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Antiproliferative Activity of *trans*-Avicennol from *Zanthoxylum chiloperone* var. *angustifolium* against Human Cancer Stem Cells

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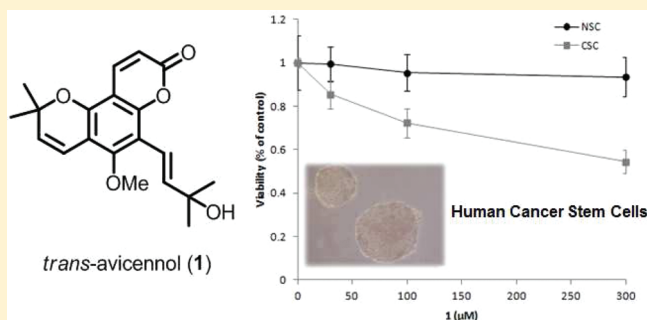
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Supporting Information

ABSTRACT: *Zanthoxylum chiloperone* var. *angustifolium* root bark was studied with the aim of finding novel molecules able to overcome cancer stem cell chemoresistance. Purification of a methanol-soluble extract resulted in the isolation of a known pyranocoumarin, *trans*-avicennol (1). Compound 1 demonstrated antiproliferative activity on glioma-initiating cells, whereas it was inactive on human neural stem cells. *trans*-Avicennol (1) activated the MAPK/ERK pathway and was also evaluated for its ability to inhibit the enzyme indoleamine-2,3-dioxygenase.



Gliomas represent the majority of primary tumors of the central nervous system, and the prognosis for patients with high-grade tumors is generally poor, with less than a 24-month survival after diagnosis.^{1,2} A subset of high-grade primary brain tumors that generate systematically long-term self-renewing floating spheres in vitro and give rise to novel tumors following xenotransplantation has been identified.³ This subset corresponds to malignant glioneuronal tumors,⁴ which are World Health Organization (WHO) grade III or IV gliomas that present glial fibrillary acidic protein (GFAP)- and neurofilament protein (NFP)-positive tumor cells.

Studies have identified subpopulations of cells within tumors that direct tumor growth and recurrence, called cancer stem cells (CSC).^{5–8} CSC are resistant to current cancer treatments, including radiation and chemotherapy.^{3,9–14} This suggests that many cancer therapies, while killing the majority of tumor cells, may fail because they do not eliminate CSC, which survive to regenerate new tumors.

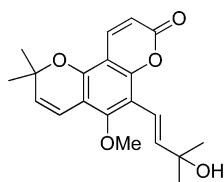
Recently, bioassay-guided investigations on plants used in traditional medicine and/or their active compounds have led to the discovery of many new antitumor agents.¹⁵ On the basis of the previous work of Itoigawa et al. and McKee et al.,^{16,17} the effects were investigated of *Zanthoxylum chiloperone* var. *angustifolium* Engl. (Rutaceae) root bark extract and one of its major components, *trans*-avicennol (1), a pyranocoumarin, on the viability of glioma cancer stem cells (gCSC). Many tumors develop the capacity to actively suppress an immune response. It has been demonstrated that most malignant tumor cells (including the CSC used in the present work) express a heme-containing dioxygenase, indoleamine 2,3-dioxygenase (IDO). This enzyme catalyzes the first and rate-limiting step in the major pathway of L-tryptophan catabolism in mammals.

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This metabolic pathway is involved not only in a variety of physiological functions but also in many diseases, with special emphasis on the important role in evasion of T-cell-mediated immune rejection.¹⁸ *trans*-Avicennol (**1**) was consequently evaluated as a possible inhibitor of IDO.

The composition of *Z. chiloperone* var. *angustifolium* root bark was analyzed by subjecting a MeOH extract to HPLC-UV-MS. This qualitative study identified the main compound present as *trans*-avicennol (**1**), which was identified by its retention time (t_R) and its mass spectrum (Figures S1 and S3, Supporting Information). Quantitative analysis was performed by an external standard method, using a calibration curve constructed using solutions containing different concentrations of a reference sample of **1**. The concentration of *trans*-avicennol (**1**) in the methanol extract of the root bark was 518 mg/g; approximately 50% of the mass of the crude extract was determined to be pure *trans*-avicennol (**1**) (Figure S4, Supporting Information).



trans-avicennol (**1**)

High-grade glioma cancer stem cells are largely characterized by their chemoresistance.^{3,9} The effects of the MeOH root extract of *Z. chiloperone* var. *angustifolium* and **1** were examined on two cultured gCSC taken from previously characterized collections, TG1 and TG10.³ TG10 cells have a p53 mutation, whereas TG1 cells do not. They were compared to human neural stem cells (NSC24) cultured as neurospheres in the same conditions. After 24 h of exposure to the MeOH extract or *trans*-avicennol (**1**), gCSC and NSC viability was determined by a WST-1 assay (water-soluble tetrazolium salt, a cell proliferation reagent). As shown in Figure S2 (Supporting Information), the methanol root bark extract of *Z. chiloperone* var. *angustifolium* led to TG1 and TG10 death: a concentration of 100 μ g/mL induced 90% of maximal viability loss in both cultures. In contrast, at the same concentration, the neural stem cell culture (NSC24) was less sensitive to the methanol root bark extract of *Z. chiloperone* var. *angustifolium* treatment, showing 30% of maximal viability loss (Figure S2, Supporting Information). The methanol root bark extract of *Z. chiloperone* var. *angustifolium* exhibited high cytotoxicity in gCSC and revealed no significant effects on NSC.

trans-Avicennol (**1**) induced pronounced cell death of gCSC (TG1 and TG10 cells), whereas it was without noticeable inhibitory effects on neural stem cells (NSC24) (Figure 1). However, the cytotoxicity induced by the active extract was only partially reproduced by pure isolated *trans*-avicennol (**1**) (Figure 1). The pure compound at a concentration of 34.2 μ g/mL led to 35% of maximal viability loss for TG1 cells and 40% for TG10 cells. Furthermore, NSC24 cells exhibited 30% of maximal viability loss when treated with the MeOH extract (100 μ g/mL) (Figure S2, Supporting Information), although *trans*-avicennol (**1**) itself (34.2 μ g/mL to 100 μ M) was inactive (Figure 1). The more potent cytotoxic activity of the MeOH extract compared to that of isolated compound **1** may be explained by the presence of other active compounds in the extract that possibly act synergistically.

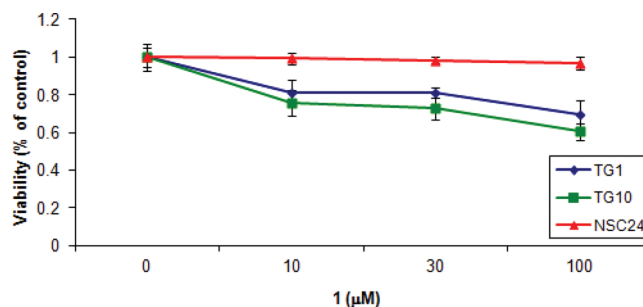


Figure 1. Effect of *trans*-avicennol (**1**) on glioma and neural stem cell viability ($n = 3$).

The MAPK/ERK signaling pathway has attracted considerable interest over the past decade, as it plays a central role in the regulation of cell proliferation and survival and contributes to oncogenic properties such as independence from growth factors. Moreover, the frequency with which this pathway is deregulated in human cancers makes this cascade an attractive target for drug development.¹⁹ Western blotting experiments were performed for phospho- and total-ERK from malignant glioma stem cells (TG1) and neural stem cells (NSC24) treated for 30 min with 100 μ M **1**. As shown in Figure 2, *trans*-

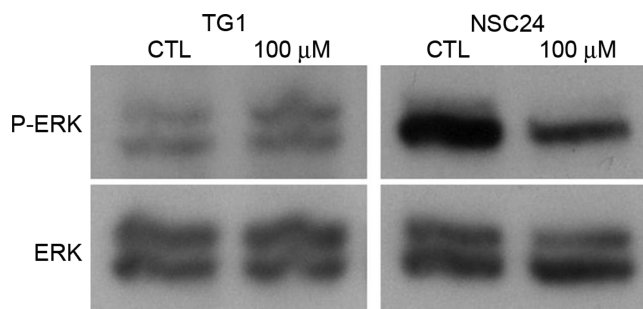


Figure 2. Effect of *trans*-avicennol (**1**) (100 μ M) on the ERK phosphorylation status of glioma and neural stem cells.

avicennol-treated TG1 cells exhibited higher levels of phospho-ERK in comparison with cells that were treated with the vehicle (DMSO). On the other hand, treatment of NSC24 cells with *trans*-avicennol (**1**) led to inhibition of ERK phosphorylation (Figure 2). These results suggest that ERK activation may participate in the effects observed for **1**.

Computer-assisted drug design has suggested that *trans*-avicennol (**1**) may target the enzyme indoleamine 2,3-dioxygenase (code PDB: 2d0t),²⁰ as it fits closely inside the active enzymatic pocket (Figure 3) and has a 3D structure very similar to that of phenylimidazole (PI), a known inhibitor of IDO. The phenyl nuclei of both PI and *trans*-avicennol (**1**) are well superimposed (Figure S5, Supporting Information). In agreement with a proposed binding model of *trans*-avicennol (**1**), a recent study by Kumar et al. showed that the four most potent synthetic PI analogues appear to establish interactions with Ser167 and Cys129.²¹ Another investigation supports this model.²² Röhrig and associates, utilizing a pharmacophore model derived from docking of known IDO inhibitors, examined the geometries of the docked ligands and concluded that a good IDO ligand should contain an aromatic fragment and be at least bicyclic, to fill the same pocket as PI, as well as have groups that can hydrogen bond to Ser167, Gly262, Ala264, and Arg231. Almost all known IDO inhibitors comply

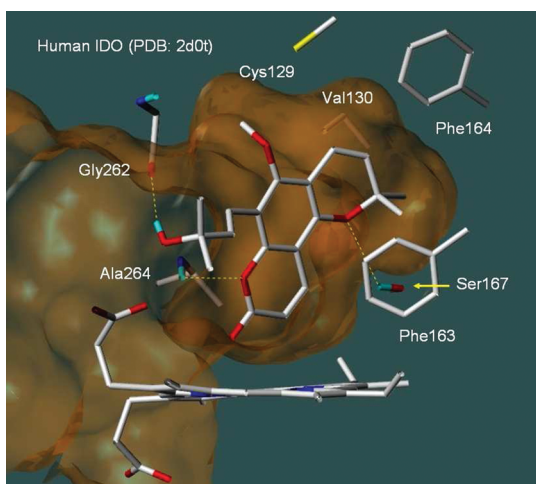


Figure 3. Docking result of the X-ray structure of indoleamine 2,3-dioxygenase (IDO) complexed with *trans*-avicennol (**1**).

with these parameters, as does **1**. As previously reported for the crystal structure of human IDO complexed with the ligand inhibitor PI at a resolution of 2.3 Å, the molecule exhibits the same interactions as PI. Phe163 interacts with **1** in a π - π stacking interaction. Ser167 forms a hydrogen bond with the oxygen of the pyran motif at 2.6 Å. Phe164, Val130, and Cys129 may also contribute via hydrophobic interactions. On the other hand, the NH group belonging to the backbone of Ala264 forms a hydrogen bond at 2.4 Å with the oxygen of the pyranone part of *trans*-avicennol (**1**). This NH, belonging to Ala269, has been proposed to interact with the ligand inhibitor cyanide when complexed with IDO at 3.4 Å.²⁰ Finally, a third hydrogen bond interaction appears between Gly262 and the hydroxy group occurring at position C-25 in **1** (Figure 3).

When tested experimentally on mouse IDO, *trans*-avicennol (**1**) showed a slight ability to competitively inhibit the enzyme, with a K_i value of 635 μ M, although this is much higher than the K_i values of known IDO inhibitors (Figure S6, Supporting Information).

EXPERIMENTAL SECTION

General Experimental Procedures. IR spectra were recorded on a Bruker Vector 22 spectrometer, and values are reported in cm^{-1} . NMR spectra were recorded on a Bruker AM-400 spectrometer and calibrated using undeuterated solvent as an internal reference. Mass spectra were recorded at the Service d'Analyse de Médicaments et Métabolites, SAMM, Châtenay-Malabry, France. Merck Kieselgel silica gel (60, particle size 40–63 μ m) was used for flash chromatography. Extraction was monitored by TLC carried out on Merck Kieselgel silica gel plates (60F-254) using UV light as the visualizing agent and vanillin–sulfuric acid or Dragendorff's reagent and heat as developing agent.

Plant Material. The root bark of *Z. chiloperone* var. *angustifolium* was collected by one of the authors (M.E.F.), in the Department of Cordillera, in January 2009 and was identified by N. Soria (Department of Botany, National University of Asuncion, Asuncion, Paraguay). A voucher specimen (MEF 234) has been deposited at the Herbarium of Chemical Sciences Faculty, San Lorenzo, Paraguay.

Extraction and Isolation. Dried, powdered roots of *Z. chiloperone* var. *angustifolium* (120 g) were basified with ammonia, and extracted in a Soxhlet apparatus with MeOH for 3 days, replacing the solvent three times in total, resulting in 6 g of crude extract (5% yield). In order to purify this compound, the MeOH extract was subjected to silica gel flash column chromatography, eluted with CH_2Cl_2 –ethyl acetate (8:2), to obtain 42 fractions. Fractions 20–35 contained *trans*-

avicennol (**1**) (2 g, >99% purity, as determined by HPLC) (2.5% yield). Physical and spectroscopic data (IR, ^1H NMR, ^{13}C NMR, MS, HRMS) for **1** were in agreement with data available in the literature.²³ The use of the basic extraction conditions and Dragendorff's reagent was dictated by a concomitant search for alkaloids.

HPLC-UV-MS Qualitative Study. A 1100 series HPLC and a 1100 series LC/MSD spectrometer were utilized in an analytical study of the MeOH extract of *Z. chiloperone* var. *angustifolium* root bark with a 150 mm \times 4.6 mm Sunfire C_{18} column (Waters). The eluents were (A) H_2O + TFA 0.1% and (B) MeOH + 0.1% TFA. Separations were performed at room temperature by solvent gradient elution from 50% A/50% B to 20% A/80% B in 20 min then to 100% B in 12 min at a flow rate of 1 mL/min. The UV measurements were made at 240 and 260 nm, and the injection volume was 5 μ L. Peak identification was performed by comparison of the retention time, the UV absorption, and the mass spectrum at different wavelengths with a reference sample of *trans*-avicennol (**1**). The results were in agreement with data available for **1**²⁴ (see full characterization in the Supporting Information).

HPLC-UV Quantitative Study. A modular system comprising a 600 E controller and pump and a 2996 PDA UV detector (Waters) was utilized in the HPLC-UV quantification experiment. The quantification of compound **1** was performed by an external standard method,²⁵ using *trans*-avicennol (**1**) (Figure S4, Supporting Information). *trans*-Avicennol (**1**) stock solutions of 10, 50, 100, 150, 200, and 300 μ g/mL were utilized. Each determination was carried out in triplicate, with an injection volume of 10 μ L (Supporting Information). The calibration curve ($r^2 = 0.999$) showed the linearity of the measurement over the range tested (10–300 μ g/mL). The regression equation is shown in the corresponding table (Supporting Information). Quantitative evaluation of the previously identified peak was performed by comparing the integrated peak area with that of the corresponding reference peak.

MGNT Stem Cell Culture. Nonadherent cultures of human MGNT stem cells (TG1 and TG10) were grown in 75 cm^2 tissue culture flasks (2500–5000 cells/ cm^2) in Dulbecco's modified Eagle's medium-F12, supplemented with B27, N2, and G5 (all from Invitrogen), as described previously.³ All cultures were maintained at 37 $^\circ\text{C}$ in a humidified atmosphere with 5% CO_2 .

Isolation and Characterization of Neural Stem Cells from Human Fetal Brain. All studies with human tissue were performed under ethical approval from the University Paris-Descartes internal review board using tissue donated with informed consent after elective termination of pregnancy. Human fetal brains at embryonic day 50–55 (Carnegie stage 19–22) were carefully dissected and mechanically dissociated into single cell suspensions. Primary cells were cultured in the form of floating spheres in NeuroCult NSC Basal Medium supplemented with NeuroCult proliferation supplements (StemCell Technologies), human epidermal growth factor (EGF) (20 ng/mL), human basic fibroblast growth factor (b-FGF) (10 ng/mL) (AbCys), and heparin (20 ng/mL) (Sigma-Aldrich). The primary culture of neural stem cells (NSC24) was established and expanded with a passage every two weeks. No culture crisis, spontaneous differentiation, or abnormal genetic derivation, as assessed by comparative genomic hybridization arrays, was detected over one year of continuous culture. Clonal properties were determined after 4 months of culture (passage 12) by manual deposition of single cells in 100 μ L into noncoated 96-well plates, followed by the addition of 50 μ L of medium every two weeks for two months. A total of $5.9 \pm 1.2\%$ of NSC24 cells and $4.5 \pm 1.6\%$ of NSC25 cells yielded spheres. Immunocytochemical analysis showed that most cells expressed the NSC markers Sox2, Bmi1, and Nestin, as well as the ESC marker Nanog, while no cells immunoreactive for synaptophysin, phosphorylated neurofilaments, or NeuN were observed.²⁶

Viability Assays. The viability of cell cultures was assessed by quantifying the reduction of the cell proliferation reagent WST-1 to a water-soluble formazan. Briefly, dissociated sphere-derived TG1, TG10, and NSC24 cells were plated in 96-well plates at 2×10^4 cells/well and treated for 24 h. Thereafter, 10 μ L/well of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfo-

nate (WST-1, Roche) was added to the culture medium and incubated at 37 °C for 3 h. The negative control groups were treated with DMSO diluted in the culture medium at the higher equivalent volume used in the treated groups and showed no significant effects on the parameters analyzed compared to cells that did not receive the vehicle. The absorbance was read at 430 nm in a microplate reader (Expert Plus V1, 4 ASYS).

Western Blotting. After the treatment of TG1 and NSC24 cells with *trans*-avicennol (**1**) for 30 min, cells were washed in cold PBS and extracts were prepared by direct cell lysis in PIPES buffer (25 mM PIPES pH 6.8, 1% Triton, 0.5 mM EDTA, 0.5 mM EGTA) in the presence of protease and phosphatase inhibitors. Cell lysates were electrophoresed in NuPAGE 4–12% Bis-Tris gels (Invitrogen). The blots were then blocked at room temperature for 2 h with TBS-T buffer (50 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween 20) containing 5% fat-free milk. Blots were exposed to primary and secondary antibodies following the manufacturer's instructions. Phospho-p42/p44 ERK antibody was purchased from Cell Signaling, and p42/p44 ERK was from Millipore. The immunoreactivity was revealed by chemiluminescence (Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA).

Statistical Analysis. The number of experimental replicates is given in the figure legends. Data obtained from independent experiments were reported as means \pm SE. Student's *t* test analysis was performed to determine statistical significance.

Molecular Modeling. The X-ray structure of indoleamine 2,3-dioxygenase complexed with PI (code PDB: 2d0t) was used for this study. First, PI was removed from the active site. Hydrogen atoms were added using Sybyl 7.3 (<http://www.tripos.com>), and energy minimization using AMBER 8.0²⁷ was performed keeping the α -carbons constrained by a constraint force of 50 kcal/mol Å², which permitted side-chains to be free to move while retaining the same secondary structures. Over the refined model the active site of IDO was defined as 10 Å around the crystallized PI. As a first test, PI was docked to the IDO active site, observing good superposition with the X-ray structure (rmsd of 0.3 Å). A docking of *trans*-avicennol (**1**) was then performed using the program GOLD v4.1.2.²⁸ The GOLD program was used with default settings for the genetic algorithm (GA) parameters. To allow poor nonbonded contacts at the start of each GA run, the maximum distance between hydrogen donors and fitting points was set to 5 Å. To further speed up the calculation, the GA docking was stopped when the top three solutions were within 1.5 Å root-mean-square deviations. It can be assumed that when these criteria are met, these top solutions represent a reproducible pose for the ligand. *trans*-Avicennol (**1**) was built with ChemDraw (<http://www.cambridgesoft.com>) and converted to 3D using the module unity translates from Sybyl 8.0. After a first energy minimization, a geometry optimization was carried out using the ab initio Hartree-Fock method, HF/6-31 basis set level, using the program Gaussian 03 (<http://www.gaussian.com>).

Mouse Indoleamine 2,3-Dioxygenase Expression. Trueclone mouse IDO cDNA was obtained from Origene and transfected into HEK 293 cells using Transfast reagent (Promega) according to the manufacturer's instructions. A HEK-expressing clone was selected using 1.2 µg/mL G418 for the enzymatic test activity.

Inhibition Assays. Indoleamine 2,3-dioxygenase activity was measured according to Matin et al. on mouse IDO-expressing cell lysates.²⁹ Cells recovered in PBS containing complete mini-protease inhibitors (Roche) were submitted to four cycles of freezing and thawing. After centrifugation at 13000 g for 10 min, the soluble fraction was collected. The protein concentration was determined using a Biorad protein assay (Marnes-la-Coquette, France), with bovine serum albumin as the standard, and 10 µg added to each test. For inhibition, **1** was solubilized in dimethylsulfoxide (DMSO) and diluted serially to 100 and 50 µM into IDO reactions in which tryptophan was titrated from 0 to 200 mM (100 µL final volume). 1-Methyltryptophan was used as a positive control inhibitor.

Plates were incubated for 2 h in a 37 °C incubator. Reactions were terminated by the addition of 20 µL of 30% trichloroacetic acid per well and further incubated 15 min at 60 °C to hydrolyze the reaction

product, *N*-formylkynurenine, to kynurenine (Figure 6, Supporting Information). After a centrifugation of 5 min at 3000 rpm in an Eppendorf tabletop centrifuge, supernatants were transferred to a flat-bottomed 96-well plate and mixed with 100 µL of Ehrlich reagent (2% 4-dimethylaminobenzaldehyde w/v in glacial acetic acid), and the reaction product was quantified at 485 nm using an Optima plate reader. Data were analyzed using Prism software.

■ ASSOCIATED CONTENT

Supporting Information

HPLC/UV/MS data of **1**, HPLC quantitative study of *Z. chiloperone* var. *augustifolium* root bark methanol extract, docking results of PI and **1** with IDO, and IDO inhibition enzymatic assay results. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

*These coauthors contributed to the work equally.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Kleihues, P.; Cavenee, W. K. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Nervous System*; IARC Press: Lyon, 2000.
- (2) Louis, D. N.; Ohgaki, H.; Wiestler, O. D.; Cavenee, W. K.; Burger, P. C.; Jouvet, A.; Scheithauer, B. W.; Kleihues, P. *Acta Neuropathol.* **2007**, *2*, 97–109.
- (3) Patru, C.; Romao, L.; Varlet, P.; Coulombel, L.; Raponi, E.; Cadusseau, J.; Renault-Mihara, F.; Thirant, C.; Léonard, N.; Bernheim, A.; Mihalescu-Maingot, M.; Haiech, J.; Bieche, I.; Moura-Neto, V.; Daumas-Duport, C.; Junier, M. P.; Chneiweiss, H. *BMC Cancer* **2010**, *10*, 66.
- (4) Varlet, P.; Soni, D.; Miquel, C.; Roux, F. X.; Meder, J. F.; Chneiweiss, H.; Daumas-Duport, C. *Neurosurgery* **2004**, *55*, 1377–1391.
- (5) Lapidot, T.; Sirard, C.; Vormoor, J.; Murdoch, B.; Hoang, T.; Caceres-Cortes, J.; Minden, M.; Paterson, B.; Caligiuri, M. A.; Dick, J. E. *Nature* **1994**, *367*, 645–648.
- (6) Reya, T. S.; Morrison, J.; Clarke, M. F.; Weissman, I. L. *Nature* **2001**, *414*, 105–111.
- (7) Singh, S. K.; Clarke, I. D.; Terasaki, M.; Bonn, V. E.; Hawkins, C.; Squire, J.; Dirks, P. B. *Cancer Res.* **2003**, *63*, 5821–5828.
- (8) Schatton, T.; Murphy, G. F.; Frank, N. Y.; Yamaura, K.; Waaga-Gasser, A. M.; Gasser, M.; Zhan, Q.; Jordan, S.; Duncan, L. M.; Weishaupt, C.; Fuhlbrigge, R. C.; Kupper, T. S.; Sayegh, M. H.; Frank, M. H. *Nature* **2008**, *451*, 345–349.
- (9) Bao, S.; Wu, Q.; McLendon, R. E.; Hao, Y.; Shi, Q.; Hjelmeland, A. B.; Dewhirst, M. W.; Bigner, D. D.; Rich, J. N. *Nature* **2006**, *444*, 756–760.
- (10) Dean, M.; Fojo, T.; Bates, S. *Nat. Rev. Cancer* **2005**, *5*, 275–284.

- (11) Diehn, M.; Clarke, M. F. *J. Natl. Cancer Inst.* **2006**, *98*, 1755–1757.
- (12) Diehn, M.; Cho, R. W.; Lobo, N. A.; Kalisky, T.; Dorie, M. J.; Kulp, A. N.; Qian, D.; Lam, J. S.; Ailles, L. E.; Wong, M.; Joshua, B.; Kaplan, M. J.; Wapnir, I.; Dirbas, F. M.; Somlo, G.; Garberoglio, C.; Paz, B.; Shen, J.; Lau, S. K.; Quake, S. R.; Brown, J. M.; Weissman, I. L.; Clarke, M. F. *Nature* **2009**, *458*, 780–783.
- (13) Eyler, C. E.; Rich, J. N. *J. Clin. Oncol.* **2008**, *26*, 2839–2845.
- (14) Woodward, W. A.; Chen, M. S.; Behbod, F.; Alfaro, M. P.; Buchholz, T. A.; Rosen, J. M. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 618–623.
- (15) Sarkar, F. H.; Li, Y. *Cancer Treat. Rev.* **2009**, *35*, 597–607.
- (16) Itoigawa, M.; Ito, C.; Tan, H. T. W.; Kuchide, M.; Tokuda, H.; Nishino, H.; Fukuyawa, H. *Cancer Lett.* **2001**, *169*, 15–19.
- (17) McKee, T. C.; Covington, C. D.; Fuller, R. W.; Bokesch, H. R.; Young, S.; Cardellina, J. H.; Kadushin, M. R.; Soejarto, D. D.; Stevens, P. F.; Cragg, G. M.; Boyd, M. R. *J. Nat. Prod.* **1998**, *61*, 1252–1256.
- (18) Muller, J.; Prendergast, G. C. *Cancer Res.* **2005**, *65*, 8065–8068.
- (19) Montagut, C.; Settleman, J. *Cancer Lett.* **2009**, *283*, 125–134.
- (20) Sugimoto, H.; Oda, S.; Otsuki, T.; Hino, T.; Yoshida, T.; Shiro, Y. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 2611–2616.
- (21) Kumar, S.; Jaller, D.; Patel, B.; LaLonde, J. M.; DuHadaway, J. B.; Malachowski, W. P.; Prendergast, G. C.; Muller, A. J. *J. Med. Chem.* **2008**, *51*, 4968–4977.
- (22) Röhrig, U. F.; Awad, L.; Grosdidier, A.; Larrieu, P.; Stroobant, V.; Colau, D.; Cerundolo, V.; Simpson, A. J.; Vogel, P.; Van den Eynde, B. J.; Zoete, V.; Michielin, O. *J. Med. Chem.* **2010**, *53*, 1172–1189.
- (23) Soriano-Agatón, F.; Lagoutte, D.; Poupon, E.; Roblot, F.; Fournet, A.; Gantier, J.-C.; Hocquemiller, R. *J. Nat. Prod.* **2005**, *68*, 1581–1587.
- (24) Cebrián-Torrejón, G.; Spelman, K.; Muñoz-Durango, K.; Torijano Gutiérrez, S.; Leblanc, K.; Ferreira, M. E.; Rojas de Arias, A.; Figadère, B.; Fournet, A.; Maciuk, A.; Grellier, P.; Cech, N. B.; Poupon, E. *Rev. Bras. Farmacogn.* **2011**, *21*, 652–661.
- (25) Ferreira, M. E.; Cebrián-Torrejón, G.; de Bilbao, N. V.; Leblanc, K.; Yaluf, G.; Schinini, A.; Serna, E.; Rolón, M.; Rojas de Arias, A.; Poupon, E.; Fournet, A. *J. Ethnopharmacol.* **2011**, *133*, 986–993.
- (26) Thirant, C.; Bessette, B.; Varlet, P.; Puget, S.; Cadusseau, J.; Dos Reis Tavares, S.; Studler, J. M.; Silvestre, D. C.; Susini, A.; Villa, C.; Miquel, C.; Bogeas, A.; Surena, A. L.; Dias-Morais, A.; Léonard, N.; Pflumio, F.; Bièche, I.; Boussin, F. D.; Sainte-Rose, C.; Grill, J.; Daumas-Duport, C.; Chneiweiss, H.; Junier, M. P. *PLoS One* **2011**, *28*, e16375.
- (27) Case, D. A.; Darden, T. A.; Cheatham, T. E., III; Simmerling, C. L.; Wang, J.; Duke, R. E.; Luo, R.; Merz, K. M.; Wang, B.; Pearlman, D. A.; Crowley, M.; Brozell, S.; Tsui, V.; Gohlke, H.; Mongan, J.; Hornak, V.; Cui, G.; Beroza, P.; Schafmeister, C.; Caldwell, J. W.; Ross, W. S.; Kollman, P. A. *AMBER8*; University of California: San Francisco, 2008.
- (28) Verdonk, M. L.; Cole, J. C.; Hartshorn, M. J.; Murray, C. W.; Taylor, R. D. *Proteins* **2003**, *52*, 609–623.
- (29) Matin, A.; Streete, I. M.; Jamie, I. M.; Truscott, R. J.; Jamie, J. F. *Anal. Biochem.* **2006**, *349*, 96–102.