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Antiangiogenic activity of deoxoartemisinin derivatives on chorioallantoic membrane

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Abstract—Non acetal-type derivatives at C-12 of artemisinin and their novel dimers including a fullerene conjugate were synthesized and some of them showed potent in vivo antiangiogenic activity on chorioallantoic membrane higher than or comparable to those of fumagillin and thalidomide.

More than 30 years ago, Folkman proposed the hypothesis that solid tumor growth was dependent on the development of tumor-associated blood vessels, a process called angiogenesis.[1] Angiogenesis or neovascularization is a complex process involving the activation, adhesion, proliferation, and transmigration of endothelial cells from preexisting blood vessels.[2] It plays a critical role in normal physiological processes but also in the growth of solid tumors.[3] Angiogenesis is considered as a potential target for anticancer chemotherapy. Strategies for regulating angiogenesis have been carried out mainly in molecular biology. However, it has been insufficiently carried out to develop antiangiogenic agents based on small molecules. It is interesting to discover the new antiangiogenic small molecules that might be suitable as clinical therapies. Artemisinin (**1**), a sesquiterpene endoperoxide isolated from *Artemisia annua* L.,[4] and its derivatives have been clinically used to treat drug-resistant malaria.[5] Their pharmacology and pharmacokinetics have been well studied.[6] Artemisinin contains an endoperoxide that could react with an iron ion to form a carbon-based free radical. Such free radical, when formed intracellularly, could cause macromolecular damages and lead to cell death. Since tumor cells uptake a large amount of iron compared to normal cells,[6g] they are more vulnerable to the cytotoxic effect of artemisinin than normal cells. As that was shown in our previous research,[7] other researchers have also reported the potential antitumor properties of artemisinin and its derivatives.[8] Some dimeric chemical structures showed especially high anticancer activities.[7a] Nonacetal 12β(C–C)-type derivatives of artemisinin showed more potent anticancer activity[7a] and 20 times more acid stability for oral administration than acetal (C–O)-type derivatives of artemisinin.[7b] Recently, artemisinin has also been reported to have antiangiogenic activity.[9] Chen et al. reported that particularly, artesunate exhibits antiangiogenesis and apoptic activity on human endothelial cells.[9b] Since the discovery of fullerenes in 1985, studies directed toward biomedical application of fullerene-based drug have demonstrated beneficial in vitro biological properties. Several reports have also recently shown that the fullerene carbon cage is relatively nontoxic[10] and it could be suitable for fitting into cavity of the target protein resulting in inhibition of the proliferation of cancer cells.

The identification and the assessment of new substances that are able to inhibit angiogenesis make use of in vivo and in vitro assays, a number of which are currently being used by many laboratories. In vitro models, although useful in delineating parts of this process, may not be representative of what occurs in vivo. One useful in vivo system that has been used extensively in angiogenesis research is the highly vascularized chorioallantoic membrane (CAM) of the chicken embryo. Chicken embryos are less expensive to use than whole animals such as rodents, making the CAM assay attractive for investigators to screen antiangiogenic substances.[11]

We report in this study the synthesis and in vivo antiangiogenic activity on chorioallantoic membrane of

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nonacetal-type derivatives at C-12 of artemisinin and their dimers. A deoxoartemisinin–C_{60} conjugate has been synthesized as a possible antiangiogenic agent designed to modify the drug delivery rate and as expected nontoxic and low dosing advantage.

Dihydroartemisinin (**2**), deoxoartemisinin (**3**), and various derivatives (**4**–**7**) as outlined in Figure 1 were prepared according to the procedure described by Jung et al.[7,12,13] It is noteworthy that C-12β nonacetal-type dimers, **9** and **10**, showed high anticancer activity against human cancer cell lines.[7] Therefore, several dimers, **9a**, **b**, and **10**, were prepared according to the known procedures,[7] respectively. Accordingly, C-13 ether dimer **8** of deoxoartemisinin was also formed in 45% yield from coupling of **7b** with 13-bromodeoxoartemisinin according to Jung's procedure.[13b] As seen in Scheme 1, a deoxoartemisinin dimer **11**[14] was prepared in 47% yield by reacting 12β-(3′-hydroxy-*n*-propyl)-deoxoartemisinin (**13**)[15] with malonyl chloride in the presence of dry pyridine at 0 °C during 1 h. Reaction of the dimer (**11**), connected with malonate linker, with fullerene (C_{60}) in the presence of DBU/I_2 in toluene (rt, 4 h) afforded the Bingel adducts[16] (**12**)[14] as a deoxoartemisinin–C_{60} conjugate in 58% yield after purification by

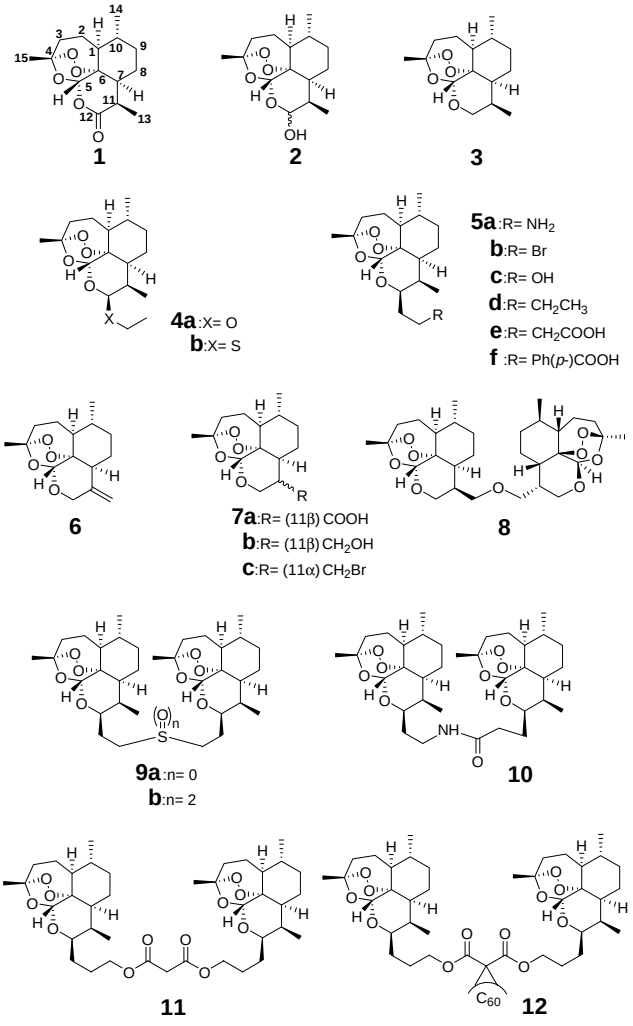
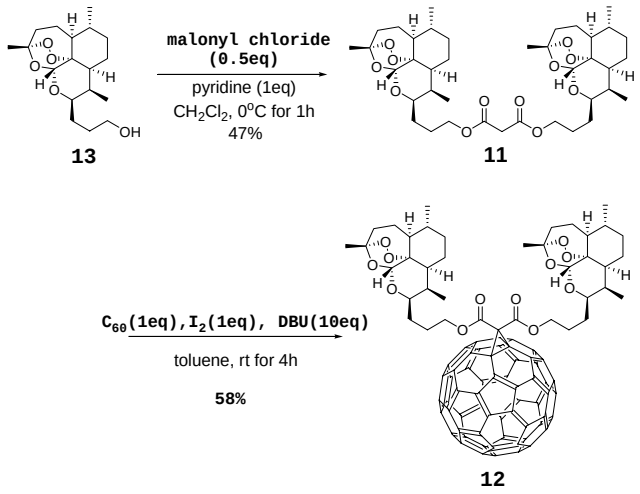


Figure 1. Structures of artemisinin and its nonacetal-type derivatives.



Scheme 1. Synthesis of novel deoxoartemisinin dimers **11** and **12**.

column chromatography and subsequent recrystallization. The ^{13}C NMR spectra showed the presence of the fullerene sp^3 carbons at δ 77.3 and 71.8, respectively, thus confirming the closed [6,6] nature of the fullerene unit. Disappearance of singlet peak corresponding to methylene protons at δ 3.37 of the dimer (**11**) further confirmed coupling of fullerene with (**11**) into dimer (**12**).

To determine in vivo antiangiogenic activity of deoxoartemisinin derivatives and dimers, a CAM assay was performed as previously described.[11] Fertilized eggs (Pulmuone Co., Kyungki-do, Korea) were incubated at 37 °C with 80–90% relative humidity. At day 2, a portion of albumin was removed and a window was made on day 3. At day 4.5 of incubation, test samples loaded on a quarter size Thermanox coverslip (Nunc, Roskilde, Denmark) were applied on the CAM of individual embryos in a concentration of 5 nmol/egg.

After another 2-day incubation, a 20% fat emulsion was injected into the CAM for observation of the inhibition avascular zone and calculated as the number of positive eggs to the total number of eggs tested. If avascular zone in about 3–6 mm diameter indicated with arrow in Figure 2 is observed, then it is evaluated as effective

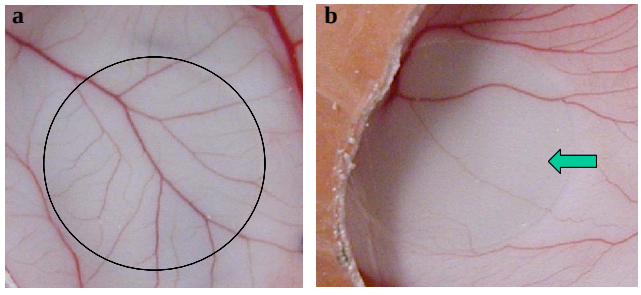


Figure 2. Antiangiogenic effect of deoxoartemisinin derivatives on the chick CAM. Membranes were treated with (a) control; (b) deoxoartemisinin derivatives in a concentration of 5 nmol/egg.

Table 1. Inhibitory effect of artemisinin and its nonacetal-type derivatives on CAM angiogenesis in a concentration of 5 nmol/egg

Compound	Positive eggs/eggs tested	Inhibition effect[a]	% inhibition
1	2/8		25
2	2/8		25
3	2/7 (1)[b]		29
4a	2/7 (1)		29
4b	1/6 (2)		17
5a	2/7 (1)		29
5b	2/7 (1)		29
5c	5/8	++	63
5d	5/8	++	63
5e	4/8	+	50
5f	2/7		29
6	2/7 (1)		29
7a	1/5 (3)	Low toxic	20
7b	2/7 (1)		29
7c	6/8	++	75
8	5/7	++	71
9a	0/0 (8)	Toxic	—
9b	0/2 (6)	Toxic	—
10	3/7		43
11	1/7		14
12	4/8	+	50
(−)-Fumagillin	4/7		57
(−)-Thalidomide	4/8		50
Control[c]	0/8		0

[a] Inhibition effect; antiangiogenic effect of plus (+) is similar to that of thalidomide or fumagillin, and double plus (++) is stronger.
[b] Number in parentheses displays egg that embryo dies.
[c] Control, solvent only (chloroform) to embryo.

inhibition on neovascularization (Fig. 2). The results are listed in Table 1.

As shown in Table 1, artemisinin (**1**), dihydroartemisinin (**2**), deoxoartemisinin (**3**), and C-12β derivatives (**4a**, **4b**, **5a**, **5b** and **5f**) showed a weak inhibitory effect in a concentration of 5 nmol/egg.

In particular, C-12β deoxoartemisinin derivatives **5c**–**e** showed similar or stronger antiangiogenic activity than fumagillin. Compounds **6**, **7a** and **7b** of C-11 deoxoartemisinin derivatives exhibited weak activity. However, compound **7c** and C-13 ether dimer **8** showed the highest antiangiogenic activity and more potency than fumagillin and thalidomide.[17] Interestingly, C-12β sulfur-linker dimer (**9a**, **9b**) that has potent antitumor activity displayed toxicity that most tested chicken embryos died at the given concentration, while amide-linker dimer **10** showed a moderate activity. The new malonate-linked C-12 dimer–C_{60} complex (**12**) showed inhibitory effect on angiogenesis as comparable to that of thalidomide, but dimer itself (**11**) has very weak activity. It is interesting to note that **5c**, **d**, and **7c**, monomers with poor cytotoxicity, have potent antiangiogenic activity in this assay.

In summary, nonacetal-type derivatives of artemisinin and their novel dimers were synthesized and some of them showed potent in vivo antiangiogenic activity.

Among the 21 synthetic compounds tested, **5c**, **5d**, **7c**, and **8** showed the most potent antiangiogenic activity and was 10–15 times more potent than artesunate with complete inhibition at 80 nmol/egg,[9b] while **5e** and **12** showed activity similar to that of fumagillin. Several C-11 and C-12 nonacetal-type derivatives of artemisinin showed stronger inhibitory effects than those of thalidomide and fumagillin known as antiangiogenic agents. The requirement for the presence of the peroxide bond for antiangiogenesis needs to be determined by preparation and in vivo screening of desoxy derivatives of deoxoartemisinin. Evidence that acetal-type analogs at C-12 are more neurotoxic in animal studies than nonacetal-type analogs is also emerging,[18] and may thus lead to the future abandonment of the currently clinically used acetal-type drugs (artemisinin, artemether, arteether, dihydroartemisinin, and artesunate). Therefore, nonacetal 12β (C–C)-type derivatives of artemisinin deserve further evaluation as possible anticancer drug candidates for oral administration because of their high acid stability,[7b] low toxicity, and high in vivo antiangiogenesis.

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14. Spectral data for compound **11**: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.29 (2H, s), 4.24–4.16 (6H, m), 3.37 (2H, s, malonyl CH_2), 2.64 (2H, m), 2.38–2.25 (2H, m), 1.40 (6H, s), 0.96 (6H, d, J = 5.7 Hz), 0.78 (6H, d, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ 166.8 (C═O), 103.3, 89.2, 81.3, 74.9, 65.6, 52.4, 44.4, 41.7, 37.5, 36.7, 34.6, 30.4, 26.7, 26.2, 26.0, 25.0, 24.9, 20.3, 13.1. FTIR (KBr) ν_{max} 2924, 2853, 1735 (C═O), 1456, 1377, 1330, 1251, 1188, 1144, 1104, 1056, 1012, 878 (O–O), 755, 415 cm^{-1}. Compound **12**: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.32 (2H, s), 4.59–4.50 (4H, m), 4.26–4.20 (2H, m), 2.70–2.62 (2H, m), 2.38–2.26 (2H, m), 1.51 (6H, s), 0.96 (6H, d, J = 5.6 Hz), 0.80 (6H, d, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ 163.8 (C═O), 145.5, 145.4, 145.3, 145.0, 144.8, 144.7, 144.0, 143.1, 142.3, 142.0, 141.1, 139.1, 103.3, 89.3, 81.2, 77.4 (Csp^3C_{60}), 74.8, 71.8 (Csp^3C_{60}), 67.5, 52.4, 44.4, 37.6, 36.7, 34.6, 30.5, 26.9, 26.3, 26.1, 25.1, 24.9, 20.3, 13.2. FTIR (KBr) ν_{max} 2925, 2916, 2852, 1746 (C═O), 1464, 1456, 1377, 1235, 1206, 1187, 1103, 1056, 1012, 875 (O–O), 755, 702, 525 cm^{-1}.
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