Regular Article

Semisynthetic Esters of 17-Hydroxycativic Acid with *in Vitro* Cytotoxic Activity against Leukemia Cell Lines

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A collection of sixteen semisynthetic 17-hydroxycativic acid esters with alcohols containing a tertiary amine group was evaluated for their *in vitro* cytotoxicity against two human cancer cell lines, THP-1 and U937, and for their effects on the cell cycle and cell death. While 17-hydroxycativic acid itself is not cytotoxic, all the esters displayed cytotoxic activity, with 50% growth inhibition (GI_{50}) values ranging between 3.2 and 23.1 μM. In general, the most potent compounds in both cell lines were esters with four carbon long alcohol residues. There was no clear relationship between the identity of the terminal secondary amine and the activity of the compound. Experiments using the 6-(pyrrolidin-1-yl)pentyl ester, 2c, revealed that this compound activates caspases-3/7 and causes poly(ADP-ribose)polymerase 1 (PARP-1) fragmentation in THP-1 and U937 cells, indicating the induction of apoptotic cell death. These results suggest that further investigation into the anticancer activity of diterpene derivatives and other labdane diterpenes may be fruitful.

Key words diterpenoid; 17-hydroxycativic acid; cytotoxic activity; human cancer cell; apoptosis

Cancer is a major cause of death and one of the most expensive diseases to treat. The number of new cases and the number of individuals living with cancer are both continuously increasing. Consequently, cancer imposes a huge socio-economic burden on human society.[1)] Despite significant advances in cancer care, fatal incidences of tumor recurrence remain common. Therefore, safe and effective new treatments for cancer are still desperately needed.

Throughout the ages, natural products have served and continue to serve as an unparalleled source of effective therapeutic agents for the treatment of a wide spectrum of diseases, including cancer. Approximately 50% of clinically approved anticancer drugs are secondary metabolites found in nature or derivatives of them.[2)] Notably, many semisynthetic analogs of such natural compounds possess more advantageous properties in terms of availability, efficacy, toxicity or absorption, distribution, metabolism, and excretion (ADME) than the original natural product.[3,4)]

Terpenoids are one of the largest and most abundant classes of natural compounds. They have been extracted from higher plants, mosses, algae and lichens, and are also present in insects, microbes and marine organisms. The bicyclic terpenes known as labdanes have attracted scientific interest because of their diverse biological activities.[5–10)] Some natural labdanes and their derivatives (see Fig. 1 for examples) have shown remarkable antiproliferative and cytotoxic activities. Andrographolide is perhaps the most intensively studied *ent*-labdane in this group; there have been extensive efforts to modify its structure in order to identify new analogs with greater activity.[11–14)] Most of the available information on the anticancer activity of other labdanes derives from simple *in vitro* experiments, as in the case of derivatives of the natural diterpene grindelic acid, which has shown cytotoxic activity against a panel of human solid tumor cell lines.[15)] *In vivo* anticancer activity of labdanes has only been documented for a few compounds, including andrographolide[16)] and synthetic derivatives of the natural labdane hispanolone, which were found to promote apoptosis through the death receptor pathway in tumor cell lines by activating caspase-8 with subsequent mitochondrial membrane depolarization.[17)]

A screening study reported by our group revealed that extracts of the endemic Argentinean species *Grindelia ven-*

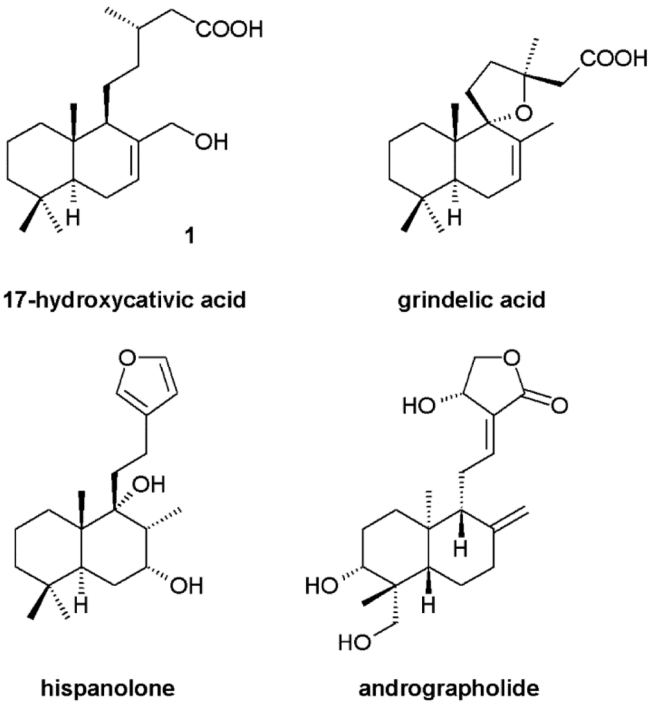

Fig. 1. 17-Hydroxycativic Acid and Some Related Diterpenes

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tanensis A. Bartoli & Tortosa exhibit acetylcholinesterase inhibitory activity.[18] Bioactivity-guided fractionation of these extracts led to the isolation of 17-hydroxycativic acid (**1**), a normal labdane diterpenoid. Compound **1** and its semisynthetic derivatives have shown potent inhibitory activity towards acetyl- and butyrylcholinesterases, potential enzyme targets in the treatment of Alzheimer's disease.[18]

Continuing our investigations of plant natural products and their derivatives with interesting biological activities, the aim of the present study was to screen a small library of semisynthetic 17-hydroxycativic acid derivatives for *in vitro* cytotoxicity towards two human leukemia cell lines, THP-1 and U937. In addition, to gain some insights into the cellular mechanisms of action of the compounds, the cytotoxic effects of two of the most potent derivatives (**2c**, **4b**) were investigated in more detail.

MATERIALS AND METHODS

Isolation and Semisynthesis The isolation of 17-hydroxycativic acid from *G. ventanensis*, the procedures for the synthesis of the derivatives tested here, and their characterization have been described previously.[18]

Cell Maintenance and Cytotoxicity Assay The cells were cultured in RPMI-1640 medium (supplemented with 10% fetal calf serum, 4 mM glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin) in a humidified CO_2 incubator at 37°C and then seeded into 96-well microtiter plates at appropriate densities for their cell sizes and growth rates. After a preincubation period, the cells were treated with the test compounds in triplicate at six different concentrations, and incubated for 72 h. A solution of calcein AM was then added and the fluorescence of the living cells was measured at 485 nm/538 nm (ex/em) with a Fluoroskan Ascent microplate reader (Labsystems). Fifty percent growth inhibition (GI_{50}) values, *i.e.*, the compound concentrations required to reduce the number of viable cells by 50% relative to an untreated control, were determined from dose–response curves as described previously.[19]

Cell Cycle Analysis THP-1 and U937 cells were treated with compounds **2c** and **4b** at different concentrations for 24 h and then fixed with ice-cold 70% ethanol. Cells were washed with phosphate buffered saline (PBS), stained with propidium iodide and then analyzed by flow cytometry using a 488 nm laser (FACSVerse, BD) as described previously.[19]

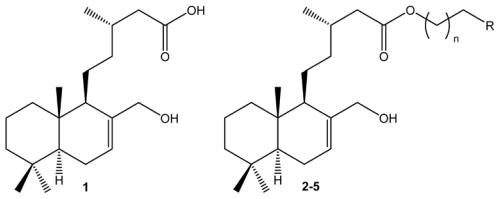
Caspase-3/7 Activity Assay THP-1 and U937 cells were seeded into 96-well microtiter plates at appropriate densities. After a preincubation period, the cells were treated with the test compounds at different concentrations for 24 h. The reaction buffer (150 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 150 mM KCl, 450 mM NaCl, 30 mM $MgCl_2$, 1.2 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 30% sucrose, 1.5% Nonidet P40, 0.3% CHAPS, 30 mM DTT, 3 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4) containing 150 μM peptide substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was then added to the wells. The fluorescence of the product was measured after 4 h of incubation using a Fluoroskan Ascent microplate reader (Labsystems) at 346/442 nm (ex/em) as described earlier.[19]

Immunoblotting For direct immunoblotting, total cellular protein lysates were prepared by harvesting cells in hot Laemmli electrophoresis sample buffer. Proteins were then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 10% gel, and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% low-fat milk and 0.1% Tween 20 in PBS for 1 h and probed overnight with specific monoclonal antibodies. After washing, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h, and peroxidase activity was measured using the ECL kit (Thermo Scientific). Specific antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. (Anti-poly(ADP-ribose)polymerase (PARP-1), anti-Bcl-2 and anti-Mcl-1) and Sigma-Aldrich (peroxidase-labeled secondary antibodies).

RESULTS AND DISCUSSION

This study examined a collection of previously reported compounds: the natural labdane 17-hydroxycativic acid (**1**) and a series of sixteen semisynthetic derivatives (**2a**–**d**, **3a**–**d**, **4a**–**d** and **5a**–**d**) prepared by modifying the C-15 carboxyl group

Table 1. Cytotoxicity of 17-Hydroxycativic Acid and Its Derivatives against Leukemic Cell Lines



Compound	R	n	GI_{50} (μM) ± SD	
			THP-1	U937
1	-	-	>100	>100
2a		3	15.0 ± 1.1	13.2 ± 1.7
2b		4	8.9 ± 0.1	7.4 ± 0.4
2c		5	16.3 ± 0.8	10.5 ± 1.3
2d		6	20.3 ± 3.0	8.7 ± 1.3
3a		3	19.9 ± 4.0	6.4 ± 2.1
3b		4	10.9 ± 0.1	23.1 ± 0.5
3c		5	16.0 ± 2.6	11.9 ± 1.2
3d		6	11.6 ± 0.5	10.0 ± 1.1
4a		3	15.7 ± 3.0	3.2 ± 0.4
4b		4	5.0 ± 1.3	7.9 ± 1.8
4c		5	12.6 ± 1.6	10.5 ± 1.1
4d		6	16.4 ± 1.6	18.5 ± 1.7
5a		3	12.2 ± 2.1	13.0 ± 1.0
5b		4	13.2 ± 1.1	6.1 ± 1.2
5c		5	15.7 ± 2.0	12.6 ± 1.5
5d		6	13.4 ± 1.7	18.9 ± nd
andrographolide	-	-	31.2	13.4

Values presented are means ± S.D. of three independent experiments. The maximum vehicle (DMSO) content in the medium was 0.1%; this level was shown not to interfere with cell growth (data not shown).

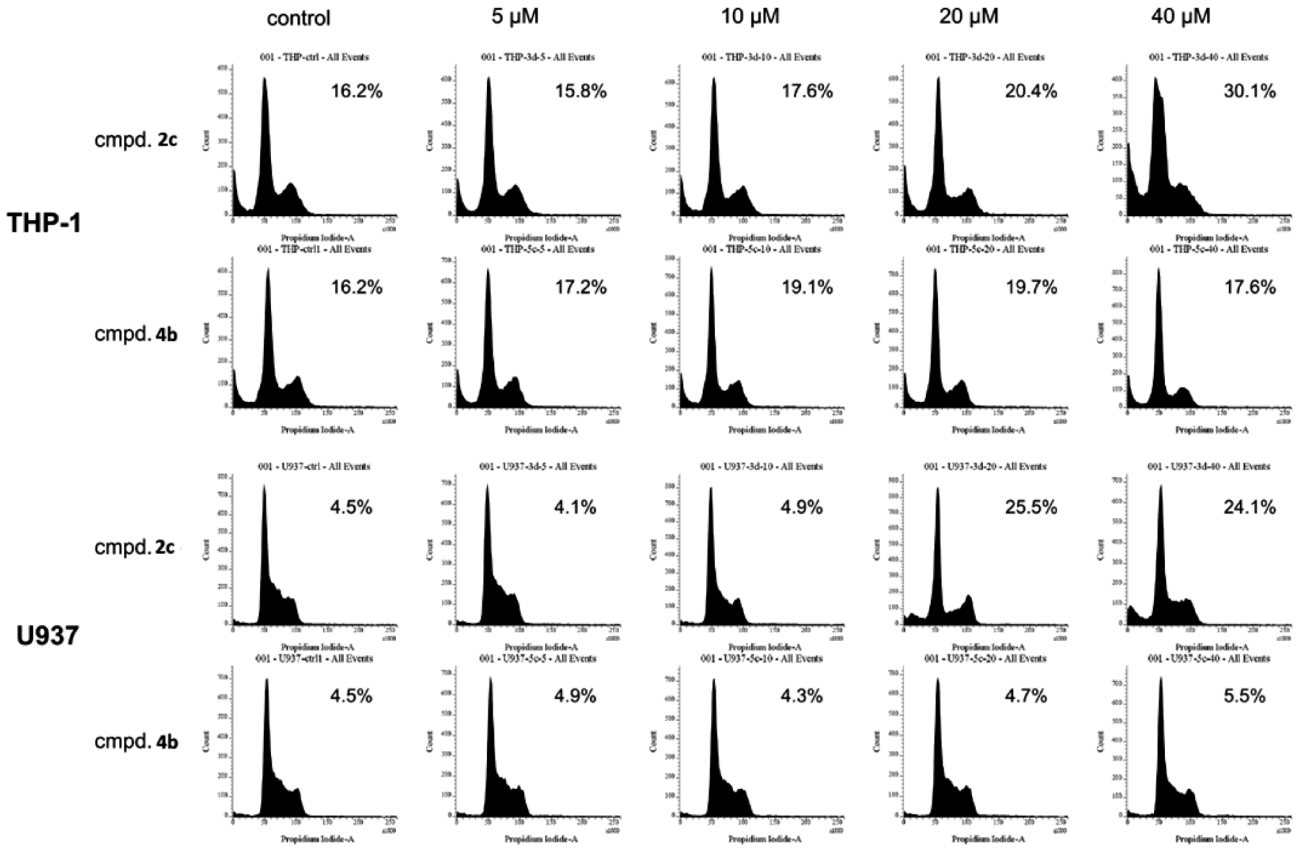


Fig. 2. Cell Cycle Profiles for THP-1 and U937 Cells Treated with Compounds **2c** and **4b**

Numbers indicate the percentage of subdiploid cells. Quantification was performed using a ModFit LTsoftware (Verity Software House).

with the introduction of a C2-C5 linker and a tertiary amine group.[18] Although compound **2b** had no detectable effect on the viability of SH-SY5Y human neuroblastoma cells over 24 h at 5 μM,[18] recent reports of labdane diterpenes with potent cytotoxic activity[11,12,15–17] and the lack of information regarding the biological activity of **1** encouraged us to study the activity of this compound and its derivatives against monocytic leukemia cell lines that were recently reported to display high sensitivity. In addition, it has been observed in compounds with different chemical structures, that the incorporation of a nitrogen-containing hydrophilic substituent has improved not only the oral bioavailability and druggability, but also the antitumoral activity.[20–22] To the best of our knowledge, this study represents the first report of compounds of this class cytotoxic activity towards cancer cells.

To investigate their anticancer potential, exponentially growing cultures of THP-1 and U937 monocytic leukemia cell lines were exposed to increasing concentrations of the chosen compounds and the cell viability was evaluated after 72 h using the calcein-AM assay. Half maximal cytotoxic concentration (GI_{50}) values were determined from dose–response curves by subtraction (Table 1). Andrographolide was assayed as a control and yielded GI_{50} values similar to those reported previously.[11]

All of the derivatives exhibited cytotoxic activity, with GI_{50} values in the single digit micromolar range in the best cases. In contrast, the parent compound **1** was inactive at the tested concentrations. The compounds with the highest activities towards THP-1 cells were **2b**, **3b** and **4b**, which exhibited GI_{50} values of 5.0–10.9 μM; the compounds with the highest activities towards U937 cells were **3a**, **4a** and **5b**, with GI_{50} values of 3.2–6.4 μM. In general, the most potent compounds in both cell lines were derivatives with four carbon alcohol chains (**2b** and **4b**). There is no clear relationship between the identity of the terminal secondary amine and the activity of the compound.

Similar results were observed for amide derivatives of the related compound grindelic acid, which were also much more cytotoxic than the parental compound; the most active derivative achieving single digit micromolar GI_{50} values.[15] It has been determined that in general, the absorption of drugs containing polar groups like carboxylic acids can be improved by masking the carboxyl group by forming simple esters.[23] Functional derivatization of the carboxylic group at C15 may thus be an important determinant of cellular cytotoxicity, since parent compound **1**, with free carboxylic group, resulted inactive. Furthermore, it is important to consider that if esterases cleave the ester bond under physiological conditions releasing parent compound *in vivo*, the ester analogue may be considered as a prodrug.[11]

The mechanistic details of the antiproliferative and cytotoxic effects of labdane diterpenoids have not previously been investigated. However, cell cycle dysregulation and apoptosis play key roles in the initiation and development of cancer, and thus represent potential targets for chemotherapeutic agents.[24] To better understand the effects of the tested compounds on cell growth, the cell cycle, and the induction of apoptosis, further experiments were performed. Even though the most

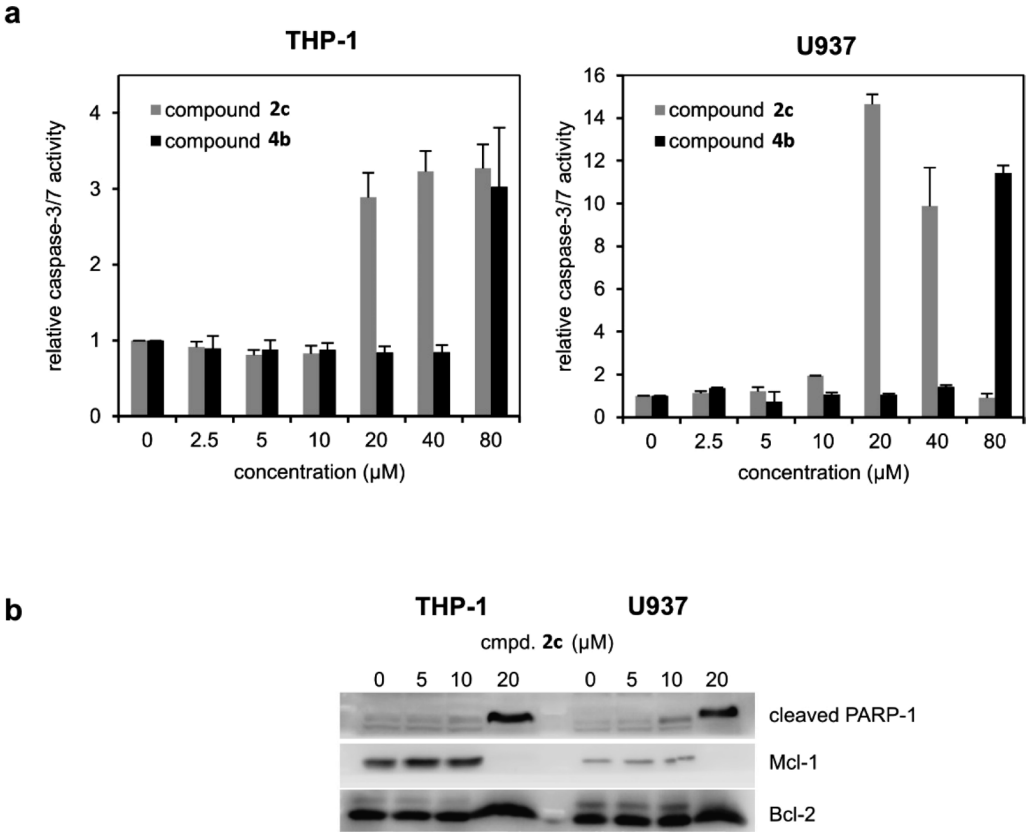


Fig. 3. (a) Relative Caspase-3/7 Activity in THP-1 and U937 Cells Treated with Different Concentrations of Compounds **2c** and **4b** for 24 h and (b) Levels of Cleaved PARP-1 (89 kDa), Mcl-1 and Bcl-2 in THP-1 and U937 Cells Treated with Different Concentrations of **2c**

active compounds against both cell lines were **2b** and **4b**, only **4b** was chosen for further investigations because **2b** presented poor solubility in the culture medium. Instead, structurally related compound **2c**, with one carbon longer linker and good cytotoxicity against both cell lines, was chosen for mechanistic studies.

Asynchronously growing THP-1 and U937 cells were treated for 24 h with **2c** or **4b** at various concentrations, and their status was monitored by flow cytometry. Figure 2 shows DNA content histograms for untreated cells and cells treated with different concentrations of **2c** and **4b**. Exposure to the test compounds for 24 h had only marginal effects on the cell cycle profiles. Compound **2c** reduced the S-phase population in U937 cells (when applied at a concentration of 20 μM) and significantly increased the sub-G1 populations in both cell lines at concentrations of 20 μM and above. Compound **4b** was less potent and did not induce any changes.

An increased sub-G1 population is an accepted indicator of ongoing apoptotic cell death. We therefore analyzed the activity of caspases-3/7, which are activated during cell death[25] in the treated cells. Relative caspase-3/7 activity was measured in lysates of the THP-1 and U937 cell lines that had been treated with different concentrations of **2c** and **4b** for 24 h (Fig. 3a). Treatment with **2c** at concentrations of 20 μM and above induced caspase-3/7 activity in both cell lines, in keeping with the increased sub-G1 populations observed under the same conditions. Compound **4b** also induced caspase activity, although the minimum concentration required to achieve a detectable effect was four times higher than for **2c**. The apparently lower sensitivity of both cell lines to induction of apoptosis by **4b** is consistent with the flow cytometry results presented in Fig. 2. Induction of caspase activity at high concentrations and therefore, low apoptosis induction was also observed for other tested compounds (data not shown). Figure 3a also demonstrate that compound **2c** showed in higher doses a decrease in caspase-3/7 activities in lysed U937 cells. This could be explained due to **2c** stronger cytotoxicity thus resulting in reduced number of intact cells at the end of the incubation period.

Considering the above-mentioned results, the effect of **2c** on the expression of apoptotic proteins in both cell lines was investigated by immunoblotting. As shown in Fig. 3b, treatment with 20 μM **2c** for 24 h caused strong cleavage of poly(ADP-ribose)polymerase-1 (PARP-1), a well-known substrate of caspase-3. The levels of the antiapoptotic protein Mcl-1 also fell, but those of the pro-apoptotic protein Bcl-2 remained unchanged. Such changes are generally considered indicative of ongoing apoptosis, and have been observed in cells treated with a wide range of unrelated cytotoxic compounds.[19,26–29]

Although **2c** and **4b** are structurally similar to previously studied labdanes, it is possible that they might induce cell death by a different mechanism. For the most part, the direct targets of labdane diterpenoids have not yet been identified. For example, andrographolide induces apoptotic cell death in various cell lines,[14,27,28] but the event or process that initiates apoptosis remains unknown. This may be partly due to the pleiotropic behavior of andrographolide, which has been shown to depolarize the mitochondrial membrane,[30] stimulate production of reactive oxygen species,[14] inhibit the phosphati-

dylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway, and induce autophagy and apoptosis.[31] This behavior was partly explained by a recent proteomic study that identified andrographolide as a highly promiscuous compound able to covalently modify numerous cellular targets in a cell type-specific manner.[32] Its reactivity is due to its α,β-unsaturated carbonyl moiety, which functions as a Michael-type acceptor; no such moiety is present in 17-hydroxycativic acid (**1**) or any of the derivatives considered in this work.

It is therefore possible that **1** and its derivatives exert their cellular effects *via* a more specific mechanism. Derivatives of another labdane, hispanolone, also appear to exhibit greater specificity: they have been shown to inhibit the inhibitor of κB kinase (IKK) kinase, leading to inhibition of the nuclear translocation of the nuclear factor-kappaB (NF-κB) transcription factor.[33] Although the NF-κB pathway is not directly linked to cell death, its inhibition may potentiate the therapeutic efficacy of ionizing radiation and some chemotherapeutic compounds.[34] It seems that labdanes may advantageously target cancer cells *via* multiple mechanisms.

In summary, we have found that 17-hydroxycativic acid derivatives show promising anticancer activity towards human leukemia cell lines, with GI_{50} values in the micromolar range. We have also demonstrated that esterification of 17-hydroxycativic acid with a nitrogen-containing aliphatic alcohol increases its cytotoxicity. Even though, exact underlaying mechanism of action of derivatives remain unknown, biochemical analyses suggest that compound **2c** induces apoptotic cell death in treated cells. Chemical and enzymatic stability studies of derivatives may be important to determine if they could be acting as prodrugs. Results suggest that further investigation into the anticancer activity of diterpene derivatives and other labdane diterpenes may be rewarding, and that these compounds may represent a class of natural-like agents with pharmacologically useful anticancer properties.

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Conflict of Interest The authors declare no conflict of interest.

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