CHEMMEDCHEM

DOI: 10.1002/cmdc.201000305

Design, Synthesis, and Biological Evaluation of the First Podophyllotoxin Analogues as Potential Vascular-Disrupting Agents

Raphaël Labruère,[a, b] Benoît Gautier,[b] Marlène Testud,[a] Johanne Seguin,[c] Christine Lenoir,[b] Stéphanie Desbène-Finck,[a] Philippe Helissey,[a] Christiane Garbay,[b] Guy G. Chabot,[c] Michel Vidal,[b] and Sylviane Giorgi-Renault*[a]

We designed and synthesized two novel series of azapodophyllotoxin analogues as potential antivascular agents. A linker was inserted between the trimethoxyphenyl ring E and the tetracyclic ABCD moiety of the 4-aza-1,2-didehydropodophyllotoxins. In the first series, the linker enables free rotation between the two moieties; in the second series, conformational restriction of the E nucleus was considered. We have identified several new compounds with inhibitory activity toward tubulin polymerization similar to that of CA-4 and colchicine, while displaying low cytotoxic activity against normal and/or cancer cells. An aminologue and a methylenic analogue were shown to disrupt endothelial cell cords on Matrigel at subtoxic concentrations, and an original assay of drug washout allowed us to demonstrate the rapid reversibility of this effect. These two new analogues are promising leads for the development of vascular-disrupting agents in the podophyllotoxin series.

Introduction

The recognition that tumor vasculature can be used as a specific target for cancer therapy has been a breakthrough for cancer treatment.[1] Indeed, tumor vasculature is needed to provide oxygen and nutrients to tumor cells, and is also the main route for metastatic spread. In addition, because a single vessel can support the survival of millions of tumor cells, the targeting of tumor vasculature is crucial for killing the maximum number of tumor cells.

The antivascular approach aims to selectively cause the collapse of newly formed capillaries by using small-molecule vascular-disrupting agents (VDAs), as opposed to antiangiogenic therapy, which aims to prevent the formation of new tumor blood vessels from the pre-existing vasculature. The specificity of VDAs is mainly due to major differences between normal and tumor vessels, as the latter are highly disorganized. Tumor vessels are fragile, have abnormal permeability and diameter, and are characterized by unstable endothelial intercellular junctions.[2]

Existing small-molecular-weight VDAs are predominantly tubulin-binding agents.[3] However, some derivatives, including flavone acetic acid and ASA404, belong to a second class that acts through a pro-inflammatory pathway. Microtubules are essential structural components of the cytoskeleton and are involved in several important cellular processes such as mitosis, vesicular transport, cell signaling, and shape maintenance. Microtubules are dynamic structures, formed of α- and β-tubulin heterodimers.[4] Historically, the search for tubulin-binding agents has attracted much attention for the development of anticancer drugs. Anti-microtubule agents can be divided into two classes: inhibitors of tubulin polymerization that bind to either the colchicine site or the vinca alkaloid site, and inhibitors of tubulin depolymerization that bind to the taxane site.[5] Early discovered VDAs are active only at doses near their maximum tolerated dose (MTD). Agents that disrupt the tumor vasculature at doses well below their MTD are currently undergoing preclinical and clinical investigations, including the phosphate prodrugs of combretastatin A-4 (fosbretabulin) and A-1 (Oxi4503), AVE8062, and ABT-751, among others.[6] These compounds are used in combination with cytotoxic chemotherapy or radiotherapy.

Although the mechanism of action of VDAs has not been fully elucidated, it involves a rapid cytoskeletal remodeling of endothelial cells through interphase microtubule disruption, leading to shutdown of tumor blood flow within 2–6 h post-treatment. Simultaneous activation of the RhoA/Rho signaling

[a] Dr. R. Labruère, M. Testud, Dr. S. Desbène-Finck, Dr. P. Helissey, Prof. Dr. S. Giorgi-Renault
Laboratoire de Chimie Thérapeutique, UMR CNRS No. 8638
Université Paris Descartes
Faculté des Sciences Pharmaceutiques et Biologiques
4 avenue de l'Observatoire, 75270 Paris Cedex 06 (France)
Fax: (+33) 1-43-29-14-03
E-mail: sylviane.giorgi-renault@parisdescartes.fr

[b] Dr. R. Labruère, Dr. B. Gautier, C. Lenoir, Prof. Dr. C. Garbay, Prof. Dr. M. Vidal
Laboratoire de Pharmacochimie Moléculaire et Cellulaire, INSERM U648
Université Paris Descartes, UFR Biomédicale
45 rue des Saints Pères, 75006 Paris (France)

[c] J. Seguin, Dr. G. G. Chabot
Laboratoire de Pharmacologie Chimique, Génétique et Imagerie
INSERM U 1022-CNRS UMR 8151
4 avenue de l'Observatoire, 75270 Paris Cedex 06 (France)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201000305.

pathway allows endothelial cells to adopt a rounded morphology and form actin stress fibers, promoting disruption of VE cadherin junctions and membrane blebbing. These changes enhance endothelial monolayer permeability and cause blood leakage and vessel collapse, leading to blood flow shutdown and central necrosis of the tumor.[7]

Identification of microtubule-binding drugs with greater therapeutic antivascular selectivity, relative to their cancer cell cytotoxicity, is an important objective for the next generation of VDAs.[3a] In this paper, we present our investigation of a series of nitrogen analogues of podophyllotoxin (Figure 1).

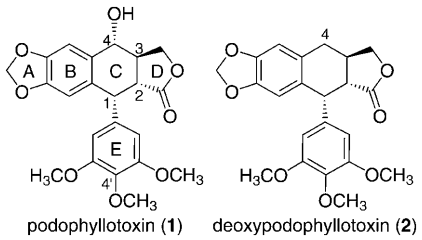


Figure 1. Structures of podophyllotoxin and deoxypodophyllotoxin.

Podophyllotoxin (**1**), a naturally occurring cyclolignan isolated from *Podophyllum* species, is a well-known cytotoxic derivative that acts as a potent anti-microtubule agent.[8] In spite of potential uses as a medicinal drug, human trials with podophyllotoxin were discontinued due to its systemic toxicity.[9] Over the last 20 years, extensive structural modifications of podophyllotoxin have led to the synthesis of etoposide, a glycosylated epimer of 4′-demethylpodophyllotoxin, which is presently in clinical use for the treatment of small cell lung cancer, testicular carcinoma, acute leukemia, and lymphoma, among others. However, etoposide is a topoisomerase II inhibitor and, therefore, has an entirely different mechanism of action than that of the parent compound. Many structural podophyllotoxin analogues have been described, although research in this area has mainly focused on the development of new topoisomerase II inhibitors.[10]

Podophyllotoxin acts at the colchicine binding site of tubulin.[11] The binding mode of colchicine was recently confirmed by an X-ray structure of α,β-tubulin complexed with DAMA-colchicine.[12] Colchicine and podophyllotoxin bind to β-tubulin at its interface with α-tubulin, with the trimethoxyphenyl nucleus hidden within the β-subunit.

Vascular disruption properties reported for antitubulin agents have surprisingly not been exploited to date in podophyllotoxin analogue studies. Recently, the antiangiogenic activity of deoxypodophyllotoxin (**2**) (Figure 1), a naturally occurring analogue of **1** without a hydroxy group at the C4 position, was reported.[13] Compound **2** inhibits the tube-like formation of human umbilical vein endothelial cells (HUVEC) at non-cytotoxic concentrations. Moreover, deoxypodophyllotoxin is several-fold more potent against HUVEC than against several cancer cell lines (A549, SK-OV, SK-Mel-2, HCT15, and B16F10).[13] These results suggest that podophyllotoxin analogues may be of value as potential new VDAs.

We have synthesized aza-analogues of podophyllotoxin (**1**) as a new class of antimitotic agents, with 4-aza-2,3-didehydropodophyllotoxin S 26711 (**3**) and *N*-methyl analogue S 26390 (**4**) as the hit compounds for this cytotoxic series (Figure 2).[14]

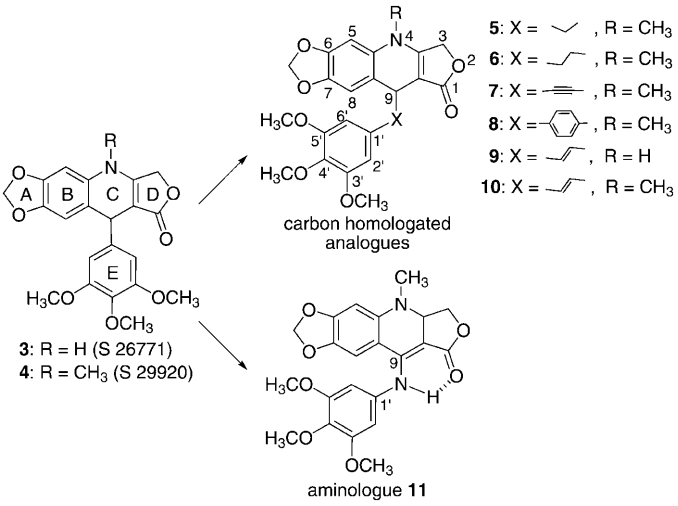


Figure 2. Design of carbon homologated analogues **5**–**10** and aminologue **11** as potential VDAs.

From a chemical point of view, the 4-aza-2,3-didehydro analogues present the advantages of possessing only one chiral center and a stable, unsaturated, lactone ring. Indeed, under physiological conditions, epimerization at C2 of the *trans*-fused γ-lactone of podophyllotoxin leads to the thermodynamically stable but inactive *cis*-epimer. We therefore aimed to design and synthesize novel 4-azapodophyllotoxin analogues as potential selective antivascular agents that maintain tubulin affinity but exhibit lower cytotoxicity against normal and/or cancer cells, in other words, compounds that are active as VDAs at subtoxic concentrations.

Within the 4-aza-2,3-didehydropodophyllotoxin series,[14] several derivatives were found to exhibit low cytotoxicity and high tubulin affinity, making them good candidates for antivascular purposes. Taking into account these results and the SAR study in the podophyllotoxin series,[10c] coupled with the X-ray structure of the α,β-tubulin–podophyllotoxin complex,[12] we designed new azapodophyllotoxins with the E ring farther from the ABCD tetracycle, with complete or partial conformational restriction of the E nucleus.

The aim of the present work was to study two new series of azapodophyllotoxins. In the first series, the linker inserted between the E and ABCD moieties enabled free rotation around single bonds C9-X and X-C1′ (Figure 2). Modifications to these carbon homologues involved bond length and degree of bond saturation, resulting in homologues **5** and **6**, ethynologue **7**, benzologue **8**, and vinylogues **9** and **10**. In the second series, conformational restriction of the E nucleus was considered, resulting in aminologue **11**,[15] in which the C9 hybridization is

sp2 and rotation is blocked by creation of an additional pseudocycle (hydrogen bond between the amino moiety and the lactone carbonyl, Figure 2). A majority of analogues **5**–**11** are *N*-methylated for synthetic and stability purposes, as *N*-substitution of the 1,4-dihydroquinoline nucleus prevents aromatization. In the first study, the methylenedioxy moiety, the lactone, and the three methoxy groups of the E ring were maintained in order to compare biological results with parent compounds **3** and **4**. The potential antivascular activity of the synthesized compounds was assessed by several biological tests, including in vitro cytotoxicity, a tubulin polymerization inhibition assay, analysis of endothelial cell morphology, and a cord disruption/reorganization assay.

Results and Discussion

Synthesis

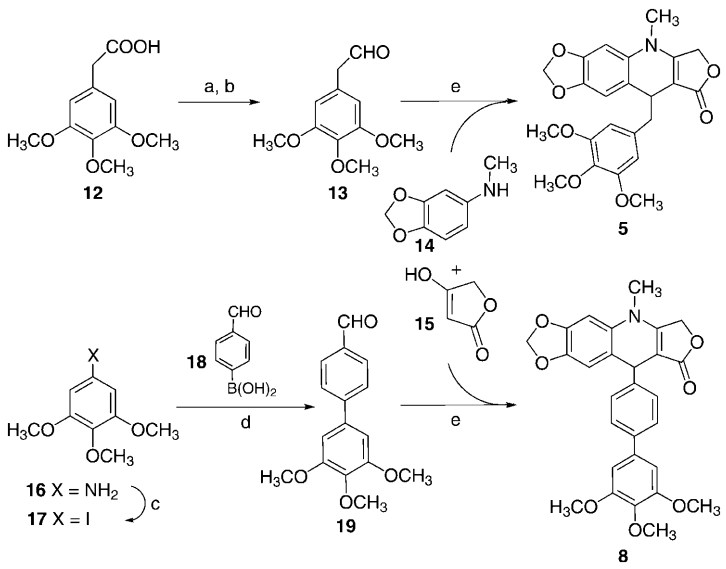
We previously reported an original three component one-pot reaction for the synthesis of 4-aza-2,3-didehydropodophyllotoxins.[16] The notable advantages of this method are mild conditions without activation, fast reaction times, and tolerance for structural diversity. Therefore, we decided to use this reaction for the preparation of the new carbon homologated analogues using the corresponding aldehydes. Using this one-pot procedure, involving tetronic acid **15**, *N*-methyl-3,4-methylenedioxyaniline (**14**), and either aldehyde **13** or **19**, the monomethylenic analogue **5** and benzologue **8** were synthesized in 50% and 97% yields, respectively (Scheme 1).

Trimethoxyphenylethanal **13** was prepared according to a two-step sequence developed by Nicolaou et al.[17] from commercially available trimethoxyphenylacetic acid **12**: reduction in the presence of $LiAlH_4$ to the corresponding alcohol, with subsequent oxidation by Dess–Martin periodinane. Benzologue **8** was obtained in four steps from commercially available trimethoxyaniline **16**. Diazotation of **16**, followed by substitution with potassium iodide, yielded trimethoxyiodobenzene **17**.[18] Carbaldehyde **19** was then synthesized by a palladium-catalyzed biaryl coupling between iodobenzene **17** and boronic acid **18**.[19]

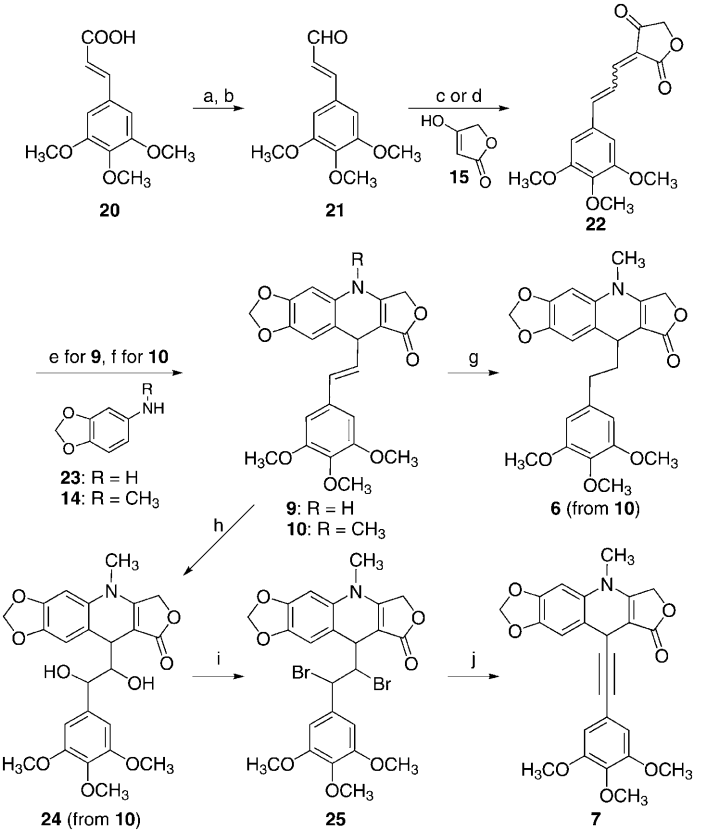
Vinylogue derivative **10** was used as a common precursor for both analogues **6** and **7**. Application of our one-pot, three component procedure to the preparation of vinylogues **9** and **10** resulted in poor yields, and subsequent isolation of pure compounds was very difficult. To circumvent this hurdle, we split this procedure into two distinct steps: condensation of trimethoxycinnamaldehyde **21** with tetronic acid **15**, followed by reaction of diene **22** with the aniline.

The preparation of **21** involved two-carbon homologation of the 3,4,5-trimethoxybenzaldehyde using either a Wittig reaction[20] or an aldolization-crotonization with the acetic acid vinyl ester.[21] We were unable to reproduce the latter synthesis, even under a variety of experimental conditions. We optimized the preparation of aldehyde **21** adopting the procedure described for the 3,4-dimethoxy analogues,[22] that is, reaction of the commercially available acid **20** with thionyl chloride, followed by reduction of the resulting acyl chloride by $LiAlH(OtBu)_3$ in dry THF. In a second step, three equivalents of **21** were reacted with one equivalent of tetronic acid **15** in hydrochloric acid, according to a procedure described for the unsubstituted cinnamaldehyde.[23] Benzylidene **22** was obtained as a mixture of stereoisomers and in only 10% yield, likely due to the instability of the compound in acidic medium. Indeed, stoichiometric condensation between **15** and **21** under neutral conditions afforded **22** in 69% yield, but as a single stereoisomer which was not further identified. Further reaction of **22** (as a mixture or as a single stereoisomer) and aniline **23** in ethanol at reflux led to vinylogue **9** in 24% yield. The *Z* conformation was established by NMR spectroscopy, in particular, by the 16 Hz ^3J coupling constant between the two ethylenic hydrogens ($\delta =$ 6.20 and 6.30 ppm). To avoid formation of the corresponding quinoline, the reaction was stopped immediately after disappearance of the starting materials as determined by TLC. Dihydroquinoline **9** was purified by recrystallization from a dichloromethane/methanol mixture, due to aromatization of the compound during flash column chromatography on silica gel or alumina.

For *N*-methylated analogue **10**, the yield was enhanced to 50% by treating aldehyde **22** with aniline **14** at reflux in dichloromethane instead of ethanol (Scheme 2). The two-carbon homologated analogue **6** was obtained in a very good yield by palladium-catalyzed hydrogenation of the extracyclic double bond of vinylogue **10** without any reduction of the lactone double bond (Scheme 2). This regioselectivity can be explained by the strong electron delocalization of the vinylogous carbamate function, which



Scheme 1. Synthesis of azapodophyllotoxin analogues **5** and **8**: a) $LiAlH_4$, anhydrous THF, 0–5 °C, 3 h, 90%; b) Dess–Martin periodinane, CH_2Cl_2, 25 °C, 1 h, 65%; c) $NaNO_2$, KI, H_2SO_4/H_2O, 50 °C, 1 h, 70%; d) **18**, $Pd(PPh_3)_4$, K_2CO_3, DMF, 65 °C, 15 h, 80%; e) EtOH, reflux, 1 h, 50% for **5** and 4 h, 97% for **8**.



Scheme 2. Synthesis of azapodophyllotoxin analogues **6**, **7**, **9**, and **10**: a) $SOCl_2$, 1,2-dichloroethane, reflux, 2 h; b) $LiAlH(OtBu)_3$, anhydrous THF, 0–5 °C, 2 h, 59% over two steps; c) **15**, HCl trituration, 25 °C, 15 min, 10%, **22** (obtained as a mixture of stereoisomers); d) **15**, EtOH, reflux, 10 h, 69%, **22** (obtained as a single stereoisomer); e) **23**, EtOH, reflux, 5 min, 24%; f) **14**, CH_2Cl_2, reflux, 1 h, 50%; g) 10% Pd/C, 10 bar H_2, MeOH, 25 °C, 2 h, 98%; h) OsO_4, NMO, CH_3CN/H_2O: 4/1, 25 °C, 24 h, 80%; i) MsCl, Et_3N, anhydrous CH_2Cl_2, 0 °C, 15 min, then LiBr, 25 °C, 48 h, 60%; j) *t*BuOK, anhydrous THF, 25 °C, 3 h, 71%.

causes partial loss of the ethylenic character of the intracyclic double bond.

In regards to ethynologue **7**, the general 4-azapodophyllotoxin synthetic route, either in one step or in two sequential steps, was unsuccessful for its preparation from vinylogue **10**. Attempts at direct deshydrogenation of **10** into **7** using manganese dioxide, selenium dioxide, or dichlorodicyanoquinone as oxidizing agents failed. Consequently, we elaborated an original pathway involving an addition-elimination procedure for the synthesis of ethynologue **7** (Scheme 2). In the first step, **10** was combined with osmium tetroxide to give diol **24** without hydroxylation of the intracyclic double bond for the same reason as mentioned above. An in situ, two-step procedure, i.e., mesylation of diol **24** and subsequent addition of LiBr, resulted in a 60% yield of compound **25** upon optimization of the reaction conditions. Finally, treatment of **25** with potassium *tert*-butoxide in THF at room temperature afforded ethynologue **7** in a 71% yield. Amino analogue **11** was synthesized according to our previously reported procedure.[15] Two structurally relevant synthetic intermediates of this process, quinoline **26**[15] and imine **27**[15] (Figure 3), were also biologically evaluated.

Biological evaluation

In order to determine the vascular-disrupting potential of these novel azapodophyllotoxin analogues, we first evaluated their inhibitory activity toward tubulin polymerization. These inhibition of tubulin polymerization (ITP) values are presented in Table 1 and are expressed as the ratio of the IC_{50} value of a given derivative over the IC_{50} value of colchicine. The inhibitory activities of combretastatin A-4 (CA-4) and S 26711 (**3**) are also presented for comparison purposes. As expected, reference compounds CA-4 (ITP = 0.72) and **3** (ITP = 0.47) were both strong anti-microtubule agents, with IC_{50} values lower than that of colchicine. Compounds **5**–**8**, **10**, and **27** were considered inactive for tubulin polymerization inhibition because they presented ITP values greater than 40. In the carbon homologue series, only *N*-unsubstituted vinylogue **9** showed significant anti-microtubule activity (ITP = 0.75). Insertion of a double bond between the tetraline moiety and the trimethoxyphenyl ring of **3** is therefore possible, whereas introduction of both vinylogation and *N*-methylation (**10**, ITP > 40) resulted in complete loss of anti-microtubule activity. Furthermore, the combination of both a spacer group and *N*-methylation led to a complete loss in ITP activity, as shown for derivatives **5**–**8** and **10**. In contrast, with an amino spacer, *N*-methylation was well tolerated as aminologue **11** (ITP = 2.89) was found to exhibit significant ITP activity. Collectively, these results suggest that the tetracyclic *N*-substitution is not, in itself, detrimental to tubulin binding. In

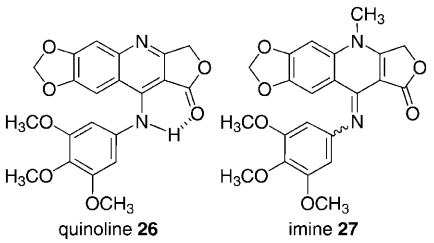


Figure 3. Structures of quinoline **26** and imine **27**.

this series, it is noteworthy that the presence of a hydrogen bond between the amino spacer and the lactone carbonyl seems to be critical for tubulin inhibition activity (e.g. aminologue **11** and quinoline **26**, as compared with inactive imine **27**).

Recently, a common pharmacophore for colchicine site inhibitors has been proposed to explain the structure-inhibition of tubulin assembly relationships.[24] Six pharmacophoric features have been identified for podophyllotoxin (**1**), such as two hydrophobic centers (trimethoxyphenyl and methylenedioxy groups), one planar group (phenyl nucleus from the tetracycle), two hydrogen bond acceptors (the lactonic C=O and

Table 1. Inhibition of tubulin polymerization, cytotoxicity, and morphological effects on EA.hy 926 endothelial cells caused by azapodophyllotoxin analogues.

Compd	ITP[a]	Cytotoxicity: IC_{50} [μM][b]			Rounding [μM][c]
		B16	NIH 3T3	EA.hy 926	
CA-4	0.72	0.010 ± 0.003	0.020 ± 0.005	0.03 ± 0.01	0.01
3 (S 26711)	0.47	0.016 ± 0.001	0.15 ± 0.07	0.15 ± 0.05	0.01
5	**> 40**	**> 30**	**> 30**	**> 30**	**2.35**
6	> 40	> 30	> 30	> 30	28.46
7	> 40	> 30	> 30	> 30	> 30
8	> 40	> 10	> 10	> 10	> 10
9	0.75	0.41 ± 0.21	0.08 ± 0.02	0.77 ± 0.05	0.12
10	> 40	14.1 ± 2.9	7.5 ± 2.1	19.1 ± 1.8	2.29
11	**2.89**	**38.4 ± 10.1**	**2.1 ± 0.8**	**8.6 ± 2.4**	**0.12**
26	1.17	0.40 ± 0.01	0.5 ± 0.1	2.8 ± 1.5	1.95
27	> 40	> 30	> 30	> 30	> 30

[a] Inhibition of tubulin polymerization (ITP) is expressed as the ratio of $IC_{50\ compd}/IC_{50\ colchicine}$. $IC_{50\ compd}$ is the concentration of compound required to inhibit 50% of the rate of microtubule assembly (average of three experiments), and the average IC_{50} value for colchicine was 0.36 μM under our conditions. [b] Concentration of compound corresponding to 50% growth inhibition after 48 h incubation (average of three experiments ± SEM). [c] Morphological effects (rounding up) on modified HUVEC (EA.hy 926) are expressed as the lowest concentration at which cell rounding was observed following a 2 h incubation period with the test compound; experiments were done in triplicate.

the *meta*-OCH_3 from the trimethoxyphenyl ring), and one hydrogen bond donor (hydroxy group).

Our new derivatives display most of the pharmacophoric features reported for podophyllotoxin. In a recent publication, using isothermal titration calorimetry, this pharmacophoric proposition was only partially confirmed, with the hydrogen bond between the hydroxy group and Thr179 of α-tubulin found to be unnecessary for the podophyllotoxin–tubulin interaction.[25] This is in agreement with our observations that both *N*-methylated analogues **4** (data not shown) and **11** retained significant tubulin affinity. This series of novel 4-azapodophyllotoxin analogues is the first demonstration that a spacer group can be introduced between the trimethoxyphenyl and tetracycle ABCD (vinylogue **9** and aminologue **11**). As the trimethoxyphenyl group of podophyllotoxin is buried within a hydrophobic pocket of β-tubulin, the additional double bond may reinforce the van der Waals interactions between vinylazapodophyllotoxin **9** and tubulin.

Our overall goal was to obtain microtubule-binding drugs with high therapeutic antivascular selectivity relative to their cancer cell and/or normal cell cytotoxicity. In order to achieve the necessary results, the cytotoxicity of the new derivatives was evaluated using a solid tumor-derived murine B16 melanoma cell line. These results for each compound were compared with the cytotoxicity against a murine fibroblast NIH 3T3 cell line as an example of normal cells and against the EA.hy 926 cell line, which is considered one of the best immortalized HUVE cell (HUVEC) lines, because these cells express most of the biochemical markers of parental HUVEC.[26] The results presented in Table 1 reveal that the cytotoxic activities of reference compounds CA-4 and compound **3** are in the nanomolar range, while the new podophyllotoxin analogues **9**–**11** and **26** are less toxic (IC_{50} values in the micromolar range), as expected. The compounds showed similar activity toward B16 melanoma cells and in the other cell lines tested (i.e., NIH 3T3 and EA.hy 926 cells). We did not observe preferential selectivity toward a particular cell line, with the possible exception that fibroblast NIH 3T3 cells were more vulnerable to compounds **9**, **10**, and **11**, as compared to B16 and EA.hy 926 cells. It is also noteworthy that ITP and cell growth inhibition values correlated relatively well for most of the new compounds, with the exception of vinylogue **10**, which was significantly cytotoxic against the three cell lines but devoid of ITP effect. Therefore, the cytotoxicity of **10** is likely due to binding to a different cellular target.

The active compounds were less cytotoxic than reference compounds CA-4 and **3**. Indeed, the antiproliferative effects of vinylogue **9** and quinoline **26** against B16 cells were 40-fold lower than those of CA-4, whereas their anti-microtubule activities were similar. Notably, aminologue **11** displayed high ITP activity associated with poor B16 cytotoxicity. This profile (good ITP activity and low cytotoxicity) is considered a good indicator for antivascular activity.[3] Indeed, this could indicate that the cytotoxicity of our new derivatives could be attributed to specific binding to tubulin, rather than binding to several other targets which activate apoptotic pathways, or could be due to rapidly reversible kinetic binding to tubulin.

The new derivatives were also tested for their effects on the morphology of endothelial cells after a brief exposure time. Successful antivascular agents were shown to induce rapid endothelial cell retraction, due to both tubulin and actin cytoskeleton remodeling, leading to neovessel destructuring in vivo at concentrations below those required to block mitosis.[27] The morphological effects of the new compounds on EA.hy 926 endothelial cells is presented in Table 1 and was expressed as the lowest concentration at which cell rounding up was observed following a 2 h-incubation period with test compounds. As expected, both cytotoxic reference compounds CA-4 and **3** caused the rounding up of endothelial cells at nanomolar concentrations. Among the newly synthesized derivatives, compounds **5**, **9**–**11**, and **26** were found to change the morphology of EA.hy 926 endothelial cells after a 2 h-exposure period at either non toxic or sub-cytotoxic concentrations in the low micromolar range for the same cell line (Table 1). Figure 4 shows representative photographs of EA.hy 926 cells exposed to the morphologically active compounds. Control cells exposed to the solvent (1% DMSO) were not affected and presented an elongated morphology, whereas active compounds induced the typical rounding up effect observed with antivascular agents. Vinylogue **9** and aminologue **11** caused rounding up at concentrations 6- and 72-fold lower than their IC_{50} values against this cell line, respectively. Interestingly, homologues **5**

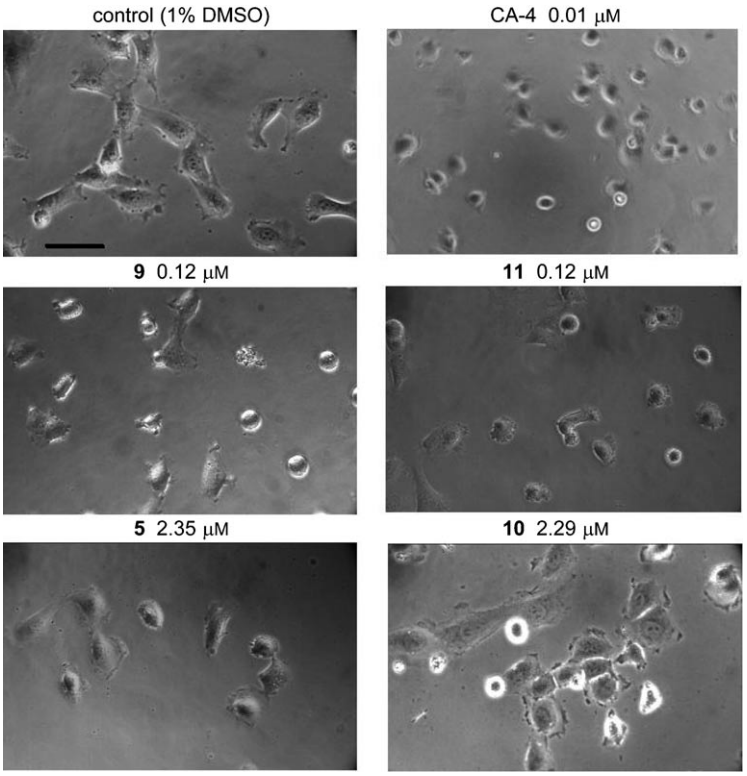


Figure 4. Morphological effects of selected azapodophyllotoxin analogues on EA.hy 926 endothelial cells. Exponentially growing cells were exposed to the indicated compound and incubated at 37 °C for 2 h at the indicated concentrations. Representative photographs were taken at 360× magnification. Scale bar, 20 μm.

and **10** produce a potent morphological effect which is not related to their antitubulin activity. In addition, the methane bridge homologue **5** was less morphologically active (2.35 μM) but was devoid of cytotoxic activity for the three cell lines. In contrast, quinoline **26**, which was effective toward EA.hy 926 rounding up, could be considered as a cytotoxic agent. In fact, cytotoxic concentration in EA.hy 926 cells ($IC_{50} = 2.8$ μM) was in the same range as the rounding up effect ($IC_{50} = 1.95$ μM).

To further evaluate the antivascular potency of the four new podophyllotoxin analogues **5**, **9**, **10**, and **11**, their ability to disrupt newly formed endothelial cell cords on Matrigel was evaluated, as well as the reorganization of the tube-like network following drug washout. The latter assay is an important parameter, because it is recognized that rapidly reversible binding would lead to prompt drug clearance in vivo.[28] The dose-effect response of the selected 4-azapodophyllotoxins toward in vitro capillary tube disruption was evaluated by exposing pre-plated HUVEC on Matrigel to increasing concentrations of the compounds for 3 h. After 3 h exposure to various drug concentrations (shown in Supporting Information) covering the cytotoxic IC_{50} range of the B16 cell line (up to 20 μM and below the limit of solubility), the lowest dose causing tube-like disruption was determined for each derivative (Figure 5). Reference compound CA-4 was used as a cord disruptive control. Among the new azapodophyllotoxins, the amino analogue **11** was the most potent, altering the HUVEC cord network at 1 μM, with complete inhibition at 10 μM. Homologues **5** and **9** were also quite effective, leading to tube disruption at 10 μM. Treatment with **10** at concentrations up to 20 μM did not modify endothelial cell tubular structure (Figure 5 and Supporting Information). It is noteworthy that compounds **5** and **11** significantly disorganized HUVEC assembly at sub-cytotoxic doses in B16 cells.

With regard to the reorganization of tube-like structures 3 h after drug washout of unbound compound, a rapid network reconstruction was noted for compounds **5** and **11** (Figure 5). This particular reorganization effect was observed with the same intensity at higher concentrations (20 μM) for analogue **5**, whereas the amino analogue **11** was a very potent disruptor at 10 μM and, therefore, only partial reconstruction was observed after 3 h (see supporting information). Compound **9** also disrupted the cords at a highly cytotoxic concentration (10 μM), and its effect was not reversible after drug washout. This suggests that **9** and **11** may exhibit different tubulin binding kinetics which cause distinct vascular disruption activity. It has been shown that, unlike CA-4, colchicine and podophyllotoxin can elicit their effects on neovasculature at doses close to the maximum tolerated dose.[29] The wider therapeutic window of CA-4 has, therefore, been attributed to differences in the association/dissociation rates of tubulin binding[30] and to the pseudo-irreversibility of tubulin binding for colchicine and podophyllotoxin.[25,31] It is noteworthy that methylenic analogue **5** displayed a reversible antivascular effect in vitro, even though this compound is not a tubulin binding agent and is devoid of cytotoxicity at concentrations as high as 30 μM.

Conclusions

We have designed and synthesized two novel series of azapodophyllotoxin analogues as potential antivascular agents. In the first series, the linker inserted between the E and ABCD moieties enabled free rotation around single bonds C9-X and X-C1′. In the second series, conformational restriction of the E nucleus was pursued. The potential antivascular activity of the synthesized compounds has been assessed by several biological tests, including a tubulin polymerization inhibition assay, in vitro cytotoxicity, analysis of endothelial cell morphology, and a cords disruption/reorganization assay. Our results allowed identification of the first two podophyllotoxin analogues, **5** and **11**, as potential vascular disrupting agents with low toxicity. Compound **5** was of particular interest due to its activity toward endothelial cord disruption, while it was surprisingly found inactive toward tubulin polymerization. Aminologue **11** was shown to be particularly active as a potential new antivascular compound, as it displayed excellent ITP activity, low cytotoxicity, and a rapidly reversible endothelial cell cord disruption effect at subtoxic doses, as hypothesized. Combined, these results demonstrate that 4-azapodophyllotoxin analogues are

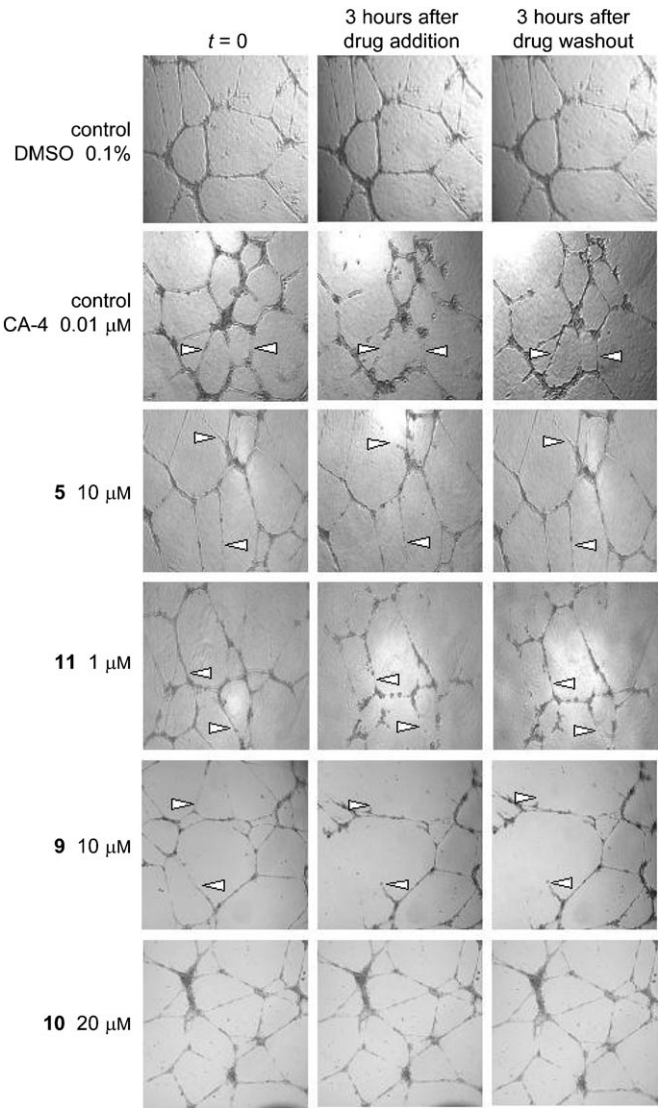


Figure 5. After allowing HUVE cells to form cords on Matrigel for 16 h, various concentrations of tested compounds were added to the media. The same microscopic field was recorded 3 h after drug addition and again 3 h after drug washout. A reversible effect in vitro was observed for **5**, **11**, and CA-4. The effect induced by **9** was not reversible, and **10** was not effective against tubular structures. Arrowheads indicate disrupted or reformed cords.

promising new leads for the development of selective and nontoxic vascular-disrupting agents.

Experimental Section

Chemistry

Commercial reagents (Fluka, Aldrich) were used without further purification, except for 3,4-methylenedioxyaniline which was recrystallized from cyclohexane. Solvents were distillated prior to use. Thin layer chromatography analyses were carried out on Merck GF 254 silica gel plates. Flash chromatography was carried out on silica gel 70 (30–70 μm). Melting points were determined with a Köfler apparatus and are uncorrected. IR spectra were recorded on a PerkinElmer 1600 spectrometer. NMR spectra were recorded on a Bruker AC spectrometer at 300 MHz for ^{1}H and at 75 MHz for ^{13}C. Elemental analyses were performed at the CNRS Analysis Laboratory (Gif-sur-Yvette, France) and were found to be within ±0.4% of theoretical values. Compounds **11**, **26**, and **27** were prepared as previously described.[15]

(9*RS*)-4-Methyl-6,7-(methylenedioxy)-9-(3,4,5-trimethoxybenzyl)-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (5). An equimolar mixture (1 mmol) of tetronic acid **15**, aldehyde **13**,[17] and aniline **14** in EtOH (10 mL) was held at reflux for 1 h. The resulting solid was removed by filtration and recrystallized from MeOH to afford **5** as a white powder (217 mg, 50%): R_f = 0.13 (CH_2Cl_2/EtOAc 9:1); mp: 200 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.80 (s, 3H), 2.85 (dd, J = 13 and 5 Hz, 1H), 3.00 (dd, J = 13 and 5 Hz, 1H), 3.65 (s, 6H), 3.80 (s, 3H), 4.25 (t, J = 5 Hz, 1H), 4.55 (d, J = 15 Hz, 1H), 4.65 (d, J = 15 Hz, 1H), 5.90 (s, 2H), 5.95 (s, 2H), 6.35 (s, 1H), 6.65 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 33.2, 36.2, 43.4, 55.9, 61.3, 65.0, 94.7, 99.7, 101.5, 106.7, 109.8, 118.6, 133.6, 134.3, 136.4, 143.9, 147.2, 152.2, 159.9, 173.1 ppm; IR (KBr): $\tilde{\nu}$ = 1742, 1662, 1590, 1500, 1483, 1421, 1245, 1230, 1209, 1128, 1035, 1007 cm^{-1}; Anal. calcd for $C_{23}H_{23}NO_7$·0.5 H_2O: C 63.59, H 5.57, N 3.22; found: C 63.83, H 5.37, N 3.28.

(9*RS*)-4-Methyl-6,7-(methylenedioxy)-9-(3′,4′,5′-trimethoxybiphenyl-4-yl)-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (8). An equimolar mixture (0.73 mmol) of tetronic acid **15**, aldehyde **19**,[19] and aniline **14** in EtOH (7 mL) was held at reflux for 4 h. The resulting solid was removed by filtration, purified by flash chromatography (EtOAc/CH_2Cl_2, 1:9), and then recrystallized from EtOH to afford **8** as a white solid (345 mg, 97%): R_f = 0.54 (EtOAc); mp: > 260 °C; ^{1}H NMR (300 MHz, [D_6] DMSO): δ = 3.25 (s, 3H), 3.87 (s, 3H), 3.90 (s, 6H), 4.80 (d, J = 15 Hz, 1H), 4.90 (d, J = 15 Hz, 1H), 5.10 (s, 1H), 5.90 (s, 1H), 5.95 (s, 1H), 6.55 (s, 1H), 6.60 (s, 1H), 6.70 (s, 2H), 7.25 (d, J = 8 Hz, 2H), 7.45 ppm (d, J = 8 Hz, 2H); ^{13}C NMR (75 MHz, [D_6] DMSO): δ = 33.6, 40.2, 56.1, 60.9, 65.0, 95.0, 97.5, 101.6, 104.4, 110.8, 118.6, 127.3, 128.2, 133.0, 137.0, 137.4, 139.8, 144.2, 144.9, 147.5, 153.3, 158.0, 173.2 ppm; IR (KBr): $\tilde{\nu}$ = 1748, 1663, 1589, 1482, 1345, 1249, 1204, 1126, 1036, 1002 cm^{-1}; Anal. calcd for $C_{28}H_{25}NO_7$: C 68.98, H 5.17, N 2.87; found: C 68.90, H 5.17, N 2.89.

(2*E*)-3-(3,4,5-Trimethoxyphenyl)prop-2-enal (21). A solution of (*E*)-3,4,5-trimethoxycinnamic acid (**20**) (2.38 g, 10 mmol) and $SOCl_2$ (2.2 mL, 30 mmol) in 1,2-dichloroethane (100 mL) was held at reflux for 2 h. The solvent was eliminated under reduced pressure, and the crude resulting yellow solid was dissolved in anhydrous THF (100 mL). A suspension of LiAlH(O*t*Bu)$_3$ (2.8 g, 11 mmol) in anhydrous THF (50 mL) was added dropwise to the stirred reaction mixture at 0–5 °C. The reaction mixture was then warmed to room temperature and stirring was continued for another 2 h. After addition of a solution of 1% aqueous HCl (300 mL), the mixture was extracted by CH_2Cl_2 (3×50 mL). The combined organic layers were dried over Na_2SO_4 and evaporated to give a crude residue, which was purified by flash chromatography (CH_2Cl_2/EtOAc, 9:1), followed by recrystallization from heptane to afford **21** as a white powder (1.3 g, 59%): mp: 110–111 °C; spectra and melting point are identical to published values.[32]

(3*E*) or (3*Z*)-3-[(2*E*)-3-(3,4,5-Trimethoxyphenyl)prop-2-enylidene]-furane-2,4(3*H*,5*H*)-dione (22). A suspension of aldehyde **21** (2.22 g, 10 mmol) and tetronic acid **15** (1 g, 10 mmol) in EtOH (10 mL) was held at reflux for 10 h. The resulting solid was removed by filtration, washed with EtOH, and recrystallized from EtOH to afford **22** as a red powder (2.16 g, 69%): R_f = 0.25 (CH_2Cl_2/EtOAc 95:5); mp: 172 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.95 (s, 9H), 4.60 (s, 2H), 6.90 (s, 2H), 7.40 (d, J = 15 Hz, 1H), 7.70 (d, J =

12 Hz, 1H), 8.15 ppm (dd, $J=15$ and 12 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=56.4$, 61.1, 72.0, 107.0, 114.7, 122.0, 130.0, 142.5, 151.6, 153.6, 155.9, 168.2, 195.5 ppm; IR (KBr): $\tilde{\nu}=1752$, 1706, 1601, 1578, 1560, 1509, 1500, 1458, 1420, 1374, 1327, 1249, 1166, 1131, 1044, 1002 cm^{-1}; Anal. calcd for $C_6H_{16}O_6\cdot 0.5\ H_2O$: C 61.34, H 5.47; found: C 61.39, H 5.43.

(9*RS*)-6,7-(Methylenedioxy)-9-[(1*E*)-2-(3,4,5-trimethoxyphenyl)-ethenyl]-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (9). A suspension of **22** (939 mg, 3 mmol) and 3,4-methylenedioxyaniline **23** (411 mg, 3 mmol) in EtOH (30 mL) was stirred under reflux for 5 min. The resulting solid was removed by filtration and recrystallized from a 7:3 mixture of CH_2Cl_2/MeOH to afford **9** as a white powder (300 mg, 24%): $R_f=0.36$ (CH_2Cl_2/EtOAc, 8:2); mp: $>260\,^{\circ}$C; ^{1}H NMR (300 MHz, [D$_6$] DMSO): $\delta=3.60$ (s, 3H), 3.75 (s, 6H), 4.50 (d, $J=7$ Hz, 1H), 4.80 (d, $J=15$ Hz, 1H), 4.90 (d, $J=15$ Hz), 5.90 (s, 1H), 5.95 (s, 1H), 6.20 (dd, $J=16$ and 7 Hz, 1H), 6.30 (d, $J=16$ Hz, 1H), 6.50 (s, 1H), 6.65 (s, 2H), 6.70 (s, 1H), 9.80 ppm (s, 1H); ^{13}C NMR (75 MHz, [D$_6$] DMSO): $\delta=38.5$, 56.9, 61.1, 65.9, 93.5, 102.3, 104.6, 110.7, 116.3, 129.4, 131.7, 133.1, 133.5, 138.0, 144.4, 144.7, 154.0, 159.6, 173.3 ppm; IR (KBr): $\tilde{\nu}=3280$, 1718, 1647, 1624, 1560, 1508, 1483, 1347, 1248, 1192, 1129, 1035, 1007 cm^{-1}; Anal. calcd for $C_{23}H_{21}NO_7$: C 65.24, H 5.00, N 3.31; found: C 65.21, H 4.92, N 3.30.

(9*RS*)-4-Methyl-6,7-(methylenedioxy)-9-[(1*E*)-2-(3,4,5-trimethoxyphenyl)ethenyl]-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (10). A solution of **22** (1.25 g, 4 mmol) and *N*-methyl-3,4-methylenedioxyaniline **14** (604 mg, 4 mmol) in CH_2Cl_2 (40 mL) was held at reflux for 1 h. The solvent was removed, and the residue was purified by flash chromatography (CH_2Cl_2/EtOAc, 9:1) to afford **10** as a white powder (910 mg, 50%): $R_f=0.43$ (CH_2Cl_2/EtOAc, 8:2); mp: $>260\,^{\circ}$C; ^{1}H NMR (300 MHz, [D$_6$] DMSO): $\delta=3.10$ (s,3H), 3.60 (s, 3H), 3.75 (s, 6H), 4.50 (d, $J=7$ Hz, 1H), 4.95 (d, $J=15$ Hz, 1H), 5.05 (d, $J=15$ Hz, 1H), 5.95 (s, 1H), 6.00 (s, 1H), 6.20 (dd, $J=16$ and 7 Hz, 1H), 6.30 (d, $J=16$ Hz, 1H), 6.65 (s, 2H), 6.80 (s, 1H), 6.90 ppm (s, 1H); ^{13}C NMR (75 MHz, [D$_6$] DMSO): $\delta=34.7$, 38.5, 56.9, 61.1, 66.2, 94.0, 97.1, 102.5, 104.6, 110.9, 118.4, 129.4, 133.0, 133.4, 134.0, 138.1, 144.4, 148.1, 154.0, 161.4, 173.3 ppm; IR (KBr): $\tilde{\nu}=1733$, 1654, 1612, 1582, 1507, 1484, 1418, 1332, 1242, 1193, 1123, 1038, 1009 cm^{-1}; Anal. calcd for $C_{24}H_{23}NO_7\cdot H_2O$: C 63.29, H 5.53, N 3.07; found: C 62.95, H 5.37, N 3.07.

(9*RS*)-4-Methyl-6,7-(methylenedioxy)-9-[2-(3,4,5-trimethoxyphenyl)ethyl]-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (6). A suspension of **10** (210 mg, 0.46 mmol) in MeOH (200 mL) was hydrogenated for 2 h under 10 bar pressure and in the presence of 10% Pd/C (100 mg). The reaction mixture was filtered through a pad of Celite, and the solvent was removed. The crude solid was recrystallized from Et_2O to afford **6** as a white powder (205 mg, 98%): $R_f=0.81$ (CH_2Cl_2/EtOAc 8:2); mp: 198 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=1.90$ (m, 1H), 2.25 (m, 1H), 2.45 (m, 2H), 3.10 (s, 3H), 3.80 (s, 3H), 3.85 (s, 6H), 4.30 (t, $J=5$ Hz, 1H), 4.70 (s, 2H), 5.95 (s, 1H), 6.00 (s, 1H), 6.30 (s, 2H), 6.50 (s, 1H), 6.70 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=31.7$, 33.4, 33.9, 37.9, 56.1, 60.7, 65.0, 94.9, 96.4, 101.6, 105.3, 109.3, 118.9, 133.8, 135.9, 137.7, 144.1, 147.2, 152.9, 159.6, 173.2 ppm; IR (KBr): $\tilde{\nu}=3310$, 1735, 1685, 1654, 1560, 1508, 1478, 1458, 1420, 1239, 1128, 1034 cm^{-1}; Anal. calcd for $C_{24}H_{25}NO_7\cdot H_2O$: C 63.01, H 5.95, N 3.06; found: C 62.90, H 5.89, N 2.67.

(9*RS*)-9-[(1,2-Dihydroxy-2-(3,4,5-trimethoxyphenyl)ethyl]-4-methyl-6,7-(methylenedioxy)-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (24). A 2.5% solution of OsO_4 in *t*BuOH (500 μL, 0.05 mmol) was slowly added to a suspension of alkene **10** (300 mg, 0.66 mmol) and *N*-methylmorpholine-*N*-oxide (154 mg, 1.32 mmol) in a 4:1 mixture of CH_3CN/H_2O (150 mL). The reaction mixture was stirred at room temperature for 24 h, and then extracted with EtOAc (3×40 mL). The combined organic layers were dried over Na_2SO_4 and concentrated. The crude solid was purified by flash chromatography (CH_2Cl_2/EtOAc, 6:4 to 0:10), followed by recrystallization from MeOH to afford **24** as a white powder (248 mg, 80%): $R_f=0.32$ (EtOAc); mp: 192 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.05$ (s, 3H), 3.75 (s, 3H), 3.80 (s, 6H), 3.85 (m, 2H), 4.00 (d, $J=9$ Hz, 1H), 4.30 (d, $J=5$ Hz, 1H), 4.50 (m, 1H), 4.90 (d, $J=15$ Hz, 1H), 5.00 (d, $J=15$ Hz, 1H), 5.90 (s, 1H), 5.95 (s, 1H), 6.25 (s, 1H), 6.35 (s, 2H), 6.80 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=33.6$, 39.4, 56.1, 60.7, 66.5, 74.7, 81.5, 93.5, 95.2, 101.7, 104.7, 110.3, 116.5, 132.7, 135.4, 137.5, 144.2, 147.3, 152.7, 160.4, 173.3 ppm; IR (KBr): $\tilde{\nu}=3400$, 1717, 1657, 1508, 1487, 1419, 1324, 1244, 1199, 1126, 1044 cm^{-1}; Anal. calcd for $C_{24}H_{25}NO_9$: C 61.14, H 5.34, N 2.97; found: C 61.37, H 5.74, N 2.66.

(9*RS*)-9-[1,2-Dibromo-2-(3,4,5-trimethoxyphenyl)ethyl]-4-methyl-6,7-(methylenedioxy)-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (25). Et_3N (500 μL, 3.6 mmol) was added to a stirred solution of compound **24** (170 mg, 0.36 mmol) maintained under nitrogen in the minimal quantity of anhydrous CH_2Cl_2. Methanesulfonyl chloride (280 μL, 3.6 mmol) was added dropwise at 0 °C while stirring. A solution of LiBr (313 mg, 3.6 mmol) in anhydrous acetone (2 mL) was added after 15 min, and the reaction was stirred at room temperature for another 48 h. The reaction mixture was poured into water and then extracted with CH_2Cl_2. The combined layers were dried over Na_2SO_4 and concentrated. The crude solid was purified by flash chromatography (CH_2Cl_2/EtOAc, 9:1) to afford **25** as a white powder (129 mg, 60%): $R_f=0.60$ (EtOAc); mp: 210 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.10$ (s, 3H), 3.80 (s, 3H), 3.85 (s, 6H), 3.95 (m, 1H), 4.10 (d, $J=7$ Hz, 1H), 4.65 (s, *2*H), 5.10 (d, $J=7$ Hz, 1H), 5.95 (s, 1H), 6.00 (s, 1H), 6.50 (s, 1H), 6.60 (s, 2H), 6.80 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=33.6$, 38.7, 56.1, 60.8, 65.0, 80.2, 87.5, 93.6, 95.1, 101.7, 104.4, 111.0, 116.0, 134.0, 134.6, 137.6, 144.0, 147.7, 153.1, 161.2, 173.2 ppm; IR (KBr): $\tilde{\nu}=1735$, 1654, 1508, 1486, 1238, 1193, 1155, 1126, 1037, 1007 cm^{-1}; Anal. calcd for $C_{24}H_{23}Br_2NO_7$: C 48.26, H 3.88, N 2.35; found: C 48.45, H 3.76, N 2.36.

(9*RS*)-4-Methyl-6,7-(methylenedioxy)-9-[2-(3,4,5-trimethoxyphenyl)ethynyl]-4,9-dihydrofuro[3,4-*b*]quinolin-1(3*H*)-one (7). A suspension of *t*BuOK (24 mg, 0.2 mmol) and compound **25** (50 mg, 0.084 mmol) in anhydrous THF (10 mL) was stirred under nitrogen atmosphere for 3 h. After dilution with H_2O, the reaction mixture was extracted with CH_2Cl_2. The combined layers were dried over Na_2SO_4 and concentrated. The crude product was purified by preparative thin-layer chromatography on silica gel (EtOAc) to afford **7** as a white powder (26 mg, 71%): $R_f=0.46$ (EtOAc); mp: $>260\,^{\circ}$C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=3.20$ (s, 3H), 3.80 (s, 9H), 4.80 (d, $J=15$ Hz, 1H), 4.85 (d, $J=15$ Hz, 1H), 5.00 (s, 1H), 5.95 (s, 2H), 6.40 (s, *2*H), 6.56 (s, 1H), 6.57 ppm (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=26.8$, 33.4, 56.0, 60.6, 64.6, 84.5, 86.9, 93.6, 94.1, 101.9, 104.2, 110.8, 115.7, 134.5, 134.6, 137.4, 143.8, 147.5, 152.9, 161.0, 173.0 ppm; IR (KBr): $\tilde{\nu}=2211$, 1737, 1686, 1638, 1561, 1544, 1509, 1477, 1460, 1128 cm^{-1}; Anal. calcd for: $C_{24}H_{21}NO_7$: C 66.20, H 4.86, N 3.22; found: C 65.91, H 4.84, N 3.11.

Biology

Inhibition of tubulin polymerization assay. Tubulin assembly in microtubules was evaluated using the fluorescent dye DAPI (4′,6-diamidino-2-phenylindole)[33] in 96-well black plates and observed using a Victor plate reader as previously described by Barron

et al.[34] and Bane et al.[35] The standard assay was performed as follows: wells were charged with tubulin (Cytoskeleton, 97% pure, final concentration: 1 mg mL^{-1}) in PME buffer (100 mM PIPES, 1 mM $MgSO_4$, 2 mM EGTA) with 10 μM DAPI and varying concentrations of compounds or colchicine as an internal control. After preincubation for 45 min at room temperature, 5 μL of 1 mM GTP was added to each well to initiate tubulin polymerization, and the plate was then transferred to the temperature-controlled Victor plate reader at 37 °C for an additional 2 h. Fluorescence was read at excitation and emission wavelengths of 360 nm and 450 nm, respectively. Percent inhibition was determined as follows: $[1-\Delta F_{sample}/\Delta F_{control}]\times 100$, where $\Delta F_{control} = F_{no\ inhibition} - F_{complete\ inhibition}$, and $\Delta F_{sample} = F_{sample} - F_{complete\ inhibition\ with\ colchicine}$. The IC_{50} for drug-induced inhibition of tubulin polymerization is the concentration of drug at which the extent of polymerization inhibition is 50% of the maximum value as determined from the semi-logarithmic plot of percent inhibition as a function of the drug concentration using the nonlinear regression software SigmaPlot (Jandel Scientific). The results are presented as the inhibition of tubulin polymerization (ITP), which is the ratio of the IC_{50} of the compound of interest over the IC_{50} of colchicine. In our experimental conditions, the average IC_{50} for the ITP of colchicine was 0.36 μM. Because the intrinsic fluorescence of compounds could interfere with this assay, fluorescence readings were routinely taken at the same concentrations as used in the tubulin polymerization assay.

Cytotoxicity assay. Murine B16 melanoma cells, murine fibroblasts NIH 3T3, and EA.hy926 endothelial cells [originally obtained from Dr. Cora-Jean S. Edgell and used with her permission][36] were grown in Dulbecco's modified essential medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg mL^{-1} streptomycin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were plated onto 96-well plates at 5000 cells per well in 200 μL DMEM. After 24 h, the cells were exposed to the solvent alone (DMSO) or to the compounds of interest at the indicated concentrations for an additional 48 h. Viability was assessed using the MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium) test, and absorbance was read at 562 nm in a microplate reader (BioKinetics Reader, EL340).[37] Stock solutions were prepared in dimethyl sulfoxide (DMSO) and kept at 4 °C in the dark. Experiments were run in triplicate and repeated three times. Results are presented as the inhibitory concentrations for 50% of cells (IC_{50}) for a 48 h exposure time. The IC_{50} values were determined using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

Endothelial cell morphology. To assess the effects of compounds on the morphology of endothelial cells, exponentially growing EA.hy926 cells in DMEM containing 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg mL^{-1} streptomycin were plated onto 96-well plates at 5×10^3 cells/100 μL per well. 24 h after plating, the medium was aspirated, and 100 μL of medium containing the test compound was added to the wells containing the cells (in triplicate) in 10-fold dilutions, and incubated for 2 h at 37 °C and 5% CO_2 atmosphere. After the 2 h incubation period, representative central areas of each well were photographed at 360× magnification. Combretastatin A-4 was included in the experiments as a positive internal standard.

HUVEC tube disruption and reorganization assay. HUVEC (Lonza, Verviers, Belgium) was cultured in EGM-2 supplemented with hEGF, hydrocortisone, gentamicin, amphotericin-B, VEGF, hFGF-B, R^3-IGF-1, ascorbic acid, heparin (EGM-2 Bulletkit), and fetal bovine serum. HUVECs were used from the second to the fifth passages for the experiments and the medium was changed every two days. HUVECs in 100 μL EGM-2 (2×10^4 cells well^{-1}) were plated in 96-well plates on a thick layer of Matrigel (Becton Dickinson; 75 μL well^{-1}) and incubated for 16 h at 37 °C in a 5% CO_2 atmosphere to allow the cells to form tube-like structures. Test compounds were dissolved in DMSO (less than 0.1% in each preparation; 100 μL well^{-1}) at various concentrations and were added to the formed cords and incubated for a 3 h period. Each well was then carefully washed with medium and incubated for an additional 3 h. The effects of compounds on capillary tube disruption and eventual reorganization was evaluated by light microscopy (40× magnification) at three indicated times (0 h, 3 h after addition of compound, and 3 h after washout). Experiments were done in triplicate.

Dose–effect responses of CA-4 and four new azapodophyllotoxins (**5**, **9**, **10**, and **11**) on endothelial tube-like disruption/reorganization are provided in the Supporting Information.

Acknowledgements

R.L. was supported by the Ministère de l'Education Supérieure et de la Recherche. The authors thank Dr. Claude Monneret for providing us with combretastatin A-4. This study was supported by a grant from the Institut National du Cancer (INCa, Boulogne-Billancourt, France). C.G. thanks the Ligue Nationale contre le Cancer for financial support.

Keywords: antitumor agents · antivascular agents · azapodophyllotoxins · nitrogen heterocycles · podophyllotoxin analogues

[1] a) J. Denekamp, *Br. J. Radiol.* **1993**, *66*, 181–196; b) F. J. Burrows, P. E. Thorpe, *Pharmacol. Ther.* **1994**, *64*, 155–174.
[2] a) G. M. Tozer, C. Kanthou, B. C. Baguley, *Nat. Rev. Cancer* **2005**, *5*, 423–435; b) D. M. Patterson, G. J. S. Rustin, *Clin. Oncol.* **2007**, *19*, 443–456.
[3] a) E. L. Schwartz, *Clin. Cancer Res.* **2009**, *15*, 2594–2601; b) A. M. Gaya, G. J. S. Rustin, *Clin. Oncol.* **2005**, *17*, 277–290; c) J. W. Lippert III, *Bioorg. Med. Chem.* **2007**, *15*, 605–615.
[4] K. H. Downing, E. Nogales, *Curr. Opin. Cell Biol.* **1998**, *10*, 16–22.
[5] M. A. Jordan, L. Wilson, *Nat. Rev. Cancer* **2004**, *4*, 253–265.
[6] C. Kanthou, G. M. Tozer, *Int. J. Exp. Pathol.* **2009**, *90*, 284–294.
[7] C. Kanthou, G. M. Tozer, *Drug Discovery Today: Ther. Strategies* **2007**, *4*, 237–243.
[8] M. Gordaliza, P. A. García, J. M. M. del Corral, M. A. Castro, M. A. Gómez-Zurita, *Toxicon* **2004**, *44*, 441–459.
[9] T. F. Imbert, *Biochimie* **1998**, *80*, 207–222.
[10] a) H. Stähelin, A. von Wartburg, *Prog. Drug Res.* **1989**, *33*, 169–266; b) L. Bohlin, B. Rosen, *Drug Discovery Today* **1996**, *1*, 343–351; c) S. Desbène, S. Giorgi-Renault, *Curr. Med. Chem. Anti-Cancer Agents* **2002**, *2*, 71–90.
[11] D. L. Sackett, *Pharmacol. Ther.* **1993**, *59*, 163–228.
[12] R. B. Ravelli, B. Gigant, P. A. Curmi, I. Jourdain, S. Lachkar, A. Sobel, M. Knossow, *Nature* **2004**, *428*, 198–202.
[13] Y. Kim, S. B. Kim, Y. J. You, B. Z. Ahn, *Planta Med.* **2002**, *68*, 271–274.
[14] H.-P. Husson, S. Giorgi-Renault, C. Tratrat, G. Atassi, A. Pierré, P. Renard, B. Pfeiffer, (Adir et Compagnie) EP 1.103.554, **2000** [*Chem. Abstr.* **2001**, *135*, 5535u].
[15] R. Labruère, P. Helissey, S. Desbène-Finck, S. Giorgi-Renault, *J. Org. Chem.* **2008**, *73*, 3642–3645.
[16] C. Tratrat, S. Giorgi-Renault, H.-P. Husson, *Org. Lett.* **2002**, *4*, 3187–3189.
[17] K. C. Nicolaou, J. A. Pfefferkorn, F. Schuler, A. J. Roecker, G.-Q. Cao, J. E. Casida, *Chem. Biol.* **2000**, *7*, 979–992.
[18] H. Erdtman, G. Eriksson, T. Norin, S. Forsen, *Acta Chem. Scand.* **1963**, *17*, 1151–1156.
[19] T. Guo, K.-K. Ho, E. McDonald, R. E. Dolle, K. W. Saionz, S. G. Kultgen, R. Liu, G. Dong, P. Geng, A. E. P. Adang, N. E. P. Adang, N. C. R. Van Straten,

(Pharmacopeia, INC) WO 02/070493A1, **2002** [*Chem. Abstr.* **2002**, *137*, 216961j].
[20] H. J. Bestmann, K. Roth, M. Ettlinger, *Angew. Chem.* **1979**, *91*, 748.
[21] P. K. Mahata, O. Barun, H. Ila, H. Junjappa, *Synlett* **2000**, *9*, 1345 – 1347.
[22] A. I. Vedernikov, S. P. Gromov, *Synthesis* **2001**, *6*, 889 – 892.
[23] H. Zimmer, W. W. Hillstrom, J. C. Schmidt, P. D. Seemuth, R. Vögeli, *J. Org. Chem.* **1978**, *43*, 1541 – 1544.
[24] T. L. Nguyen, C. McGrath, A. R. Hermone, J. C. Burnett, D. W. Zaharevitz, B. W. Day, P. Wipf, E. Hamel, R. A. Gussio, *J. Med. Chem.* **2005**, *48*, 6107 – 6116.
[25] S. Gupta, L. Das, A. B. Datta, A. Poddar, M. E. Janik, B. Bhattacharyya, *Biochemistry* **2006**, *45*, 6467 – 6475.
[26] D. Bouïs, G. A. Hospers, C. Meijer, G. Molema, N. H. Mulder, *Angiogenesis* **2001**, *4*, 91 – 102.
[27] G. Micheletti, M. Poli, P. Borsotti, M. Martinelli, B. Imberti, G. Taraboletti, R. Giavazzi, *Cancer Res.* **2003**, *63*, 1534 – 1537.
[28] P. D. Davis, G. J. Dougherty, D. C. Blakey, S. M. Galbraith, G. M. Tozer, A. L. Holder, M. A. Naylor, J. Nolan, M. R. L. Stratford, D. J. Chaplin, S. A. Hill, *Cancer Res.* **2002**, *62*, 7247 – 7253.
[29] D. J. Chaplin, G. J. Dougherty, *Br. J. Cancer* **1999**, *80*, 57 – 64.
[30] J. Griggs, J. C. Metcalfe, R. Hesketh, *Lancet Oncol.* **2001**, *2*, 82 – 87.
[31] C. M. Lin, H. H. Ho, G. R. Pettit, E. Hamel, *Biochemistry* **1989**, *28*, 6984 – 6991.
[32] S. Koul, J. L. Koul, S. C. Taneja, K. L. Dhar, D. S. Jamwal, K. Singh, R. K. Reen, J. Singh, *Bioorg. Med. Chem.* **2000**, *8*, 251 – 268.
[33] D. Bonne, C. Heusele, C. Simon, D. Pantaloni, *J. Biol. Chem.* **1985**, *260*, 2819 – 2825.
[34] D. M. Barron, S. K. Chatterjee, R. Ravindra, R. Roof, E. Baloglu, D. G. Kingston, S. Bane, *Anal. Biochem.* **2003**, *315*, 49 – 56.
[35] S. L. Bane, R. Ravindra, A. Zaydman in *Microtubule Protocols* (Ed.: J. Zhou), Humana, Totowa, **2007**, pp. 281 – 288.
[36] C. J. Edgell, C. C. McDonald, J. B. Graham, *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 3734 – 3737.
[37] J. Carmichael, W. G. DeGraff, A. F. Gazdar, J. D. Minna, J. B. Mitchell, *Cancer Res.* **1987**, *47*, 936 – 942.

Received: July 22, 2010
Revised: September 17, 2010
Published online on October 26, 2010

