91.14 (17). Anal. Calcd for $C_{34}H_{49}N_3O_3$ C, 74.55; H, 9.02; N, 7.67. Found: C, 74.15; H, 9.42; N, 7.62.

4.2. Biology assays

4.2.1. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder and dimethylsulfoxide (DMSO) were obtained from Sigma Aldrich Co (St. Luis, MO). The compounds tested for biological activity were dissolved in DMSO for stock solutions, stored at −80 °C. The working solutions were prepared in medium. Antibody to poly-(ADP-ribose)-polymerase (PARP) was obtained from Boehringer Mannheim (Mannheim-Waldhof, DE), to $p21^{waf1}$ from BD Biosciences (San Jose, CA), to Mcl-1 and p53 from Santa Cruz Biotechology, Inc., (Santa Cruz, CA), to caspase 9, cleaved caspase 9, caspase 3 and cleaved caspase 3 from Cell Signaling, to NOXA from Abcam.

4.2.2. Cell culture

AsPC-1, MIA PaCa-2, PANC-1, Hep G2, PC-3 and A549 cells were cultured in RPMI 1640 supplemented with 10% of FBS. MCF7 cells were cultured in DMEM supplemented with 5% FBS and 5 μg/mL insulin.

4.2.3. Determination of cell growth inhibition

AsPC-1, MIA PaCa 2, PANC-1, Hep G2, A549, PC-3, MCF7 cells were plated with 1.0×10^3 cells/well in 96-well plates in 100 μl medium. The tested compounds with variant concentrations in 100 μl were added after 24 h after seeding and the cells were continued to culture for 3 days. Fifty μl of MTT (0.5 mg/ml) was added to each well and incubated for 4 h. Supernatant was removed and the formazan precipitated was dissolved in DMSO (100 μl). The density of absorbance was measured at 570 nm on a multiple plate reader. Concentrations that inhibited cell growth by 50% (IC_{50}) were determined by nonlinear regression with GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA).

4.2.4. Cell cycle analysis

Cell cycle was assessed by flow cytometry using a fluorescence activated cell sorter (FACS). AsPC-1 cells were treated with diverse concentrations of compound **32** for 24 h, and then fixed with ice-cold 70% ethanol at a density of 1×10^6 cells/mL overnight. The cells were treated with PI/RNase solution according to the manufactures protocol. DNA content was quantitated by a flow cytometry (Becton Dickinson, San Jose, CA) with an excitation wavelength of 488 nm and an emission wavelength of 625 nm.

4.2.5. Western blot analysis

Protein extracts (50 μg) of cells treated with compound **32** at variant concentrations for 24 h were prepared in lysis buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 μmol/L leupeptin, and 2 μg/mL aprotinin (pH, 8.0) were separated on 8% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to nitrocellulose membranes and blocked with 5% nonfat milk. The membranes were incubated with specific antibodies overnight at 4 °C. Immunocomplexes were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, NJ).

Acknowledgments

Ana S. Leal is supported by Fundação para a Ciência e Tecnologia (SFRH/BD/41566/2007). Jorge A.R. Salvador is supported by Universidade de Coimbra. Yongkui Jing is supported by The Samuel Waxman Cancer Research Foundation. NMR data was obtained at the Nuclear Magnetic Resonance Laboratory of the Coimbra Chemistry Centre (www.nmrccc.uc.pt), Universidade de Coimbra, supported in part by grant REEQ/481/QUI/2006 from FCT, POCI-2010 and FEDER, Portugal. Elemental analysis was obtained at the C A C T I Centro de Apoio Científico e Tecnolóxico á Investigación of the Universidade de Vigo.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.010.

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