Synthesis of Honokiol Analogues and Evaluation of their Modulating Action on VEGF Protein Secretion and Telomerase-Related Gene Expressions

María Sánchez-Peris, 1 Juan Murga,*1 Eva Falomir,*1 Miguel Carda,1 and J. Alberto Marco2

1 Department of Inorganic and Organic Chemistry, University Jaume I, E-12071 Castellón, Spain.
2 Department of Organic Chemistry, University of Valencia, Dr. Moliner, 50, E-46100 Burjassot, Spain.

* Corresponding authors: E-Mails: jmurga@uji.es (J.M.); efalomir@uji.es (E.F.); Tel.: +34-964728243, (J.M.); Fax: +34-964728214 (J.M.).

Abstract: A group of 36 biphenyl derivatives structurally related to honokiol were synthesised by means of Suzuki coupling reactions. Their cytotoxicities were evaluated and compared to that of honokiol. Some of the compounds were then evaluated for their ability to downregulate the secretion of the VEGF protein and the expression of the VEGF, hTERT and c-Myc genes, the two latter involved in the activation of telomerase in tumoral cells. Some of the synthesized derivatives showed promising pharmacological features as they exhibited IC50 values in low micromolar range, good therapeutic margins and a multiple mode of action on tumor cells based on the inhibition of VEGF and, at the same time, of the expression of genes related to the activation of telomerase.

Keywords: honokiol analogues, angiogenesis, gene regulation, VEGF, telomerase, hTERT, c-Myc

Molecules containing biaryl moieties are relatively common within natural products. For their preparation, Nature has developed an ample array of biosynthetic strategies (1). A number of these biaryl natural products belong to the biogenetic class of lignans (2). Honokiol (Figure 1), has aroused a great degree of interest because of their various pharmacological properties. This product and many derivatives thereof, both of natural and synthetic origin, have been reported to display anti-oxidative, anti-inflammatory, anti-tumor, anti-diabetic, anti-microbial, anti-neurodegenerative, anti-depressant, pain control, gastrointestinal, cardiovascular and liver protective properties, among others (3,4).

We have been investigating a range of analogues of natural products (5) for their cytotoxicity and potential value in anticancer therapy (6). The latter feature may be related to the ability of the compounds to disrupt microtubule dynamics (7), to inhibit the angiogenesis process (8) or to inhibit the expression of genes related to telomerase activation (9) among other alternative mechanisms.

Thanks to the intense studies on genes that mediate cancer progression and therapeutic resistance, many gene targets that regulate apoptosis, proliferation and cell signaling have been identified.
Multiple cumulative genetic and/or epigenetic changes are needed to cause cancer. Molecules that can inhibit expression of such genes are powerful tools in cancer research (10). In this sense, methodologies focused on sequence-specific gene suppression strategies involving antisense oligonucleotides and ribozymes or else involving gene silencing using RNA interference (RNAi) have been developed (11). However, their adaptation as broadly applicable functional genomic and therapeutic tools has proven difficult because of problems regarding stability and poor efficiency of delivery.

Figure 1. Structure of honokiol

Many experiments have shown that targeting a single gene can inhibit the growth and proliferation of tumor cells (12). However, interference targeting a single gene has limitations in the prevention and treatment of cancer as it is known that tumorigenesis results in many cases from multiple gene mutation. Therefore, therapies targeting multiple genes may have better effects on malignant tumors.

In most solid tumors, angiogenesis is an important process for tumor growth and metastasis (13). Many different mediators are involved in this process, including VEGF, which has been shown to play a critical role in pathological angiogenesis (14). VEGF levels in serum are tightly associated to a more aggressive disease state and may serve as a marker to evaluate diagnosis. Blocking VEGF expression can inhibit tumor growth and prevent metastasis (15).

Most cancer cells also exhibit telomerase activity. The latter maintains the length of the telomeres, thus preserving genomic stability (16). Telomerase is a ribonucleoprotein composed of two main subunits which, in the case of human beings, are called human telomerase RNA (hTR) and human telomerase protein (hTERT). Many studies have demonstrated that interference in the expression of the hTERT gene can efficiently inhibit the growth and tumorigenicity of cancer cells (17), as the hTERT gene is a rate-limiting factor in telomerase synthesis and activity. Equally important is the c-Myc gene, which has been found to be amplified in various types of human cancers such as, for example, lung carcinoma (18), breast carcinoma (19) and colon carcinoma (20). The result of the expression of this gene, the c-Myc protein, is a transcriptional factor with an important role in cell proliferation, differentiation, invasion and adhesion of tumor cells (21). It is also involved in the activation of hTERT gene transcription (17).

Since on one hand tumoral cell secretion of VEGF is an important factor in metastasis and, on the other hand, telomerase is responsible for the immortality of tumoral cells, we consider that the potential multiple ability (22) of some compounds to perturb microtubule dynamics and, at the same time, to inhibit VEGF secretion by tumoral cells and the expression of the VEGF, hTERT and c-Myc genes is a goal worth pursuing.
As the natural biphenyl derivative honokiol displays valuable anticancer activity, we decided to prepare a number of synthetic biphenyl derivatives, such as 1-36 (Figure 2), and to investigate their behavior in each of the aforementioned three types of biological activities. In fact, many of these compounds can be viewed as analogues of honokiol that differ in the relative positions of the C-allyl and OH groups. In some cases, hydroxyl functions have been replaced by OMe groups. The C-allyl groups were introduced by means of Claisen rearrangement (23) in precursors having O-allyl groups. The latter have also been used in the biological evaluations. It is worth noting that the biphenyl moiety present in all compounds can be considered a privileged structure (24) that may prove useful in the development of lead compounds. Indeed, small molecules have always aroused interest in cancer therapy (25).

Figure 2. Structure of biphenyl derivatives investigated in this study.
The starting materials for the synthesis of compounds 1-36 were hydroxylated biphenyl derivatives prepared by means of palladium-catalyzed Suzuki coupling (26). The phenolic functions were then O-allylated and the resulting O-allyl derivatives subjected to Claisen rearrangement (Scheme 1) under Lewis acid catalysis (23). Compounds with O-allyl groups ortho or para to the other benzene ring (1, 2, 5, 8-11, 14-17) gave single compounds in the Claisen rearrangements. Those having O-allyl groups meta to the other benzene ring (3, 4, 6, 7, 12, 13, 18 and 36) gave mixtures of regioisomeric rearrangement products. In the case of compounds 6, 12 and 18, the mixtures could be chromatographically resolved into their individual components. These were then subjected to biological evaluation. However, compounds 3, 4, 7, 13 and 36 gave mixtures which could not be separated into pure components. No biological evaluation of such mixtures was carried out.

Scheme 1. Synthesis and structures of biphenyl derivatives.

Methods and Materials

Chemistry

Conditions for O-allylations (27)

A mixture of the appropriate phenol (10 mmol) and K₂CO₃ (2.07 g, 15 mmol) was dissolved in acetone (15 mL). Allyl bromide (1.1 mL, ca. 12.5 mmol) was then added dropwise and the reaction mixture was stirred at reflux for 5 h. The mixture was then cooled, and the volatiles removed under reduced pressure. Addition of a 10% aqueous NaOH solution (10 mL) was followed by extraction with Et₂O (3 x 15 mL). The organic layers were then washed with brine and dried on anhydrous MgSO₄, followed by removal of all volatiles under reduced pressure. This afforded an oily material which was subjected to column chromatography on silica gel (hexane-EtOAc mixtures) to yield the desired O-allylated derivative. Yields were in the range between 50 and 85%.

Conditions for Claisen rearrangements (28)

A mixture of the appropriate O-allylated derivative (4 mmol) in dry hexane (40 mL) was treated dropwise under N₂ with a 1M solution of Et₂AlCl in dry hexane (8 mL for compounds having one O-allyl group and 16 mL for compounds having two O-allyl groups). The mixture was then stirred at
room temperature until consumption of the starting materials (ca. 2 h, TLC monitoring). The mixture was then cooled in an ice bath, followed by slow dropwise addition of a 2M aqueous HCl solution (40 mL). The mixture was poured into a separation funnel, and the organic phase was separated from the aqueous layer, which then was additionally extracted with EtOAc (4 x 15 mL). The combined organic layers were washed with brine, desiccated over anhydrous Na₂SO₄, followed by removal of all volatiles under reduced pressure. This afforded an oily material which was subjected to column chromatography on silica gel (hexane-EtOAc mixtures) to yield the desired C-allyl derivative. Yields were in the range between 30 and 60%. Crystallization of the solid products purified by means of column chromatography was performed using methanol.

Physical data of individual compounds are given in the Supporting Information section.

**Biology**

**Cell culture**

Cell culture media were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, U.K.). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis, Mo., USA). Plastics for cell culture were supplied by Thermo Scientific™ BioLite. All tested compounds were dissolved in DMSO at a concentration of 10 mg/mL and stored at –20ºC until use.

Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 µg/mL) and amphotericin B (1.25 µg/mL), supplemented with 10% FBS.

**Cytotoxicity assays**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used, as previously described (29). Some 5 x 10³ cells of HT-29, MCF-7 or HEK-293 cells in a total volume of 100 µL of their respective growth media were incubated with serial dilutions of the tested compounds. After 3 days of incubation (37 ºC, 5% CO₂ in a humid atmosphere), 10 µl of MTT (5 mg/ml in PBS) were added to each well and the plate was incubated for further 4 h (37 ºC). The resulting formazan was dissolved in 150 µL of 0.04 N HCl/2-propanol and read at 550 nm. All determinations were carried out in triplicate.

**ELISA analysis**

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated at the concentrations showed in Table 2 of the corresponding drugs in DMSO for 72 h (Fig. 3). Culture supernatants were collected and VEGF secreted by HT-29 cells was determined using Invitrogen Human Vascular Endothelial Growth Factor ELISA Kit according to the manufacturer’s instructions.

**RT-qPCR analysis**
HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated at the concentrations showed in Table 2 of the corresponding drugs in DMSO for 48 h. Cells were collected and the total cellular RNA from HT-29 cells was isolated using Ambion RNA extraction Kit according to the manufacturer’s instructions. The cDNA was synthesized by MMLV-RT with 1-21 μg of extracted RNA and oligo(dT)15 according to the manufacturer’s instructions.

Amplification of the genes was performed by use of a StepOnePlus™ thermalcycler. Fast TaqMan Gene Expression Master Mix containing the appropriate buffer for the amplification conditions, dNTPs, thermostable DNA polymerase enzyme and a passive reference probe was used. Each of the genes were amplified using predesigned primers by Life Technologies TaqMan® Gene Expression Assays, Hs99999903-m1 (β-actin), Hs00900055-m1 (VEGF), Hs00972646-m1 (hTERT) and Hs00153408-m1 (c-Myc).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analyses were done using Microsoft Excel and GraphPad Prism®. Differences between means were determined using one-way ANOVA with Dunnett's Multiple Comparison Test, and considered to be statistically significant at \( \alpha \leq 0.05 \).

Results and Discussion

Cytotoxicity of the biphenyl derivatives

The cytotoxic ability of compounds 1-36 was measured by means of their IC\(_{50}\) values towards the tumoral cell lines HT-29 and MCF-7 and towards the non-tumoral cell line HEK-293 (see Table 1). We have also evaluated the therapeutic safety margin of each compound as expressed by means of the \( \alpha \) and \( \beta \) coefficients. These are obtained by dividing the IC\(_{50}\) value of each compound for the nontumoral HEK-293 line by those for the HT-29 (\( \alpha \)) and the MCF-7 (\( \beta \)) tumoral cell line, respectively. The higher the value of either coefficient, the higher the therapeutic safety margin for the corresponding compound.

The observed IC\(_{50}\) values are in the low to medium micromolar range. In general terms, these biphenyl derivatives exhibit the same cytotoxicity range in the HT-29 line as in the MCF-7 line. Compounds 3, 6 and 36 showed comparable or better IC\(_{50}\) values than honokiol towards the HT-29 cell line and, in addition, exhibited a markedly higher therapeutic margin than honokiol (\( \alpha > 7 \)). Compounds 4, 9, 15 and 16 exhibited better IC\(_{50}\) values than honokiol towards the MCF-7 cell line even though only 4 and 15 exhibited a higher therapeutic margin than honokiol (\( \beta > 3 \)).

Thirteen compounds (3, 6, 10, 12, 13, 15, 16, 17, 20, 22, 25, 28 and 36) were selected for further biological evaluations. The selection was made on the basis of products showing either comparatively low IC\(_{50}\) values (high cytotoxicity) or else \( \alpha > 1 \) (good safety margin).
Table 1. IC$_{50}$ values (µM) and selectivity coefficients for biphenyls 1-36.$^a$

| Compound | HEK-293 | HT-29 | MCF-7 | $\alpha^*$ | $\beta^{**}$ |
|----------|---------|-------|-------|-----------|-----------|
| Honokiol | 35 ± 7  | 25 ± 3 | 18.2 ± 0.5 | 1.5 | 1.9 |
| 1        | 320 ± 30| 227 ± 24 | 247 ± 24 | 1.4 | 1.3 |
| 2        | 67 ± 6 | 101 ± 13 | 80.0 ± 0.9 | 0.7 | 0.8 |
| 3        | 258 ± 5 | 27 ± 12 | 242 ± 4 | 9.7 | 1.1 |
| 4        | 49 ± 5 | 208 ± 14 | 14 ± 4 | 0.2 | 3.6 |
| 5        | 70 ± 30 | 210 ± 30 | 109 ± 15 | 0.3 | 0.7 |
| 6        | >100 | 11 ± 3 | 110 ± 14 | >9 | 1 |
| 7        | 104 ± 12 | 232 ± 22 | 230 ± 30 | 0.4 | 0.5 |
| 8        | 140 ± 30 | >100 | 230 ± 50 | >1.4 | 0.6 |
| 9        | 21 ± 5 | 69.5 ± 0.7 | 18 ± 4 | 0.3 | 1.2 |
| 10       | 110 ± 9 | 56.0 ± 0.7 | 228 ± 13 | 2.0 | 0.5 |
| 11       | 31 ± 6 | 180 ± 10 | 214 ± 10 | 0.2 | 0.1 |
| 12       | >100 | 70 ± 40 | 97 ± 5 | >1.5 | >1 |
| 13       | 120 ± 40 | 70 ± 30 | 210 ± 3 | 1.6 | 0.6 |
| 14       | 60 ± 30 | 219 ± 13 | 52.9 ± 2.1 | 0.3 | 1.2 |
| 15       | 26 ± 5 | 38 ± 12 | 2.9 ± 0.14 | 0.7 | 8.7 |
| 16       | 4.5 ± 2.3 | 32 ± 10 | 6.5 ± 2.2 | 0.1 | 1.1 |
| 17       | 53 ± 13 | 44 ± 6 | 130 ± 40 | 1.2 | 0.4 |
| 18       | 48 ± 3 | >100 | 187 ± 14 | 0.5 | 0.3 |
| 19       | 240 ± 11 | 290 ± 40 | 230 ± 50 | 0.8 | 1.0 |
| 20       | 138 ± 14 | 120 ± 30 | 123 ± 5 | 1.2 | 1.1 |
| 21       | 85 ± 10 | 105 ± 11 | 66 ± 6 | 0.8 | 1.3 |
| 22       | 56 ± 18 | 60 ± 30 | 46.4 ± 0.6 | 0.9 | 1.2 |
| 23       | 131 ± 13 | 175 ± 18 | 129 ± 13 | 0.7 | 1.0 |
| 24       | 81 ± 3 | >100 | 176 ± 23 | 0.8 | 0.5 |
| 25       | 93 ± 4 | 79 ± 23 | 56 ± 13 | 1.2 | 1.7 |
| 26       | 189 ± 9 | >100 | 182 ± 7 | 1 | 1.1 |
| 27       | 320 ± 70 | 23 ± 3 | 350 ± 30 | 0.6 | 0.7 |
| 28       | 121 ± 19 | 69 ± 8 | 107 ± 12 | 1.7 | 1.1 |
| 29       | 57 ± 3 | 50 ± 10 | 88 ± 14 | 0.3 | 0.3 |
| 30       | 170 ± 40 | 147.7 ± 2.1 | 282 ± 20 | 0.4 | 0.7 |
| 31       | 271 ± 8 | 290 ± 30 | 530 ± 40 | 0.5 | 0.7 |
| 32       | 260 ± 30 | 270 ± 30 | 150 ± 40 | 0.9 | 0.6 |
| 33       | 270 ± 30 | 280 ± 40 | 239 ± 6 | 0.1 | 0.2 |
| 34       | >100 | 209 ± 7 | 235 ± 3 | 0.2 | 0.2 |
| 35       | 292 ± 10 | 240 ± 50 | 303 ± 9 | 0.1 | 0.5 |
| 36       | 130 ± 30 | 19 ± 9 | 190 ± 30 | 7.1 | 0.7 |
Values are the average (± s.d.) of three different measurements performed as described in the Experimental Section. \( \alpha = IC_{50} \) (HEK-293) / IC\(_{50} \) (HT-29). \( \beta = IC_{50} \) (HEK-293) / IC\(_{50} \) (MCF-7). Values of \( \alpha \) and \( \beta \) have been rounded off to a decimal figure.

**Table 2.** Concentrations for testing selected compounds.

| Concentration | Biphenyl derivatives |
|---------------|----------------------|
| 10 µM         | 6, 36                |
| 20 µM         | Honokiol, 3, 16, 17  |
| 40 µM         | 10, 12, 13, 15, 22, 25 |
| 60 µM         | 28                   |
| 90 µM         | 20                   |

**Effect of biphenyl derivatives on VEGFA protein secretion and VEGF gene inhibition into HT-29**

The influence of the synthetic biphenyl derivatives on the secretion of the VEGFA protein and the expression of the VEGF gene was performed on the HT-29 line. The thirteen aforementioned compounds were tested at concentrations that were close to their respective IC\(_{50} \) values in the HT-29 line (see Table 2).

The amount of VEGFA protein was first determined by means of the ELISA procedure (30), as described in the Methods and Materials Section. Figure 3 shows the percentage of VEGFA secreted to the culture medium after 72 h of incubation in the presence of each of the selected compounds at concentrations showed in Table 2. The values observed when cells were treated only with DMSO were used as the control (standardized to 100%).

Most of the studied derivatives showed an ability to diminish the secretion of VEGFA (Fig. 3) (31). Compound 16 showed an activity (59%) similar to that of honokiol at the same concentration whereas 36 showed a 61% decrease in VEGFA secretion at half the concentration of honokiol. The strongest effect was observed with compounds 25 and 28, which were able to lower VEGFA protein secretion to 40% and 47% of the control value, respectively, an effect stronger than that observed for honokiol (55%). However, it should also be noted that 25 and 28 were acting at a concentration twice and three times, respectively, higher than that of the natural product.
In order to find out whether the selected biphenyl derivatives were able to downregulate the secretion of VEGFA protein by means of a mechanism based on interference at the transcriptional level, we tested the ability of the compounds to inhibit the expression of the VEGFA gene. In this case, HT-29 cells were incubated for 48 h with the selected derivatives at the concentrations shown in Table 2, as well as with DMSO as the control test. The real time quantitative PCR (RT-qPCR) methodology (32) was then used as described in the Experimental Section to determine the percentage of VEGFA gene expression related to the control value. The results are shown in Figure 4.

Seven of the thirteen studied synthetic derivatives showed an ability to diminish the expression of VEGFA gene to a noticeable degree (< 50% of the control value, with honokiol showing a 38% value). The most active compound was 6, which inhibited gene expression to 16% of the control value and at the lowest concentration of all of the tested compounds (half the concentration of honokiol). At the same concentration as the natural product, biphenyl derivatives 16 and 17 were able to decrease the expression of the VEGFA gene to less than 40% of the control value. Finally, compounds 12, 15 and 22 lowered the expression of the VEGFA to 23, 24 % and 35%, respectively, of the control value, even though at a concentration twice higher than for honokiol. We can observe that most of these compounds display one allyloxy group at one at least of the two aromatic rings and the ortho position is usually not occupied. Furthermore, the most frequent substitution patterns for the oxygen atoms in these structures are meta-meta, meta-para and para-para.
Figure 4. Expression percentage of the VEGFA gene after 48 h of incubation of HT-29 cells determined by means of the RT-qPCR methodology. At least three measurements were performed in each case. Error bars indicate standard errors of the mean. The statistical analysis was evaluated using one-way ANOVA with Dunnett's Multiple Comparison Test (P < 0.001).

It is worth mentioning here that the results discussed above do not show a good correlation between the VEGFA amount excreted to the medium and the degree of gene expression. This suggests that these compounds exert the control of VEGFA production at a phase different from that of gene transcription (33).

Effect of biphenyl derivatives on the inhibition of the hTERT and c-Myc genes

In order to determine whether the studied compounds were able to downregulate the expression of the hTERT and c-Myc genes, we have performed an RT-qPCR analysis on HT-29 tumoral cells. The cells were incubated for 48 h in the presence of DMSO (control) and the selected compounds (see Table 2). Figures 5 and 6 show the results observed for the expression of the hTERT and c-Myc genes, respectively.

Figure 5. Expression percentage of the hTERT gene after 48 h of incubation. At least three measurements were performed in each case. Error bars indicate standard errors of the mean. The statistical analysis was evaluated using one-way ANOVA with Dunnett's Multiple Comparison Test (P < 0.001).
Honokiol and several of the synthetic biphenyl derivatives were able to inhibiting \( hTERT \) gene expression to levels below 50% of the control (45% for honokiol). Again, compound 6 and 36 deserve mention as they were able to lower \( hTERT \) gene expression to 34% and 42%, respectively, of the control and at concentration one half that of the natural product (Fig. 5). Compounds 12 and 25 also diminished the expression of the gene to about 25% of the control value although at a concentration twice higher than that of honokiol. In this case, the most frequent substitution patterns for the oxygen atoms in the most active derivatives are \textit{meta-meta} or \textit{meta-para}.

As regards the downregulation of the expression of the \( c-Myc \) gene (Fig. 6), compounds 13, 15, 16, 22, 25 and 36 were able to diminish the expression of this gene to levels below 45% (the value for honokiol) of the control value. As above, compound 36 is also noteworthy as it caused a decrease of gene expression to 36% of the control at half the concentration of honokiol. Compounds 15 and 25 were also quite active, with gene expression reduced to 13% and 7%, respectively, of the control value, but at a concentration twice than that of honokiol. Another compound that showed a noticeable activity was 16, which lowered gene expression to 23% of the control at the same concentration as the natural product. Thus, the most frequent oxygen substitution patterns in this case are \textit{para-para} or \textit{meta-para}.

\textbf{Conclusions}

Thirty-six biphenyl derivatives structurally related to honokiol have been synthesized and biologically evaluated. All of them bear either \( O \)- or \( C \)-allyl groups.
IC$_{50}$ values were first determined for all these biphenyl derivatives towards the HT-29, MCF-7 and HEK-293 cell lines. Some of these derivatives exhibited comparable or lower IC$_{50}$ values than honokiol towards the HT-29 cell line or else higher therapeutic margin ($\alpha$ values) than the natural product.

Thirteen of the derivatives (3, 6, 10, 12, 13, 15, 16, 17, 20, 22, 25, 28 and 36) were then selected for further biological evaluation on the basis of showing either comparatively low IC$_{50}$ values or else $\alpha > 1$ (good safety margin). These derivatives were specifically evaluated for their ability to inhibit the secretion of the VEGFA protein and to inhibit the expression of the VEGFA, hTERT and c-Myc genes. We observed that, in general, the most active derivatives in these particular types of biological properties exhibited in many cases substitution patterns meta-meta or meta-para in their oxygen atoms.

Table 3 summarizes the results obtained for compounds that showed the highest activity in simultaneously inhibiting the VEGFA protein secretion and the expression of the VEGFA, hTERT and c-Myc genes. All these derivatives exhibit two oxygen functions (OH or OR) and also two allyl units, either of the C-allyl or the O-allyl type.

**Table 3.** Compounds with the highest activity in simultaneously inhibiting the VEGFA protein secretion and the expression of the VEGFA, hTERT and c-Myc genes.

| Biological activity | IC$_{50}$ (µM) HT-29 | % VEGFA secretion | % VEGFA gene expr. | % hTERT gene expr. | % c-Myc gene expr. |
|---------------------|----------------------|-------------------|-------------------|------------------|------------------|
| HT-29               | 25                   | 55                | 36                | 45               | 45               |
|                     | 11                   | 76                | 16                | 34               | 58               |
|                     | 38                   | 58                | 24                | 45               | 58               |
|                     | 79                   | 40                | 42                | 25               | 13               |
|                     | 19                   | 61                | 55                | 42               | 7                |
| 6                   |                      |                   |                   |                  |                  |
| Honokiol            |                      |                   |                   |                  |                  |
| 15                  |                      |                   |                   |                  |                  |
| 25                  |                      |                   |                   |                  |                  |
| 36                  |                      |                   |                   |                  |                  |

Compound 6 turns out to be a very promising derivative as a potential anticancer agent because it is able to simultaneously diminish the expression of all three targeted genes at a concentration in the low micromolar range (half the concentration used for honokiol). In addition, 6 exhibits the highest therapeutic margin of all the tested derivatives, honokiol included, and has no free hydroxyl groups.
This feature could be advantageous because, as it is not able to form glucuronide conjugates, it may exhibit a lower metabolism rate. Compounds of this type therefore deserve to be the object of further pharmacological investigation.

**Acknowledgments**

Financial support has been granted to M.C. by the Ministerio de Economía y Competitividad of Spain (project CTQ2014-52949-P), by the Consellería d’Empresa, Universitat i Ciencia de la Generalitat Valenciana (projects PROMETEO/2013/027 and ACOMP/2014/274) and by the University Jaume I (project PI-1B2011-37). M. S.-P. thanks the University Jaume I for a predoctoral fellowship.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**

1. Aldemir, H.; Richarz, R.; Gulder, T. A. M. (2014) The biocatalytic repertoire of natural biaryl formation. Angew. Chem. Int. Ed. 53:8286-8293.
2. Zhang, J.; Chen, J.; Liang, Z.; Zhao, C.(2014) New lignans and their biological activities. Chem. Biodivers. 11:1-54.
3. Shen, J.-L.; Man, K.-M.; Huang, P.-H.; Chen, W.-C.; Chen, D.-C.; Cheng, Y.-W.; Liu, P.-L.; Chou, M.-C.; Chen, Y.-H. (2010) Honokiol and magnolol as multifunctional antioxidative molecules for dermatologic disorders. Molecules 15:6452-6465.
4. Lee, Y.-J.; Lee, Y. M.; Lee, C.-K.; Jung, J. K.; Han, S. B.; Hong, J. T. (2011) Therapeutic applications of compounds in the Magnolia family. Pharmacol. Ther. 130:157-176.
5. For a recent interesting review on natural product analogues: Maier, M. E. (2015) Design and synthesis of analogues of natural products. Org. Biomol. Chem. 13:5302-5343.
6. See, for example: Vilanova, C.; Diaz-Oltra; S.; Murga, J.; Falomir, E.; Carda, M.; Redondo-Horcajo, M.; Díaz, J. F.; Barasoain, I.; Marco, J. A. (2014) Design and synthesis of pironetin analogue / colchicine hybrids and study of their cytotoxic activity and mechanisms of interaction with tubulin. J. Med. Chem. 57:10391-10403, and references to previous work cited therein.
7 Fojo T. (Ed.) (2008) The role of microtubules in cell biology, Neurobiology and Oncology, Humana Press, Totowa, New Jersey.
8. Moserle, L.; Jiménez-Valerio, G.; Casanovas, O. (2014) Antiangiogenic therapies: going beyond their limits. Cancer Discov. 4:31-41.
9. Bernardes de Jesus, B.; Blasco, M. A. (2013) Telomerase at the intersection of cancer and aging. Trends Genet. 29:513-520.
10. Fang, B. (2014) Development of synthetic lethality anticancer therapeutics. J. Med. Chem. 57:7859-7873.
11. Ren, Y.-J.; Zhang, Y. (2014) An update on RNA interference-mediated gene silencing in cancer therapy. Expert Op. Biol. Ther. 14:1581-1592.

12. See, for example: Reicht, S. J.; Fosnot, J.; Kuroki, A.; Tang, W.; Yang, X.; Maguire, A. M. (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol. Vis. 9:210-216.

13. Welti, J.; Loges, S.; Dimmeler, S.; Carmeliet, P. (2013) Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. J. Clin. Invest. 123:3190-3200. (b) Jayson, G. C.; Kerbel, R.; Ellis, L. M.; Harris, A. L. (2016) Antiangiogenic therapy in oncology: current status and future directions. Lancet, DOI: 10.1016/S0140-6736(15)01088-0.

14. Gensicka, M.; Glowacka, A.; Dzierzbicka, K.; Cholewinski, G. (2015) Inhibitors of angiogenesis in cancer therapy - synthesis and biological activity. Curr. Med. Chem. 22:3830-3847.

15. Arora, S.; Kaur, J.; Sharma, C.; Mathur, M.; Bahadur, S.; Shukla, N. K.; Deo, S. V. S.; Ralhan, R. (2005) Stromelysin 3, Ets-1, and vascular endothelial growth factor expression in oral precancerous and cancerous lesions: correlation with microvessel density, progression, and prognosis. Clin. Cancer Res. 11:2272-2284.

16. Yinnan, C.; Yanmin Z. (2016) Functional and mechanistic analysis of telomerase: an antitumor drug target. Pharmacol. Ther. 163:24-47.

17. Lamy, E.; Goetz, V.; Erlacher, M.; Herz, C.; Mersch-Sundermann, V. (2013) hTERT: another brick in the wall of cancer cells. Mut. Res. 752:119-128.

18. Little, C. D.; Nau, M. M.; Carney, D. N.; Gazdar, A. F.; Minna, J. D. (1983) Amplification and expression of the c-Myc oncogene in human lung cancer cell lines. Nature 306:194-196.

19. Mariani-Constantini, R.; Escot, C.; Theillet, C. (1988) In situ c-myc expression and genomic status of the c-myc locus in infiltrating ductal carcinomas of the breast. Cancer Res. 48:199-205.

20. Augenlicht, L. H.; Wadler, S.; Corner, G. (1997) Low-level c-myc amplification in human colonic carcinoma cell lines and tumors: a frequent, p53 independent, mutation associated with improved outcome in a randomized clinical trial. Cancer Res. 57:1769-1775.

21. (a) Huang, H.; Weng, H.; Zhou, H.; Qu, L. (2014) Attacking c-Myc: targeted and combined therapies for cancer. Curr. Pharm. Des. 20:6543-6554. (b) Chen, B.-J.; Wu, Y.-L.; Tanaka, Y.; Zhang, W. (2014) Small molecules targeting c-Myc oncogene: promising anti-cancer therapeutics. Int. J. Biol. Sci. 10:1084-1096.

22. This corresponds to the concept of polypharmacology: J.-U. Peters (2013) Polypharmacology - foe or friend? J. Med. Chem. 56: 8955-8971.

23. For a recent review of this topic, see: Rehbein, J.; Hiere, M. (2013) Claisen rearrangement of aliphatic allyl vinyl ethers from 1912 to 2012: 100 years of electrophilic catalysis. Synthesis 1121-1159.

24. Han, C.; Zhang, J.; Zheng, M.; Xiao, Y.; Li, Y.; Liu, G. (2011) An integrated drug-likeness study for bicyclic privileged structures: from physicochemical properties to in vitro ADME properties. Mol. Divers. 15:857-876.

25. Hoelder, S.; Clarke, P. A.; Workman, P. (2012) Discovery of small molecule cancer drugs: successes, challenges and opportunities. Mol. Oncol. 6:155-176.

26. Data taken from the Ph. D. Thesis of M. S.-P., Univ. Jaume I, December 2015.
27. Ma, L.; Chen, J.; Wang, X.; Liang, X.; Luo, Y.; Zhu, W.; Wang, T.; Peng, M.; Li, S.; Jie, S.; Peng, A.; Wei, Y.; Chen, L. (2011) Structural modification of honokiol, a biphenyl occurring in Magnolia officinalis: the evaluation of honokiol analogues as inhibitors of angiogenesis and for their cytotoxicity and structure activity relationship. J. Med. Chem. 54:6469-6481.

28. Tripathi, S.; Chan, M.-H.; Chen, C. (2012) An expedient synthesis of honokiol and its analogues as potential neuropreventive agents. Bioorg. Med. Chem. Lett. 22:216-221.

29. Rodríguez-Nieto, S.; Medina, M.A.; Quesada, A.R. (2001) A reevaluation of fumagillin selectivity towards endothelial cells. Anticancer Res. 21:3457-3460.

30. (a) Van Weemen, B. K.; Schuurs, A. H. W. M. (1971) Immunoassay using antigen-enzyme conjugates. FEBS Lett. 15:232-236. (b) Engvall, E.; Perlmann, P. J. (1972) Enzyme-linked immunosorbent assay, Elisa: III. Quantitation of specific antibodies by enzyme-labeled antiimmunoglobulin in antigen-coated tubes. Immunol. 109:129-135. (c) O’Beirne, A. J.; Cooper, H. R (1979) Heterogeneous enzyme immunoassay. J. Histochem. Cytochem. 27:1148-1162.

31. (a) Martí-Centelles, R.; Falomir, E.; Murga, J.; Carda, M.; Marco, J. A. (2015) Inhibitory effect of cytotoxic stilbenes related to resveratrol on the expression of the VEGF, hTERT and c-Myc genes. Eur. J. Med. Chem. 103:488-496. (b) Martí-Centelles, R.; Murga, J.; Falomir, E.; Carda, M.; Marco, J. A. (2015) Inhibitory effect of cytotoxic nitrogen-containing heterocyclic stilbene analogues on VEGF protein secretion and VEGF, hTERT and c-Myc gene expression. Med. Chem. Comm. 6:1089-1815. (c) Martí-Centelles, R.; Cejudo-Marín, R.; Falomir, E.; Murga, J.; Carda, M.; Marco, J. A. (2013) Inhibition of VEGF expression in cancer cells and endothelial cell differentiation by synthetic stilbene derivatives. Bioorg. Med. Chem. 21:3010-3015. (d) Vilanova, C.; Díaz-Oltra, S.; Murga, J.; Falomir, E.; Carda, M.; Marco J. A. (2015) Inhibitory effect of pironetin analogue/colchicine hybrids on the expression of the VEGF, hTERT and c-Myc genes. Bioorg. Med. Chem. Lett. 25:3194-3198. (e) Ali, M. A.; Choy, H.; Habib, A. A.; Saha, D. (2007) SNS-032 prevents tumor cell-induced angiogenesis by inhibiting vascular endothelial growth factor. Neoplasia 9:370-381.

32. Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L.; Vandesompele, J.; Wittwer, C. T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55:611-622.

33. (a) Nakamura, M.; Abe, Y.; Tokunaga, T. (2002) Pathological significance of vascular endothelial growth factor A isoform expression in human cancer. Pathol. Int. 52:331-339. (b) Ellrichmann, M.; Ritter, P. R.; Schrader, H.; Schmidt, W. E.; Meier, J. J.; Schmitz, F. (2010) Gastrin stimulates the VEGF-A promoter in a human colon cancer cell line. Regulatory Peptides 165:146-150.

Supporting Information

Physical data. Physical and spectral data of compounds 1-36.