Bioorganic & Medicinal Chemistry Letters 19 (2009) 2168–2171

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis and cytotoxicity of 2-cyano-28-hydroxy-lup-1-en-3-ones ☆

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ARTICLE INFO

Article history:
Received 12 February 2009
Revised 25 February 2009
Accepted 26 February 2009
Available online 3 March 2009

Keywords:
2-Cyano-28-hydroxy-lup-1-en-3-one
Triterpenoid
Betulinic acid
Betulin
Cytotoxicity

ABSTRACT

New A-ring modified betulin and dihydrobetulin derivatives possessing the 2-cyano-1-en-3-one moiety were prepared and tested for cytotoxicity in seven cancer cell lines. The most active agent **9a** synthesized in this account was further demonstrated to induce apoptosis and to activate caspases in malignant melanoma cells.

Pentacyclic triterpenoids are a class of pharmacologically active and structurally rich natural products with privileged motifs for further modifications and structure-activity relationship analyses.[1] The success by Gribble et al. in identification of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) series of oleanolic acid derivatives (e.g., **1** and **2**) as potent antiproliferative and antiinflammatory agents[2] has sparked much interest in the introduction of the 2-substituted-1-en-3-one system into the A-ring of glycyrrhetinic acid (**3** and **4**),[3] ursolic acid (**5**)[3] and betulinic acid (**6**).[4] The synthetic pentacyclic triterpenoids CDDO (**1**) and CDDO–Me (**2**) are currently in clinical trials for cancer treatment and have been shown to effectively suppress the growth of a broad spectrum of solid and hematologic cancer cell types both in vitro and in mouse models xenografted with human tumors.[2]

The mechanism of action conferred by these molecules is not fully understood. There are evidences that the electrophilic Michael acceptor moieties in these synthetic triterpenoids can form reversible adducts with reactive thiol groups in proteins such as the cytoplasmic repressor Keap-1, glutathione and mitochondrial proteins to cause protein modification and misfolding, which might be responsible for the antiproliferative, antiinflammatory and antitumor activities seen for these molecules. For example, thiol modification in Keap-1 results in Nrf2-mediated induction of the phase II antioxidant response essential for cellular protection against oxidant and inflammatory stress.[2f] Intracellular depletion of glutathione[2g] or modification of thiols in mitochondrial proteins[2c] leads to the generation of reactive oxygen species (ROS) and disruption of redox balance which are critical for apoptosis induction via caspase-dependent or caspase-independent pathways and subsequent cell death. Reactions of **1** and **2** with reactive cysteine moiety in IκBα kinase β (IKKβ) block tumor necrosis factor α (TNFα)-induced NF-κB regulation of cell proliferation and survival.[2d] In addition, activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) has been reported for 2-cyano derivatives-induced cytotoxicity in different cancer cell lines.[3b,4b]

It is generally recognized that the carboxylic acid group at C17 position of betulinic acid (**7**) is essential for its cytotoxicity,[5] as the corresponding reduced form, betulin (**8**), is void of such activity.[5f] In order to test if this would also be the case in the 2-cyano-1-en-3-one modified lupane triterpenoids, herein we wish to report the synthesis and inhibition of cell growth of the lupenone derivatives **9** in which the carboxylic moiety at the C17 is replaced by hydroxymethyl group.

The reaction sequence to introduce the 2-cyano-1-en-3-one functionality onto the A-ring of betulin (**8**) and dihydrobetulin (**10**) is shown in Scheme 1. THP-protected betulone (**11a**) and dihydrobetulone (**11b**) were prepared by selective protection of the 28-OH followed by oxidation of 3-OH under basic conditions

☆ Part of the results was presented at the 232nd American Chemical Society National Meeting, San Francisco, California, September 10–14, 2006. Abstract no. MEDI 20.

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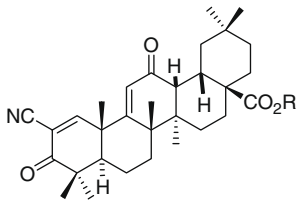
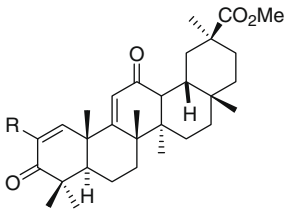
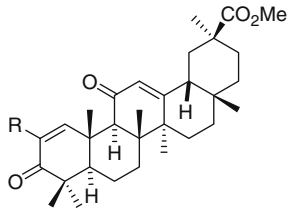
doi:10.1016/j.bmcl.2009.02.107



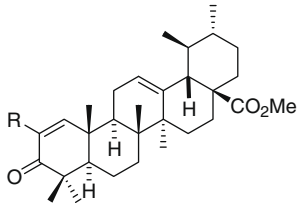
1, R=H (CDDO)
2, R=Me (CDDO-Me)



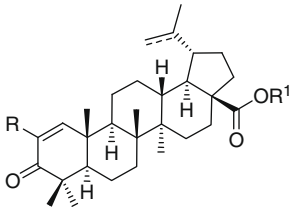
3, R=I, CN, CF_3



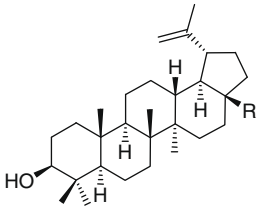
4, R=I, CN, CF_3, $S(O)_2Me$, $P(O)(OMe)_2$



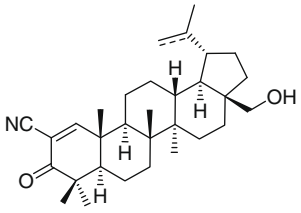
5, R=I, CN, CF_3



6, R = Cl, CN, OMe, CHO
R_1 = H, Me



7, R= CO_2H (betulinic acid)
8, R=CH_2OH (betulin)



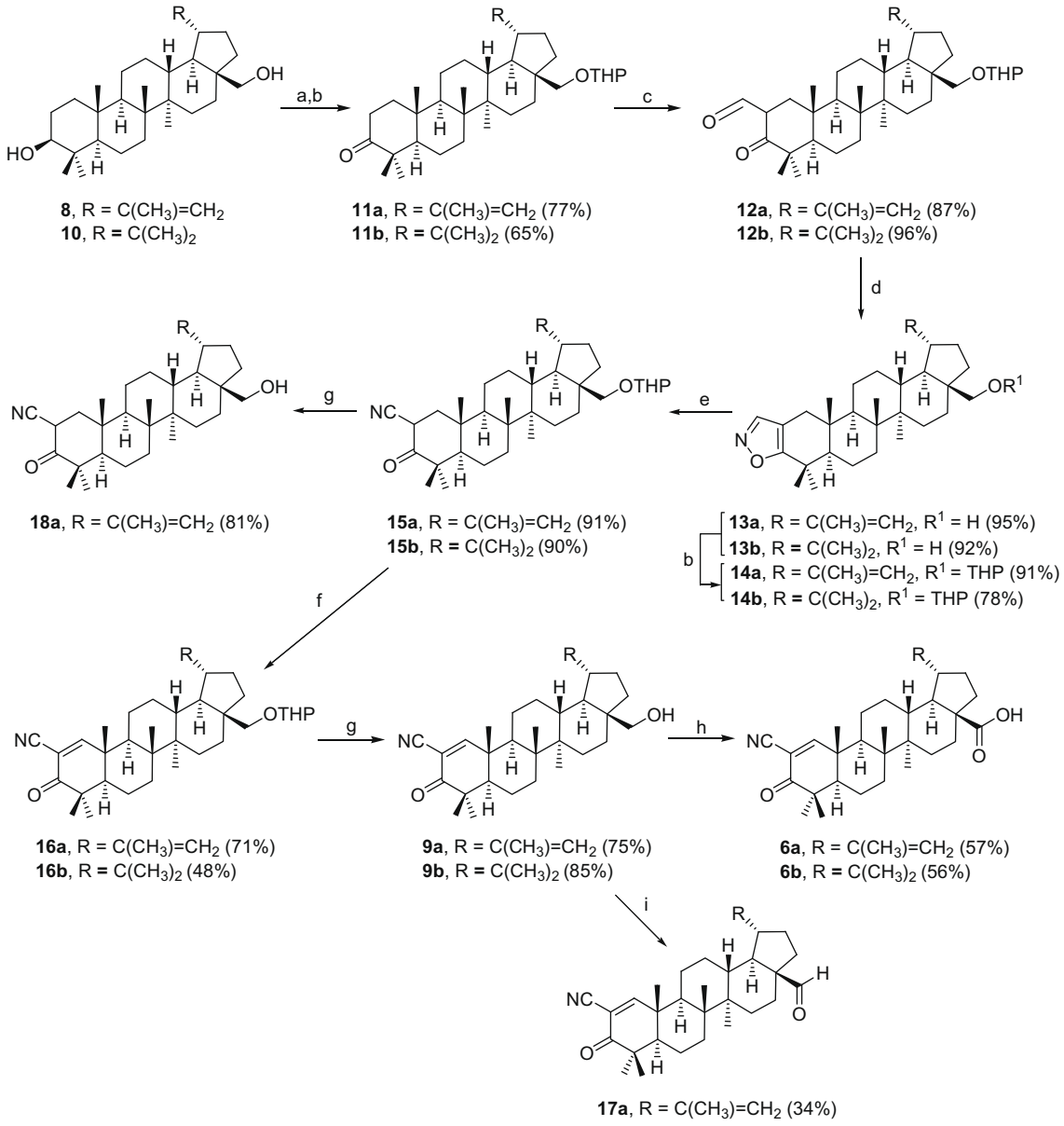
9

(CrO_3 and pyridine). Formylation at C2 was realized using NaOMe and HCO_2Et and the resulting ketoaldehydes **12a** and **12b** were cyclized into isoxazoles **13a** and **13b** by reacting with $NH_2OH{\cdot}HCl$. It is noteworthy that the THP protecting group was lost under these conditions and had to be reintroduced for the subsequent reactions to afford 28-*O*-THP-protected isoxazoles **14a** and **14b** (Scheme 1). The isoxazole ring opening between the N–O bond was prompted by NaOMe uneventfully to deliver the 2-cyano group (**15a** and **15b**). The new double bond between C1–C2 was formed by dehydrogenation with DDQ to complete the construction of the 2-cyano-1-en-3-one moiety in A-ring (**16a** and **16b**). Deprotection of the THP groups freed the 28-OH (**9a** and **9b**),[6] which can be further functionalized. For instance, Jones' oxidation of **9a** and **9b** afforded the carboxylic acids **6a** and **6b**; this provides another effect route for the synthesis of these acid derivatives, especially for **6a** which was not successfully prepared by You et al.[4a] Oxidation of **9a** under basic conditions furnished the aldehyde **17a**. In order to investigate the importance of the enone functionality in relation to the antiproliferative activities of 2-substituted lupanones, the 1,2-saturated α-cyanoketo alcohol **18a** was prepared by removal of the 28-*O*-THP group from the precursor **15a** (Scheme 1).

All the synthesized lupane triterpenoids were evaluated for their antiproliferative activity using MTS assay[7] against a panel of cancer cell lines including SK-MEL-2 and A-375 of malignant melanoma, Daoy and LN-229 of cerebellar medulloblastoma, OVCAR-3 of ovarian, HT-29 of colon and MCF-7 of breast adenocarcinoma and the results are shown in Table 1. Interestingly, the most cytotoxic compounds were found to be the targeted compounds **9a** and **9b** with IC_{50} values ranging from 4 to 9 μM against almost all tested cancer cell lines except for MCF-7 breast cells, even though they are in general less cytotoxic than CDDOs (**1** and **2**).[2] Isoxazoles **13a** and **13b** exhibited better inhibitory activity against melanoma cells than other types of cancer cells. In comparison, the 1,2-saturated analogue **18a** was much less cytotoxic. The cytotoxicity of both 2-cyano-1-en-3-one betulinic acid **6a** and the dihydrobetulinic acid derivative **6b** were less potent than reported,[4a,c] reflecting the laboratory variability.

Compound **9a** was then further investigated for apoptosis induction activity using Annexin-V assay.[8] As shown in Figure 1, **9a** induced 24% apoptosis in SK-MEL-2 after treated with 50 μM of agent for 8 h; addition of pancaspase inhibitor z-VAD, as expected, significantly reduced the apoptotic population to 7%. Furthermore, initial investigation indicated that the **9a**-induced apoptosis was mediated by caspase activation. In Roche Homogenous Caspase Assay,[9] **9a** increased caspase activation by two and eightfold in SK-MEL-2 after treated with 5 and 50 μM concentration of compound, respectively, for 8 h; in comparison, betulinic acid (**7**) activated the caspases by 70% and 2.6-fold, respectively (Fig. 2).

In conclusion, incorporation of the 2-cyano-1-en-3-one functionality into the A-ring of betulin (**8**) and dihydrobetulin (**10**) converted the non-cytotoxic molecules to potent apoptosis-inducing antiproliferative agents **9a** and **9b**. It was apparent that the presence of a carboxyl group at C17 such as in **6a** and **6b** was not critical for the cytotoxic activity while the 1(2) double bond was essential. The results reported in this account warrant further investigation of apoptotic pathways for **9a** and **9b**.



Scheme 1. Reagents and conditions with yields shown in parentheses: (a) DHP, PPTS, CH_2Cl_2 (rt); (b) CrO_3, pyridine, CH_2Cl_2 (0 °C); (c) NaOMe, MeOH, HCO_2Et (rt); (d) $NH_2OH{\cdot}HCl$, H_2O, EtOH (reflux); (e) NaOMe, ether, MeOH (0 °C to rt); (f) DDQ, benzene (reflux); (g) PPTS, EtOH (rt); (h) CrO_3, H_2SO_4, acetone (0 °C to rt); (i) CrO_3, pyridine, CH_2Cl_2 (0 °C).

Table 1
Cytotoxicity of 2-cyano-lup-1-en-3-one triterpenoids measured as IC_{50} (μM)

Compound	SK-MEL-2	A-375	DAOY	LN-229	OVCAR-3	HT-29	MCF-7
7 (betulinic acid)	33	51	31	61	59	37	50
6a (ALS 862)	>75	>75	>75	18	>75	38	>75
6b (ALS 854)	59	16	71	30	24	18	59
9a (ALS 769)	7	6	8	6	7	8	25
9b (ALS 851)	6	4	9	—[a]	8	7	>75
13a (ALS 758)	8	—[a]	8	>75	60	10	25
13b (ALS 778)	8	10	7	>75	75	75	>75
15a (ALS 764)	19	38	29	>75	>75	>75	25
15b (ALS 845)	25	48	21	45	—[a]	>75	>75
16a (ALS 765)	23	12	14	21	>75	>75	>75
17a (ALS 775)	40	7	13	>75	>75	49	>75
18a (ALS 768)	42	>75	44	>75	>75	72	68

[a] Not determined.

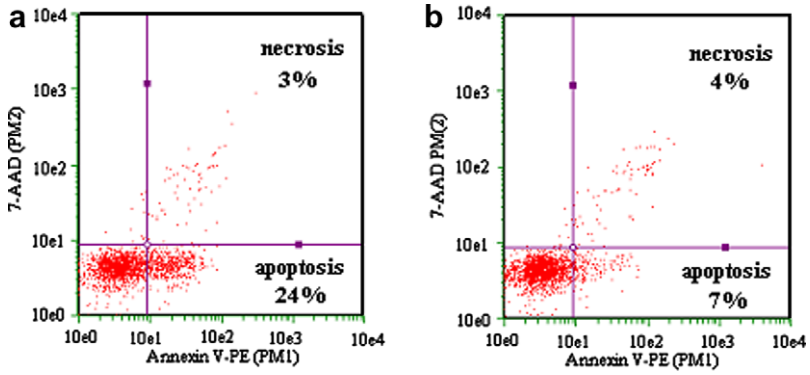


Figure 1. Annexin assay: SK-MEL-2 cells were treated with 50 μM of **9a** for 8 h in the absence (a) or presence (b) of z-VAD.

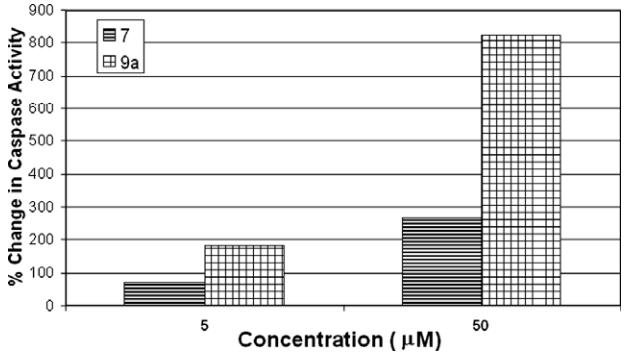


Figure 2. Caspase assay: SK-MEL-2 cells were treated with betulinic acid (**7**) or **9a** at 5 and 50 μM concentration for 8 h.

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6. The newly synthesized compounds provided satisfactory MS (ESI) and NMR spectra without exhibiting any discernible impurities and the selected analytical data are shown below. Compound **9a**: 170–175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.00 (s, 3H), 1.03–1.32 (m, 5H), 1.12 (s, 6H), 1.13 (s, 3H), 1.19 (s, 3H), 1.37–1.65 (m, 11H), 1.66–1.80 (m, 2H), 1.69 (s, 3H), 1.87 (m, 1H), 1.97 (m, 2H), 2.41 (dt, J = 5.8, 11 Hz, 1H), 3.37 (apparent dd, J = 4.0, 11.0 Hz, 1H), 3.78 (apparent dd, J = 4.0, 11.0 Hz, 1H), 4.62 (s, 1H), 4.70 (s, 1H), 7.80 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.6, 16.5, 18.4, 18.9, 21.2, 21.4, 24.9, 26.9, 27.8, 29.0, 29.6, 33.3, 33.9, 37.4, 40.7, 42.1, 43.1, 43.7, 45.0, 47.7, 47.8, 48.5, 52.5, 60.5, 110.1, 114.0, 115.0, 150.0, 170.7, 198.3. Compound **9b**: mp 168–171 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.78 (d, J = 7.0 Hz, 3H), 0.87 (d, J = 6.5 Hz, 3H), 0.97 (s, 3H), 1.13 (s, 6H), 1.14 (s, 3H), 1.20 (s, 3H), 1.06–1.40 (m, 5H), 1.44–1.76 (m, 15H), 1.80–1.96 (m, 3H), 3.34 (d, J = 11.0 Hz, 1H), 3.76 (d, J = 10.5 Hz, 1H), 7.81 (s, 1H). Compound **13a**: 130–137 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (s, 3H), 0.96–1.14 (m, 4H), 1.01 (s, 3H), 1.08 (s, 3H), 1.20 (s, 3H), 1.22–1.41 (m, 4H), 1.30 (s, 3H), 1.42–1.77 (m, 10H), 1.70 (s, 3H), 1.84–1.98 (m, 4H), 2.41 (dt, J = 6.5, 11.1 Hz, 1H), 2.46 (d, J = 14.8 Hz, 1H), 3.36,3.82 (AB-type, J_{AB} = 11.0 Hz, 2H), 4.60 (dd, J = 1.4, 2.2 Hz, 1H), 4.70 (d, J = 2.4 Hz, 1H), 7.96 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 14.7, 15.7, 16.0, 18.8, 21.2, 21.4, 22.63, 25.2, 27.1, 28.7, 29.1, 29.7, 33.2, 33.9, 34.8, 35.8, 37.4, 38.9, 40.9, 42.7, 47.7, 47.8, 48.7, 49.0, 53.5, 60.5, 108.9, 109.8, 150.3, 173.0; ESI MS m/e 466 (M+1). Compound **13b**: 139–145 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.78 (d, J = 7.0 Hz, 3H), 0.81 (s, 3H), 0.84–1.14 (m, 4H), 0.86 (d, J = 7.0 Hz, 3H), 0.99 (s, 3H), 1.09 (s, 3H), 1.16–1.34 (m, 4H), 1.20 (s, 3H), 1.31 (s, 3H), 1.45–1.78 (m, 12H), 1.80–1.98 (m, 4H), 2.49 (d, J = 15.0 Hz, 1H), 3.33,3.79 (AB-type, J_{AB} = 10.7 Hz, 2H), 7.98 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.6, 14.9, 15.7, 16.0, 18.8, 21.2, 21.4, 21.7, 23.0, 26.8, 27.0, 28.7, 29.3, 29.5, 33.3, 34.0, 34.8, 35.8, 36.9, 38.8, 41.0, 42.9, 44.6, 47.9, 48.0, 48.6, 53.5, 60.6, 108.9, 150.3, 173.1. Compound **18a**: 190–194 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (s, 3H), 0.94–1.18 (m, 4H), 0.98 (s, 3H), 1.05 (s, 6H), 1.15 (s, 3H), 1.20–1.48 (m, 10H), 1.52–1.74 (m, 7H), 1.69 (s, 3H), 1.85 (m, 1H), 1.96 (m, 2H), 2.40 (m, 1H), 3.35 (apparent d, J = 11.0 Hz, 1H), 3.79 (apparent t J = 9.2 Hz, 1H), 4.60 (s, 1H), 4.69 (s, 1H)..
7. *MTS assay*: The antiproliferative activities of the molecules were measured by using the CellTiter 96 Aqueous Non-radioactive cell proliferation assay. Cells (1×10^4 per well) were plated onto a 96-well plate the evening before treatment in triplicate. Upon treatment with the test compound solutions in growth media, they were incubated for 72 h for all the cell lines with exception for OVCAR which was treated for 120 h. Next, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2*H*-tetrazolium, inner salt) reagent was added and incubation was continued for 1.5–4 h at 37 °C. During the incubation, MTS is bioreduced to formazan by viable cells. The inhibitory effects were obtained by measuring the absorbance of the formazan at 490 nm on a Wallac Victor II plate reader and calculated by subtracting the absorbance measured at the same wavelength from DMSO-treated cells.
8. *Annexin-V assay*: Guava Nexin Assay was used to measure the percentage of cells undergoing apoptosis by treatment with the test compounds. Cells (8.75×10^5 per well) were plated in a 60 mm dish the evening before treatment. Upon treatment with the test compound solutions in growth media without FBS, the cells were incubated at 37 °C for 6 h. Next 10 % FCS was added to prevent cell damage and improve the efficiency of cell pelleting during subsequent centrifugation steps. After trypsinization and cell pelleting, the Nexin staining solution (including Annexin-V and 7-ADD) was added and incubation was continued on ice for 20 min. Apoptosis was measured on a Guava instrument by setting the gates as described in the manufactures protocol to establish quadrants and to measure the population of viable, mid apoptotic and late apoptotic/necrotic cells in each quadrant.
9. *Homogeneous caspase assay*: Cells (1×10^4 per well) were plated on black-walled, clear-bottomed 96-well plates the evening before treatment, treated with test compound solutions in growth media without FBS, and incubated at 37 °C for 8 h prior to the addition of caspase reagent (DEVD-Rhodamine 110; Roche Homogenous Caspase Assay, fluorimetric, catalog # 03 005 372 001). Upon caspase activation, DEVD-Rhodamine 110 is cleaved. Fluorescent emission of the released rhodamine 110 was measured at 535 nm on Wallac plate reader using the homogeneous caspase program with excitation wavelength at 490 nm and the emission wave at 535 nm. The percentage changes in caspase activation reported are compared to the DMSO treatment. The assay detects caspases 2, 3 and 7 to a greater extent than caspases 6, 8, 9 and 10.