New Hybrids Derived from Podophyllic Aldehyde and Diterpenylhydroquinones with Selectivity toward Osteosarcoma Cells

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Supporting Information

ABSTRACT: A new family of molecular hybrids, between cyclolignans related to podophyllic aldehyde and several diterpenyl-naphthohydroquinones (DNHQ), was prepared and its biological activity evaluated in several human solid tumor cell lines, which are representative of the most prevalent solid tumors in the 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.

KEYWORDS: Cyclolignans, hybrids, podophyllic aldehyde, diterpenyl-naphthohydroquinones, cytotoxicity, apoptosis, cell cycle, osteosarcoma

Hybridization 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 chemodiversity of druglike 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,

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Scheme 1. Structure of Podophyllotoxin (1), Podophyllic Aldehyde (2), Myrceocommunic Acid (3), and Some Diterpenylhydroquinone Derivatives (4–7) Previously Prepared by Us

particularly in cancer therapy,^{4–10} for example, to avoid resistances or discover new applications for future treatments.¹¹

Among all of 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^{14–16} and terpenylhydroquinones,^{17–19} showed a promising selectivity against colon carcinoma cell lines^{14,15} and melanoma and ovarian carcinoma cell lines^{17,18} respectively.

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 the 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 4–7¹⁹ (Scheme 1). Both precursors showed antineoplastic activity with different mechanisms of action. Podophyllic aldehyde arrests the cell cycle in the G2/M phase at submicromolar concentrations¹⁶ 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 first connected to the cyclolignan and then condensed with the DNHQ or the linker is first 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' -dibromo-*p*-xylene) have been chosen in order to analyze 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 relactonization to the corresponding *cis*-lactone during esterification, picropodophyllic acid was protected as acetone²⁴ to get the acid 8. This protecting group can be easily removed after condensation with the alkylated DNHQ 9–11 (Scheme 2).

With regard 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 workup used to remove the DDQ. In view of that, we decided to incorporate the linker before aromatization.

Scheme 2. Synthesis of the Lignohydroquinones 15–17 from the Corresponding Natural Products^a

^aReagents and conditions: (i) (1) KOH/MeOH, (2) HCl/H₂O, pH = 4, (3) PPTS, 2,2-DMP/Acetone; (ii) (1) *p*-BQ, BF₃·Et₂O, (2) Ac₂O/pyr, (3) Br-linker-Br, K₂CO₃/DMF, (4) DDQ/Toluene; (iii) K₂CO₃/DMF; (iv) (1) *p*-TsOH, H₂O/acetone, (2) Swern oxidation.

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 podophyllaldehyde, 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 conditions¹⁶ to obtain the final hybrids 15–17 in acceptable yields.

To determine the efficiency of the hybridization 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 were 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 podophyllaldehyde that has been the lead compound for this work. The precursors podophyllaldehyde, 2, DNHQ 6, and the starting cyclolignan podophyllotoxin, 1, were also included in the assays for comparison purposes.

According to the MTT assay,²⁵ all of 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). In addition, the intermediates 9–11 were cytotoxic in the micromolar 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 their potency. Lignohydroquinones with aliphatic linkers presented similar IC₅₀ values at the micromolar range in all of the three tumor cell lines tested. Interestingly, lignohydroquinone 17, with the aromatic linker, showed cytotoxicity in the MG-63 cell line at the sub-micromolar level (IC₅₀ = 0.15 μ M), being almost seven to ten times more potent against this cell line than against MCF-7 and HT-29 lines (IC₅₀ = 1.03 μ M and 1.44 μ M, respectively). These results indicated a certain degree of selectivity that could be associated with an aromatic linker; thus, it merits further studies on its mechanism of action. Since the cytotoxicity of DNHQs was also observed earlier,¹⁷ we decided to analyze 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

Table 1. Cytotoxicity (IC₅₀, μ M) of the Evaluated Compounds by MTT Assay and Flow Cytometry

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

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

precursors (Table 1, B). In this assay, only the DNHQ 6 was cytotoxic on the three 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 ($\text{IC}_{50} = 57.6 \mu\text{M}$). 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 15 $\times$ more potent than natural product 1 against MG-63 ($\text{IC}_{50} = 1.23 \mu\text{M}$ and $18.3 \mu\text{M}$, respectively), and regarding the synthesized hybrids, those with the most flexible aliphatic linkers, 15 and 16, were moderate active ($\text{IC}_{50} = 58.2$ and $44.6 \mu\text{M}$, respectively), and conjugate 17, which had a more rigid aromatic spacer, was the most potent in the assay with an IC_{50} under μM ($\text{IC}_{50} = 0.50 \mu\text{M}$). 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 to 100 μM) (Figure 1). 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 podophyllaldehyde, 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 (Figure 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 podophyllaldehyde, 2,¹⁶ induce tubulin polymerization

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 annexin + total and the corresponding ratio PI-/PI+.

inhibition what is translated in a G2/M arrest, while quinones hardly show any influence in cell cycle.

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

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 μ M 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).

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 podophyllinic aldehyde, 2, confirmed that the hybrid preserved the tubulin inhibition effect associated with 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 synthesize the hybrid. Additionally, *online* 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 (Figure S1–3). Thus, from the standpoint of the design, the hybridization appears as a good strategy to discover new drugs from the junction of different natural products.

The combination and even improvement of the precursor effects were observed for compound 17 that bears 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 reassaying would be necessary. In this sense, several studies are in progress toward the synthesis of better lignohydroquinones.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.7b00493.

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

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

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BQ, benzoquinone; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; 2,2-DMP, 2,2-dimethoxypropane; DNHQ, diterpe-

nylnaphthohydroquinones; PI, propidium iodide; PPTS, pyridinium *p*-toluenesulfonate.

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