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NEW HYBRIDS DERIVED FROM PODOPHYLLIC ALDEHYDE AND DITERPENYLHYDROQUINONES WITH SELECTIVITY TOWARDS OSTEOSARCOMA CELLS

Ángela P. Hernández[a,b], Paula Díez[c,d], Pablo A. García[a], José M. Miguel del Corral[a], Martín Pérez-Andrés[c], David Díez[b], Arturo San Feliciano[a], Manuel Fuentes[c,d]* and Mª Ángeles Castro[a]*

[a] Departamento de Ciencias Farmacéuticas, Área de Química Farmacéutica, Facultad de Farmacia, CIETUS/IBSAL, Campus Miguel de Unamuno, University of Salamanca, 37007 Salamanca, Spain.

[b] Departamento de Química Orgánica, Facultad de Ciencias Químicas, University of Salamanca, 37008 Salamanca, Spain.

[c] Department of Medicine and General Cytometry Service-Nucleus, Cancer Research Centre (IBMCC/CSIC/USAL/IBSAL). 37007 Salamanca, Spain.

[d] Proteomics Unit. Cancer Research Centre (IBMCC/CSIC/USAL/IBSAL), 37007 Salamanca, Spain

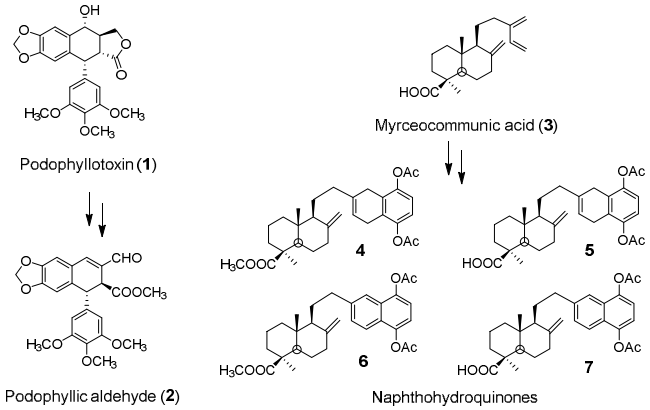
KEYWORDS Cyclolignans; hybrids; podophyllic aldehyde; diterpenylnaphthohydroquinones; cytotoxicity; apoptosis; cell cycle; osteosarcoma.

ABSTRACT: A new family of molecular hybrids, between cyclolignans related to podophyllic aldehyde and several diterpenylnaphthohydroquinones (DNHQ), was prepared and its biological activity evaluated in several human solid tumor cell lines, which are representatives of the most prevalence solid tumors in Western world. Both, cyclolignan and quinone fragments, were linked through aliphatic or aromatic spacers. The new hybrid family was evaluated for its cytotoxicity and it was found that the hybrids were several times more potent against the osteosarcoma cell line MG-63 than against MCF-7 and HT-29 cell lines. The presence of an aromatic ring in the linker gave the most potent and selective agent, improving the cytotoxicity of the parent compounds. Cell cycle studies demonstrated that this hybrid induces a strong and rapid apoptotic effect and arrests cells at the G2/M phase of the cell cycle, in the same way that the parent compound podophyllic aldehyde does.

Hybridation is a useful strategy to generate new multifunctional compounds by combining diverse chemical structures or fragments. The junction of different bioactive molecules can provide an unlimited number of combinations, which could greatly increase the chemo-diversity of drug-like molecules.[1] In this context, natural products are promising and efficient starting materials for the discovery and development of new drugs particularly in the anticancer field;[2] more than 70% of the current anticancer drugs are natural or derived of natural products with a variety of structures and mechanisms of action that would increase when they are combined into new hybrid entities.[3] The synergistic or additive effect of those new chemical entities is a promising approach in diverse diseases, particularly in cancer therapy,[4-10] for example to avoid resistances or discover new applications for future treatments.[11]

Among all the cancer types described, osteosarcoma is a very rare bone cancer that appears in children and young adults. This disease is particularly aggressive, presenting a high risk of metastasis.[12] Current treatments for this cancer combine surgery with chemotherapy. Despite the improvements related to immunomodulation or early diagnosis, selective treatments continue playing an important role in the therapy of this disease.[13] Our previous cytotoxicity studies with two different types of natural products, cyclolignans[14,15,16] and terpenylhydroquinones,[17,18,19] showed a promising selectivity against colon carcinoma cell lines[14,15] and melanoma and ovarian carcinoma cell lines[17,18] respectively.

Scheme 1: Structure of podophyllotoxin (1), podophyllic aldehyde (2), myrceocommunic acid (3), and some diterpenylhydroquinone derivatives (4-7) previously prepared by us.



This fact prompted us to explore their effects on other cancer lines, as osteosarcoma, and design a new family of hybrids, named "lignohydroquinones", by junction of both structures looking for a dual mechanism of action. Previous cyclolignan docking experiments revealed that bulky groups at C-9/C-9' position of the cyclolignan skeleton can adopt different conformations when interact with the colchicine-binding site, leaving a free space that can allow to accommodate larger groups as those designed in this work.[20, 21]

Podophyllotoxin, **1**, was the starting material for the cyclolignan fragment, from which podophyllic aldehyde, **2**, was obtained.[16] Additionally, the terpenoid myrceocommunic acid, **3**, was used previously to obtain the DNHQs **4-7**,[19] (Scheme 1). Both precursors showed antineoplastic activity with different mechanisms of action. Podophyllic aldehyde arrests the cell cycle in the G2/M phase at sub-micromolar concentrations[16] whereas DNHQs, as **6**, are supposed to work through a potent pro-oxidant effect that induces cell death.[22]

Preparation of the new lignohydroquinones and the synergistic effect and improvement of the cytotoxicity against osteosarcoma cell line MG-63 are presented here.

The general structure of the prepared lignohydroquinones is depicted in Scheme 2. As it can be seen, both precursors are connected through aliphatic or aromatic linkers by ester bonds, maintaining the α,β-unsaturated aldehyde of the cyclolignan fragment and the naphthohydroquinone diacetate moiety, due to the high relevance of these features in their antineoplastic cytotoxicity.

Thus, the natural compound podophyllotoxin, **1**, isolated from the resin of rhizome of *Podophyllum emodi* Wall. ex Hook.f. & Thomson, (fam. *Berberidaceae*)[14] and myrceocommunic acid, **3**, isolated from berries of *Juniperus oxycedrus* L. (fam. *Cupressaceae*),[17] were the very starting materials of the new family of lignohydroquinones.

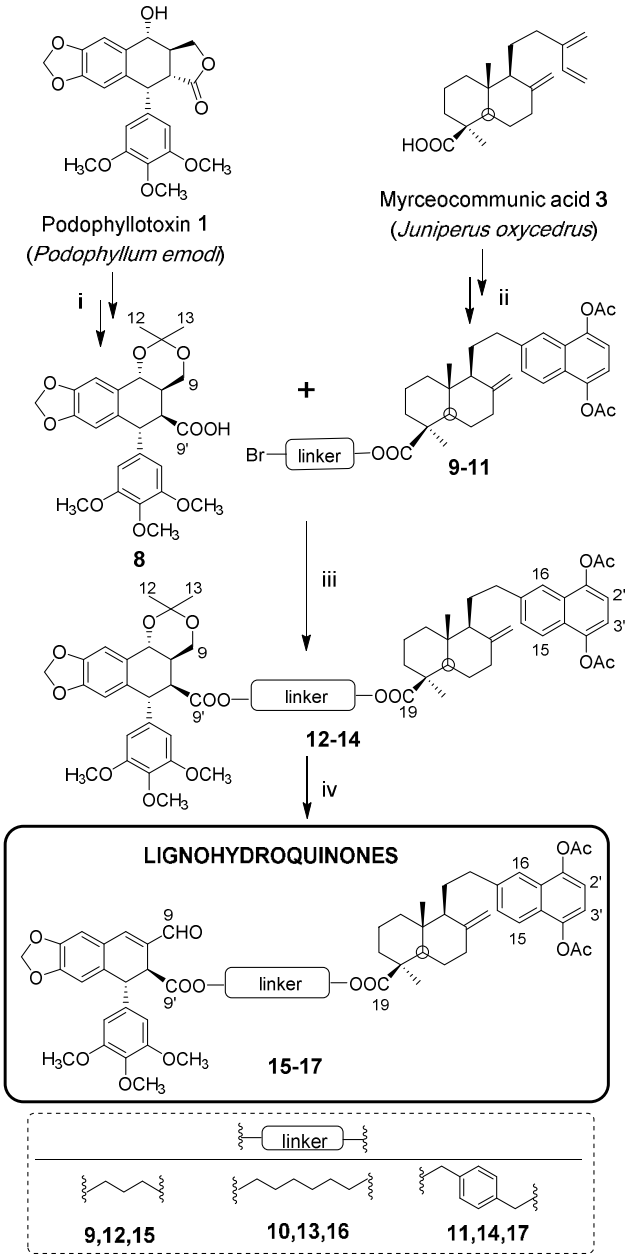
Among the possible strategies to prepare the final lignohydroquinone hybrids, two were considered depending on whether the linker is firstly connected to the cyclolignan and then condensed with the DNHQ or the linker is firstly joined to the DNHQ and condensed later with the cyclolignan. Bearing in mind our previous expertise with lignan-hybrids,[23] it was decided to choose the synthetic route that begins alkylating the DNHQ-carboxylic acid with the linker, followed by reaction with the cyclolignan **8**, leaving for the last step the formation of the aldehyde group by oxidation under Swern conditions. As linker reagents, α,ω-dibromoderivatives (1,3-dibromopropane, 1,6-dibromohexane and α,α'-dibromoxylene) have been chosen in order to analyse the effect of the spacer size and flexibility on the bioactivity of the new hybrids.

To synthesize our objective compounds, the presence of a carboxylic group in each precursor is necessary to join the linkers. About the cyclolignans, podophyllotoxin, **1**, can be easily transformed into picropodophyllic acid, as previously described by us.[16] In order to avoid the re-lactonization to the corresponding *cis*-lactone during esterification, picropodophyllic acid was protected as acetonide[24] to get the acid **8**. This protecting group can be easily removed after condensation with the alkylated DNHQ **9-11** (Scheme 2).

With regards to DNHQs, compound **5** was obtained by Diels-Alder condensation of **3** and *p*-benzoquinone, followed by acetylation.[17,19] Aromatization of **5** with DDQ gave **7** that was treated with 1,6-dibromohexane, to obtain the DNHQ **10** in low overall yield (21% from **5**). We observed that aromatization to give **7** became a limiting step, which could be due to possible interferences between the free carboxylic group and the work up used to remove the DDQ. In view of that, we decided to incorporate the linker before aromatization. Thus, DNHQ **5** was treated with the dibromoderivatives and subsequent aromatization of the naphthalene core yielded the expected DNHQs **9-11** in acceptable overall yields (43-61% from **5**).

The last steps of the synthetic route include the condensation of the two fragments of the hybrid compounds and the final transformation of the cyclolignan fragment into the α,β-unsaturated aldehyde to get the lignohydroquinones, analogues of the podophyllic aldehyde, **2**. Thus, DNHQs **9-11** reacted with the acetonide-acid **8** in DMF and potassium carbonate to get the conjugates **12-14** that were deprotected with *p*-TsOH and oxidized under Swern conditions[16] to obtain the final hybrids **15-17** in acceptable yields.

Scheme 2: Synthesis of the lignohydroquinones 15-17 from the corresponding natural products



[a]Reagents and conditions: (i) (1) KOH/MeOH (2) HCl/H_2O, pH=4 (3) $_P$PTS, 2,2-DMP/Acetone; (ii) (1) p-BQ, BF_3/Et_2O (2) Ac_2O/pyr (3) Br-linker-Br, K_2CO_3/DMF (4) DDQ/Toluene; (iii) K_2CO_3/DMF; (iv) (1) *p*-TsOH, H_2O/Acetone (2) Swern

To determine the efficiency of the hybridation strategy in the design of new drugs, the cytotoxicity of the new hybrids was evaluated by MTT assay and then, flow cytometry experiments were performed to deepen into the mechanism of action of the new conjugates. In both cases, three tumor cell lines where tested: MCF-7 (breast cancer), MG-63 (osteosarcoma) and HT-29 (colon adenocarcinoma). These hybrids maintain the α,β-unsaturated aldehyde function present in the podophyllic aldehyde that has been the lead compound for this work. The precursors podophyllic aldehyde, **2**, DNHQ **6** and the starting cyclolignan podophyllotoxin, **1**, were also included in the assays for comparison purposes.

Table 1: Cytotoxicity (IC_{50}, μM) of the evaluated compounds by MTT assay and flow cytometry.

	(A) MTT assay (72 h)			(B) Flow cytometry (24 h)		
Comp	MCF-7	MG-63	HT-29	MCF-7	MG-63	HT-29
1	0.028 ± 0.003	0.007 ± 0.005	0.066 ± 0.002	>100	18.3 ± 1.4	>100
2	0.48 ± 0.03	0.06 ± 0.02	0.029 ± 0.006	>100	1.23 ± 0.06	>100
6	0.015 ± 0.001	0.41 ± 0.08	0.018 ± 0.002	29.9 ± 0.7	1.16 ± 0.47	5.65 ± 0.07
15	2.21 ± 0.05	2.23 ± 0.40	1.51 ± 0.43	>100	58.2 ± 1.0	>100
16	1.00 ± 0.06	2.06 ± 0.72	1.91 ± 0.95	>100	44.6 ± 0.2	57.6 ± 0.1
17	1.03 ± 0.14	**0.15 ± 0.01**	1.44 ± 0.30	>100	**0.50 ± 0.29**	>100

By MTT assay,[25] all the compounds were cytotoxic on any of the three cell lines tested and several considerations of structure-activity relationship can be deduced (Table 1). The precursors, **1**, **2** and **6**, were highly cytotoxic in MTT assay (72 h of incubation), reaching even nM levels in most of the cases, which is in accordance with our previous reports[16,17](Table 1, A). Also, the intermediates 9-11 were cytotoxics are the μM range (Table S1) Considering the lignohydroquinone hybrids synthesized in this work, **15-17**, all of them were cytotoxic on the tumor cell lines evaluated, although the nature and size of the linker have influenced its potency. Lignohydroquinones with aliphatic linkers presented similar IC_{50} values at the μM range in all of the three tumor cell

lines tested. Interestingly, lignohydroquinone **17,** with the aromatic linker, showed a cytotoxicity in MG-63 cell line at the sub-μM level (IC_{50} = 0.15 μM), being almost seven to ten times more potent against this cell line than against MCF-7 and HT-29 lines (IC_{50}= 1.03 μM and 1.44 μM, respectively). These results indicated a certain degree of selectivity that could be associated with an aromatic linker and, so that it merits further studies on its mechanism of action. Since the cytotoxicity of DNHQs was also observed earlier,[17] we decided to analyse the cell growth inhibitory effect[26, 27] over a shorter time of incubation (24 h) by flow cytometry, with the aim to know if the new hybrids maintain or even enhance the properties and potency of their precursors (Table 1, B). In this assay, only the DNHQ **6** was cytotoxic on the tree cell lines tested but none of the cyclolignans or hybrids were cytotoxic on MCF-7 and HT-29 cells at the maximum concentration tested; only the hybrid **16**, with the longer aliphatic spacer, showed a moderate cytotoxicity against HT-29 cell line (IC_{50} = 57.6 μM). However, all of them showed from moderate to very good cytotoxicity against MG-63 osteosarcoma, which was the most sensitive cell line to these compounds. In fact, the lead cyclolignan **2** was nearly 15x fold more potent than natural product **1** against MG-63 (IC_{50} = 1.23 μM and 18.3 μM, respectively); and regarding the synthesized hybrids, those with the most flexible aliphatic linkers, **15** and **16**, were moderate active (IC_{50} = 58.2 and 44.6 μM, respectively), and conjugate **17**, which had a more rigid aromatic spacer, was the most potent in the assay with an IC_{50} under μM (IC_{50} = 0.50 μM). This IC_{50} value has improved those found for the precursors **1**, **2** and **6**, and highlighted the importance of the size and flexibility of the linker on the cytotoxic potency of the hybrids. The differences in potency for this lignohydroquinone respect to the other hybrids is clearly observed when the cellular viability (%) of each cell line is plotted against drug concentrations (from 0.1 μM to 100 μM) (Figure1) In order to go deeply into the mechanism of action of the new selective lignohydroquinone **17** in comparison with the parent compounds, the apoptotic ability of precursors and final hybrids was evaluated in MG-63 using the annexin/PI assay, which allowed the differentiation among cells in early apoptosis (annexin+/PI-), late apoptosis and death (annexin+/PI+), and living cells (annexin-/PI-) (Figure 2). The ratios for living cells (annexin-/PI-) and total apoptotic cells (annexin+) calculated for podophyllotoxin, **1**, and podophyllic aldehyde, **2**, were in agreement with our previous results in which a delayed apoptosis caused by inhibition of tubulin polymerization was observed.[16] In our assays (Fig 2 and Table S2), hybrids **15** and **16** lacked important apoptotic effect, while **17** induced a strong and rapid apoptosis as deduced from the percentage of total apoptotic cells (89.96 %, Table S2). Furthermore, the ratio between cells in early apoptosis (annexin+/PI-) and late apoptosis (annexin+/PI+) for compound **17** was similar as that found for **2,** (Table S2) which could be associated with similar mechanism of action but with a quicker effect, probably related with the molecule conformation because of the aromatic linker. Finally, cell cycle assays allow the characterization of drug action mechanisms when involving changes in the normal cell cycle progression. It is well known that cyclolignans as podophyllotoxin, **1**, or podophyllic aldehyde, **2**,[16] induce tubulin polymerization inhibition what is translated in a G2/M arrest, while quinones hardly show any influence in cell cycle.

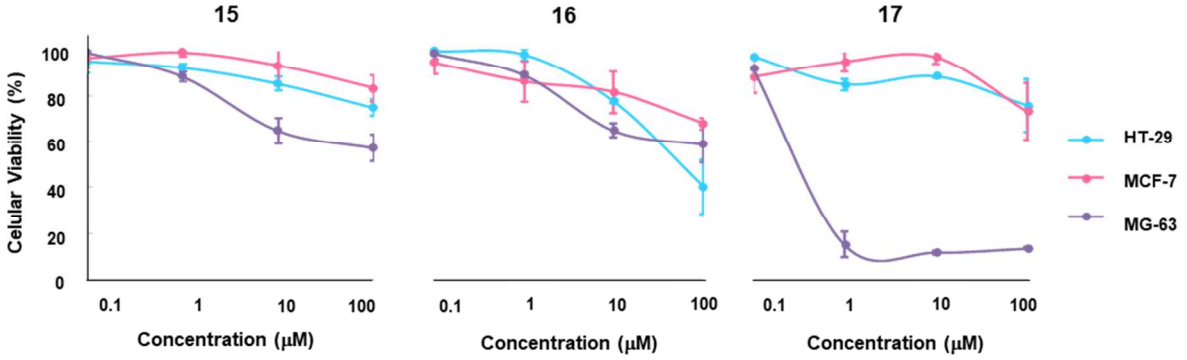


Figure 1: Cell viability results. Comparison of the effect of the lignohydroquinone hybrids (**15-17**) on the three studied tumour cell lines (HT-29, MCF-7, MG-63) at different drug concentrations (0.1-100 μM) during 24 h.

To determine the presence of significant alterations in cell cycle phases, our hybrid **17** was incubated for 24 h in MG-63 cells and compared to the precursors and the other hybrids at 1 μM concentration. The cell cycle assay revealed differential cell cycle patterns depending on the drug. These results are depicted in Figure 3 and Table S3.

The hybrid **16** showed practically the same cell cycle profile than the control while the compound **15** and the DNHQ precursor **6** present a G2/M arrest, but it is not as remarkable as the effect of hybrid **17**. Treatment with **17** demonstrated to increase G2/M cell cycle arrest by 5 folds ($P < 0.001$) compared to the control. Indeed, all the cells treated with this drug were arrested in this phase of the cell cycle. This increase in G2/M population and the complete reduction in G0/G1 and S populations in **17** and podophyllic aldehyde, **2**, confirmed that the hybrid preserved the tubulin inhibition effect associated to cyclolignans.

The comparison of the G2/M population identified after the incubation with hybrid **17** and the other compounds, **15** and **16**, highlighted again the potency of the linker used to synthetize the hybrid. Additionally, *on line* analysis of the drug-likeness/ADME/toxicity properties for the hybrid **17**, performed through PreADMET algorithms (http://preadmet.bmdrc.org/), showed interesting results related to the practical absence of carcinogenicity (mouse, rat), mutagenicity (Ames test), environmental and developmental (algae, insects, minnow fish, medaka embryos) risks, and only low cardiac risk due to hERG inhibition, while ensuring high human intestinal absorption, and adequate permeability values on Caco2 adenocarcinoma cells and through skin (Fig. S1-3). Thus, from the standpoint of the design, the hybridation appears as a good strategy to discover new drugs from the junction of different natural products.

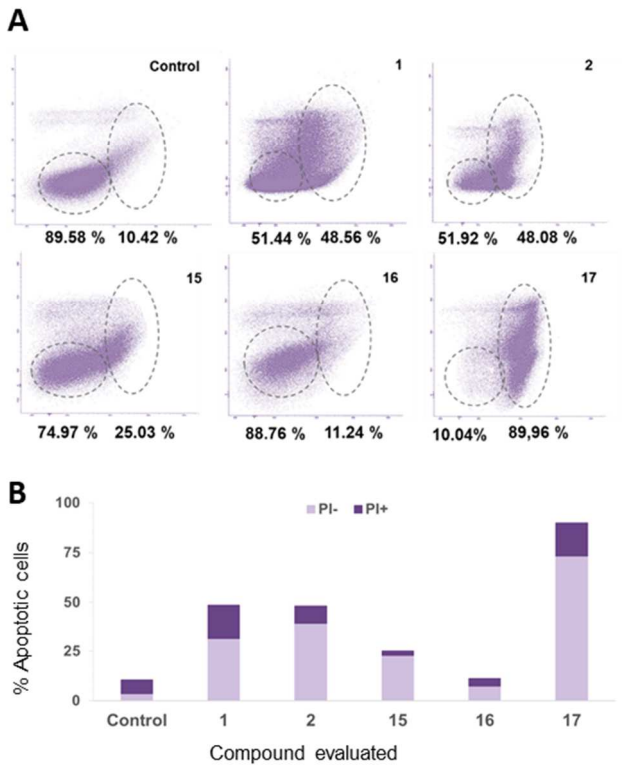


Figure 2: Effect of the cyclolignan precursors (**1** and **2**) and the final hybrids (**15**-**17**) on the apoptosis of MG-63 cells treated with 1 μM of the indicated compound and control with vehicle (DMSO, <0.001%) for 24 h. Apoptosis was determined by annexin V-PI staining by flow cytometry. (A) Annexin/PI diagrams. (B) Percentage of Anexxin+ total and the corresponding ratio PI-/PI+.

The combination and even improvement of the precursor effects were observed for compound **17** that bear an aromatic linker, particularly against osteosarcoma cells in comparison with the other cell lines tested. These results demonstrated the importance of considering different linkers to join the two fragments that form the hybrid.

Interestingly, this new family of hybrids could open new perspectives for the treatment of osteosarcoma due to the good results in terms of antineoplastic cytotoxicity and selectivity found in this work. Obviously, much more medicinal chemistry research including molecular design, calculation/prediction of physicochemical/ADME-Toxicity properties, followed by further synthesis and re-assaying would be necessary. In this sense, several studies are in progress towards the synthesis of better lignohydroquinones.

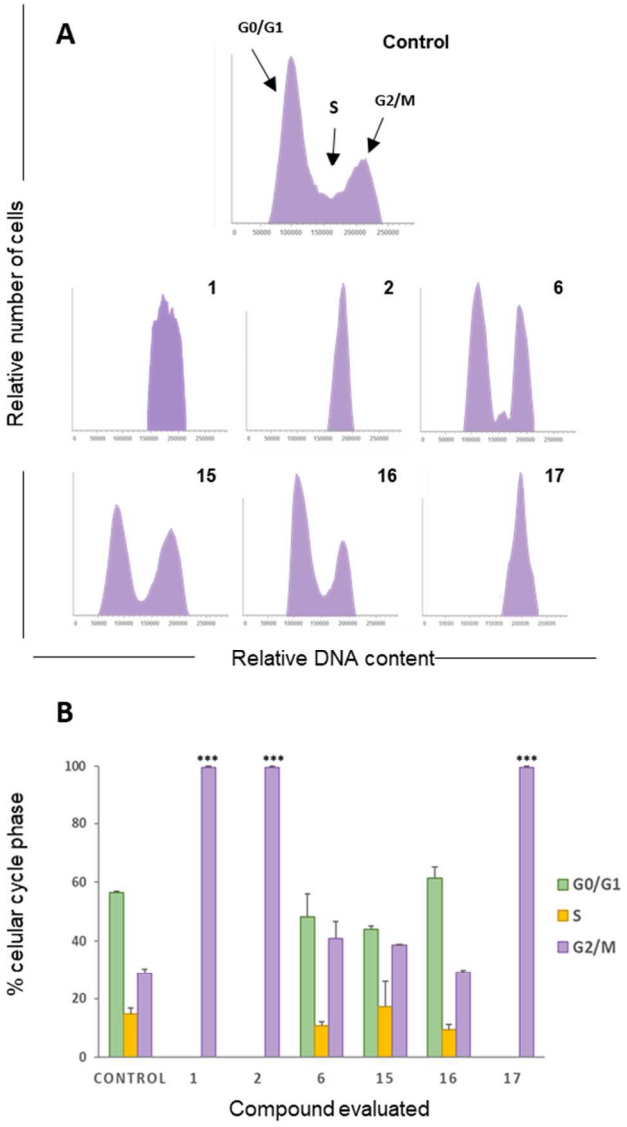


Figure 3: Effect of the precursors (**2** and **6**) and the lignohydroquinone hybrids (**15**-**17**) on DNA-ploidy determined by flow cytometric analysis in MG-63 cell cultures. The cells were incubated with 1 μM of the indicated compound and control with vehicle (DMSO, <0.001%) for 24 h. *** significantly different from control at $p < 0.001$. (A).DNA content analyzed by fluorescence flow cytometry. (B) Percentage of the cell population at the phase of the cell cycle (G0/G, S and G2/M).

ASSOCIATED CONTENT

The supporting Information is available free of charge via the Internet at http://pubs.acs.org.

Synthetic procedures, characterization data for final compounds and intermediates, biological assay methods and cell cycle analysis.

AUTHOR INFORMATION

Corresponding Author

*E-mail: macg@usal.es

Author Contribution

All the authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

BQ, benzoquinone; DDQ, 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone; 2,2-DMP, 2,2-dimethoxypropane; DNHQ, diterpenylnaphthohydroquinones; PI, propidium iodide; PPTS, pyridinium *p*-toluensulfonate.

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