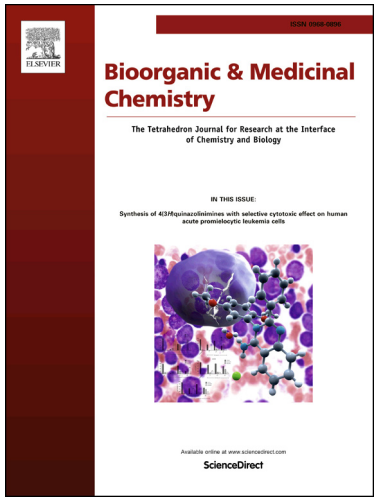


Synthesis of Novel Artemisinin Dimers with Polyamine Linkers and Evaluation of Their Potential as Anticancer Agents

George E. Magoulas, Tzoanna Tsigkou, Lina Skondra, Margarita Lamprou, Panagiota Tsoukala, Vassiliki Kokkinogouli, Evangelia Pantazaka, Dionissios Papaioannou, Constantinos M. Athanassopoulos, Evangelia Papadimitriou

PII: S0968-0896(17)30111-6
DOI: http://dx.doi.org/10.1016/j.bmc.2017.05.018
Reference: BMC 13736

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 18 January 2017
Revised Date: 25 April 2017
Accepted Date: 8 May 2017

Please cite this article as: Magoulas, G.E., Tsigkou, T., Skondra, L., Lamprou, M., Tsoukala, P., Kokkinogouli, V., Pantazaka, E., Papaioannou, D., Athanassopoulos, C.M., Papadimitriou, E., Synthesis of Novel Artemisinin Dimers with Polyamine Linkers and Evaluation of Their Potential as Anticancer Agents, *Bioorganic & Medicinal Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.bmc.2017.05.018

SYNTHESIS OF NOVEL ARTEMISININ DIMERS WITH POLYAMINE LINKERS AND EVALUATION OF THEIR POTENTIAL AS ANTICANCER AGENTS

George E. Magoulas[1,⊥,#], Tzoanna Tsigkou[2,#], Lina Skondra[2], Margarita Lamprou[2], Panagiota Tsoukala[1], Vassiliki Kokkinogouli[1], Evangelia Pantazaka[2], Dionissios Papaioannou[1], Constantinos M. Athanassopoulos[1,*], Evangelia Papadimitriou[2,**]

[1] Laboratory of Synthetic Organic Chemistry, Department of Chemistry, University of Patras, GR-26504, Patras, Greece

[2]Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, GR-26504, Patras, Greece

⊥Current address: National Hellenic Research Foundation, Institute of Biology, Medicinal Chemistry and Biotechnology, 48 Vassileos Constantinou Avenue, GR-11635 Athens, Greece

[#]They have equally contributed to this work

* Corresponding author. Tel: +302610 997909, e-mail: kath@chemistry.upatras.gr

** Corresponding author. Tel: +30 2610 962336, e-mail: epapad@upatras.gr

Abstract

The natural product artemisinin and derivatives thereof are currently considered as the drugs of choice for the treatment of malaria. At the same time, a significant number of such drugs have also shown interesting anticancer activity. In the context of the present research work, artemisinin was structurally modified and anchored to naturally occurring polyamines to afford new artemisinin dimeric conjugates whose potential anticancer activity was evaluated. All artemisinin conjugates tested were more effective than artemisinin itself in decreasing the number of MCF7 breast cancer cells. The effect required conjugation and was not due to the artemisinin analogue or the polyamine, alone or in combination. To elucidate potential mechanism of action, we used the most effective conjugates **6, 7, 9** and **12** and found that they decreased expression and secretion of the angiogenic growth factor pleiotrophin by the cancer cells themselves, and inhibited angiogenesis in vivo and endothelial cell growth in vitro. These data suggest that the new artemisinin dimers are good candidates for the development of effective anticancer agents.

Keywords: angiogenesis; artemisinin; breast cancer; conjugates; dimers; endothelial cells; polyamines

1. Introduction

Artemisinin (ART, **1**), a natural product isolated from the Chinese plant *Artemisia Annua*, and its semisynthetic derivatives dihydroartemisinin (DHA, **1a**), artemether (**1b**) and artenusate (**1c**) are a group of drugs that are standard treatment worldwide for *P. falciparum* malaria.[1-4] Chemically, ART is a sesquiterpene lactone containing an unusual peroxide bridge, whose functionality is believed to be responsible for the drug's mechanism of action.[5-7] Besides antimalarial activity, recent studies report that artemisinins are also efficacious for improving the immune system and for curing other diseases, including cancers.[8-11] Their reported anticancer activity includes strong selectivity,[12-14] reversal of multidrug resistance[7,15,16] and sensitization of cancer cells to radiation[7,17-19] and chemotherapy,[20,21] and attracts extensive attention. Many ART derivatives have been synthesized as anticancer agents and studied *in vitro* and/or *in vivo* in breast, prostate, ovarian, head and neck, lung, gastric, hepatocellular, pancreatic and kidney cancers, osteosarcomas, glioblastomas and leukemias.[7-11] Although production of reactive oxygen species (ROS) is believed to play a role in the anticancer activity of artemisinins, other mechanisms that are not necessarily connected to ROS production have been also suggested, such as apoptosis or autophagy induction, cell cycle arrest and reduced cell proliferation, alteration of tumour-associated gene expression, and inhibition of tumour angiogenesis.[7,11]

Pharmacological shortcomings of ART and derivatives include low water/oil solubility, poor bioavailability and a short half-life *in vivo*. An emerging problem with treatments containing ART and ART derivatives (ART combination therapies, ACTs) is the continuous increase of resistance to ART and ACTs. To overcome these problems, semisynthetic and fully synthetic ART-like compounds have been developed and tested. Moreover, there are studies showing that dimeric and trimeric ART derivatives have much higher antitumor activity than their monomeric

counterparts, with the nature of the linker potentially playing an important role in the anticancer activity.[11,22,23]

Polyamines (PAs) are ubiquitous organic polycationic molecules present in all living organisms, being essential in the regulation of cell proliferation and differentiation. PA transport systems control cellular import and export of PAs and have been exploited as potential targets for therapeutic intervention in cancers. Many cancer cells, such as neuroblastoma, melanoma, human lymphocytic leukemic, colonic and lung cancer cell lines, exhibit elevated PA import activity, probably due to their enhanced need for the growth supporting effect of PAs.[24,25] This property has been exploited in many published papers on PA conjugates with cytotoxic drugs, which may thus possess improved efficacy and cell selectivity.[24,26] Such a conjugate of the PA spermine (spm) with epipodophyllotoxin has proved highly effective in human breast[27] and ovarian[28] cancer xenografts, in leukemia cells,[27] in pediatric neuroblastoma and high grade glioma cell lines,[29] in head and neck squamous cell carcinoma cell lines[30] and in non-small cell lung cancer cells,[31] and is being evaluated in phase I trials for leukemias.[32] Naphthalimide coupled with PAs exhibits antineoplastic activity and excellent selectivity for transformed versus normal cells.[33-34] Other examples of PA conjugates for anticancer lead discovery include dimeric quinoline, cinnoline and phthalimide moieties,[35] *all-trans* retinoic acid,[36] chloramphenicol[37] and minoxidil.[38] Recently, conjugates of ART with the natural PA spermidine (spd) have been synthesized and their antimalarial and anticancer activity was evaluated.[39]

Prompted by the significant anticancer activity of both ART and PA analogs and conjugates, we decided to further explore the potentiality of ART-PA conjugates. We, therefore, describe herein the chemical modification of ART at positions 10 and 11 in order to bear suitable linkers, the application of the thus modified ART (compounds **2**-**4**) to the synthesis of a series of symmetric

ART-PA conjugates (compounds **5-13**, Fig. 1), and finally the evaluation of the latter as potential anticancer agents. The natural PAs putrescine (put), spd and spm were employed as the PA counterpart of the conjugates. The modified ART moiety was attached to the primary amino functions through a urethane functionality, which is hydrolytically more labile than the corresponding amide bond, and the secondary amino functions were masked with the also hydrolytically relatively labile methoxycarbonyl moiety to increase lipophilicity and protect conjugates of the **8**/**11A** type (see Fig. 1) from decomposition.[40] Finally, the diethylene glycol linker was employed to increase water solubility compared to the hydroxypropyl linker. With these new conjugates, we wished to explore the role of (a) the length of the PA chain (compare for example compounds **5**, **8** and **11**), (b) the modified ART nucleus (compare for example compounds **5**-**7**) and (c) the nature of the linker and its position (10 or 11) on the ART nucleus (compare for example compounds **5** and **6**, and **5** and **7**, respectively).

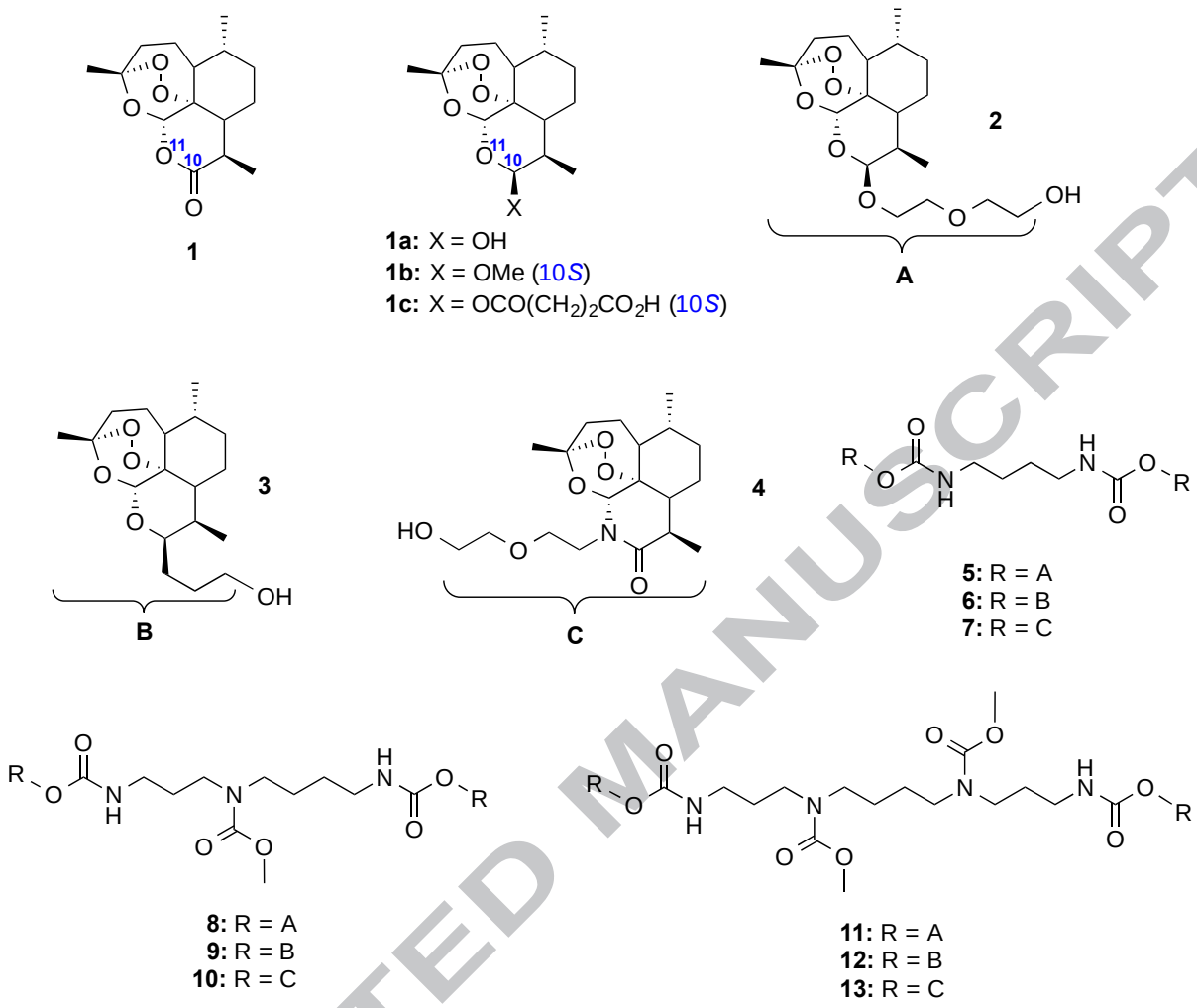


Fig. 1. Structures of compounds encountered in the present work.

2. Results and discussion

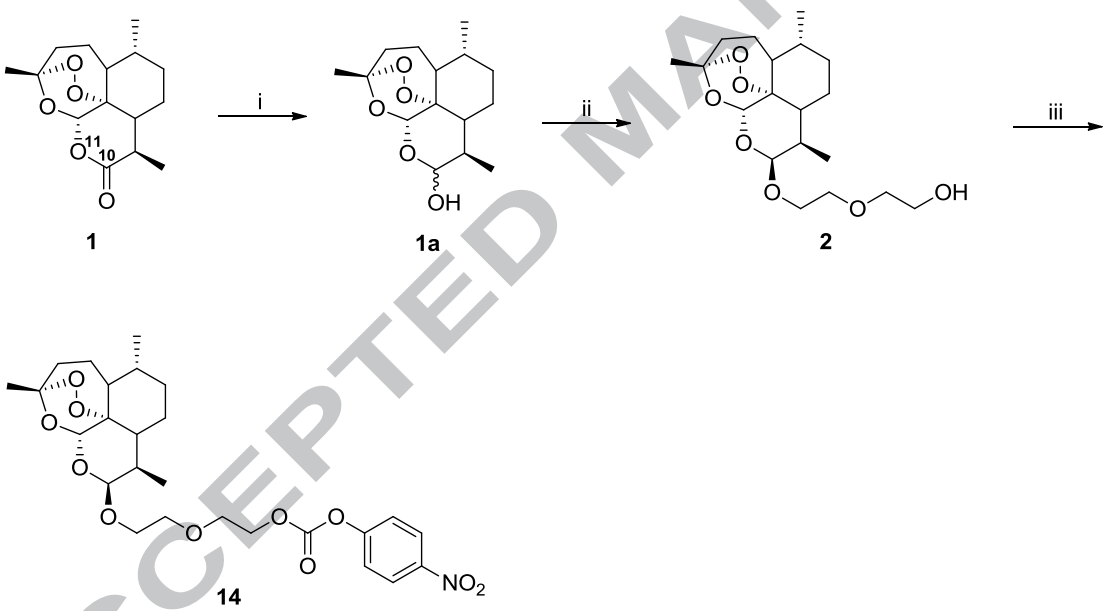
2.1. Chemistry

Dimers **5-13** consist of a PA backbone bearing two ART derivatives at their primary amino functions through a urethane bond. The parent compound, ART**,** was modified at positions 10 and 11. That way, three hydroxyl-functionalized ART derivatives (**2-4**) were synthesized as

described below, which upon activation and reaction with selectively protected PAs provided the projected dimers with the desired urethane linkages.

*2.1.1. Activated 10-oxo-ART analogue **2***

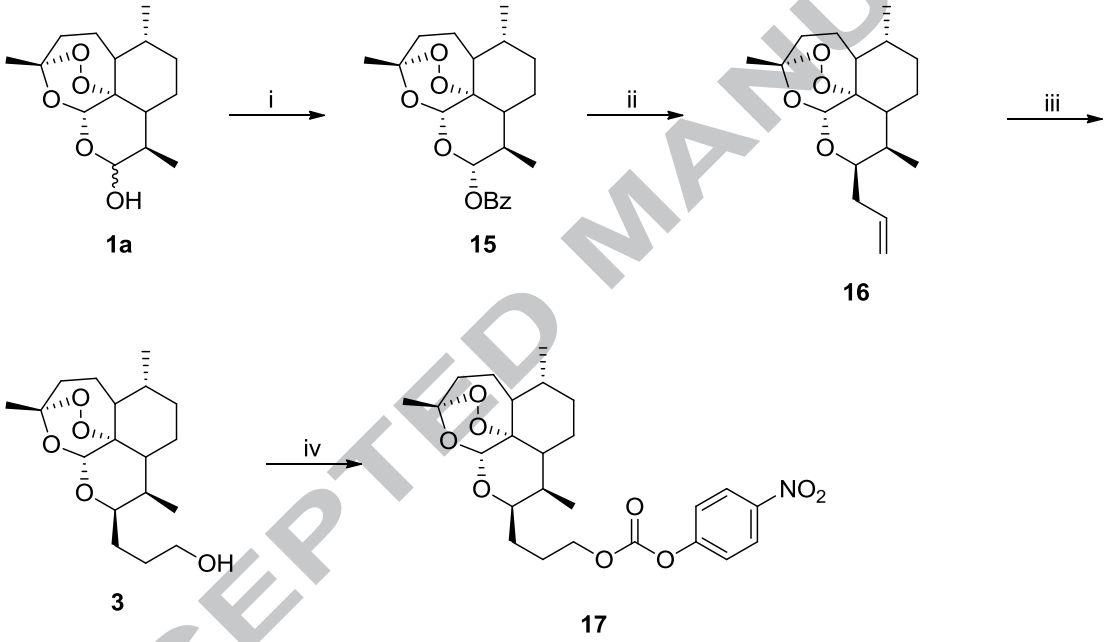
ART was subjected to $NaBH_4$-mediated reduction to afford DHA (**1a**) in quantitative yield.[41] The latter was etherified with diethylene glycol in the presence of catalytic amount of $BF_3{\cdot}Et_2O$ to furnish alcohol **2**, which was finally activated through its reaction with 4-nitrophenyl chloroformate in the presence of Et_3N to give the expected mixed carbonate active ester **14** in 90% yield (Scheme 1).



Scheme 1. Reagents and conditions: (i) $NaBH_4$, MeOH, 0°C, 3 h, 100%; (ii) diethylene glycol, $BF_3{\cdot}Et_2O$, 0 to 25°C, 1.5 h, 74%; (iii) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, 25°C, 24 h, 90%.

*2.1.2. Activated 10-carba-ART analogue **3***

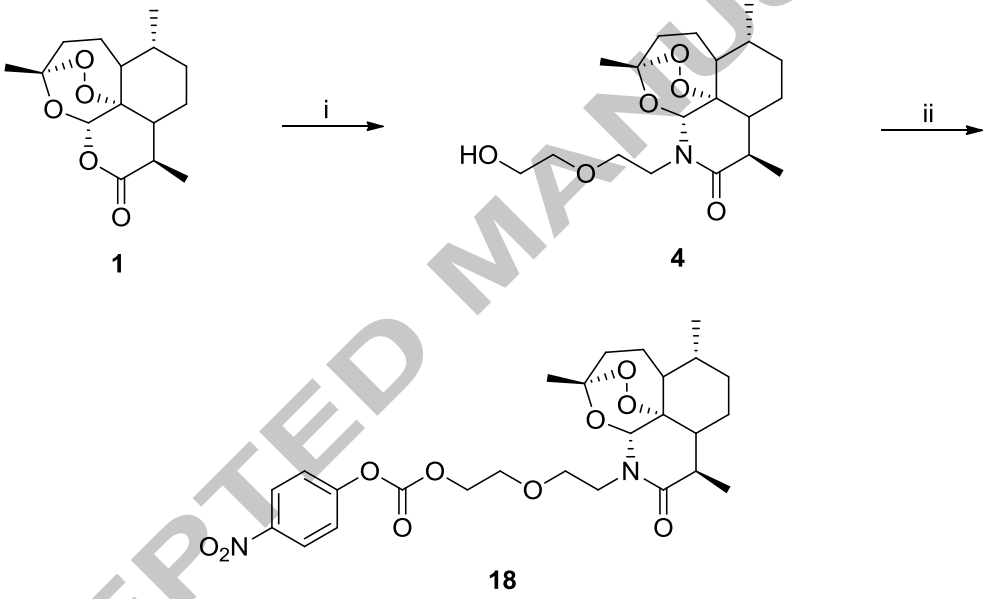
DHA **(1a)** was initially reacted with benzoyl chloride in the presence of pyridine to afford the corresponding C-10 benzoate (**15**) in quantitative yield. This, in turn, was treated with allyltrimethylsilane, using anhydrous $ZnCl_2$ as Lewis acid, in the presence of 4 Å molecular sieves to give 10*β*-allyldeoxoartemisinin (**16**) in 92% yield.[42] The latter was then subjected to a hydroboration-oxidation sequence of reactions to furnish the primary alcohol **3** in 85% yield over two steps.[5] Finally, the required mixed carbonate active ester **17** was successfully obtained through activation of alcohol **3** with 4-nitrophenyl chloroformate, as described previously, in 84% yield (Scheme 2).



Scheme 2. Reagents and conditions: (i) Benzoyl chloride, pyridine, CH_2Cl_2, 0^{o}C, 16 h, 100%; (ii) allyltrimethylsilane, $ZnCl_2$, 4 Å mol. sieves, DCE, 0^{o}C, 3 h, 92%; (iii) (a) $BH_3{\cdot}SMe_2$, THF, -20^{o}C, 2 h; (b) H_2O_2, Na_2CO_3, H_2O, 25^{o}C, 30 min, 85%; (iv) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, 25^{o}C, 24 h, 84%.

*2.1.3. Activated 11-aza-ART analogue **4***

The insertion of a nitrogen atom at position 11 of ART involves ring opening from a suitable amine, serving as a nucleophile, and then an acid-catalysed lactam ring formation according to a published procedure.[43,44] For that purpose, ART was reacted with 2-(2-aminoethoxy)ethanol and the thus obtained intermediate was treated with SiO_2/20% H_2SO_4 to provide the desired alcohol **4** in 50% yield. The latter was activated, similarly to alcohols **2** and **3**, to afford the mixed carbonate active ester **18** in 85% yield (Scheme 3).

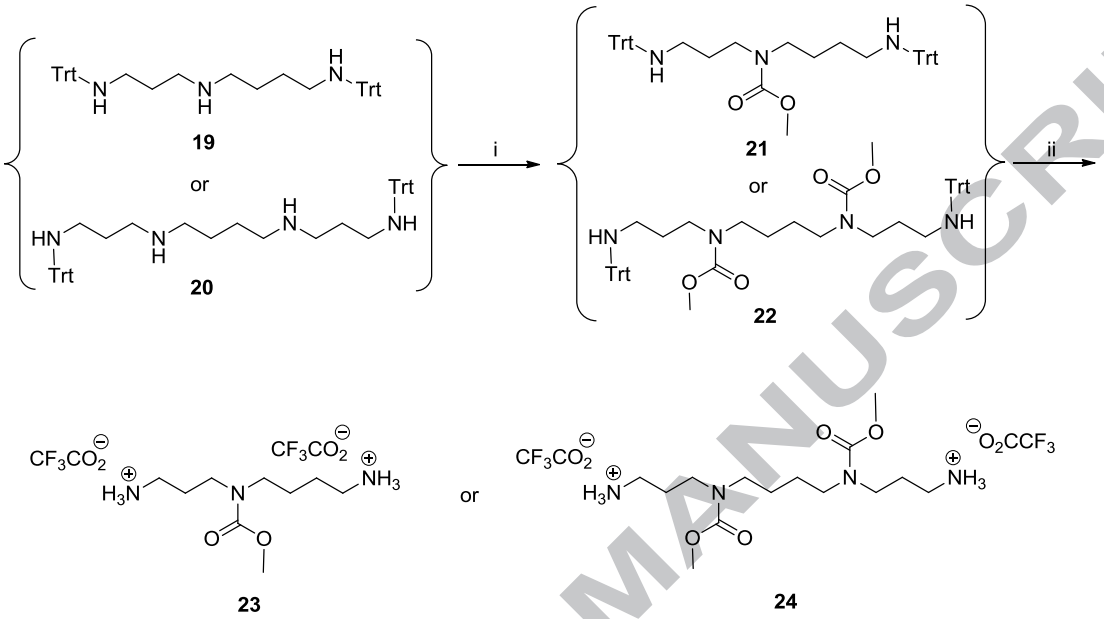


Scheme 3. Reagents and conditions: (i) (a) 2-(2-aminoethoxy)ethanol, $CHCl_3$/MeOH, 0 to 25°C, 1.5 h; (b) SiO_2/20% H_2SO_4, di-*tert*-butylphenol, $CHCl_3$, 0 to 25°C, 16 h, 50% (ii) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, 25°C, 24 h, 85%.

2.1.4. Selectively protected PAs

N^1,N^8-ditritylspermidine (**19**)[45] and N^1,N^{12}-ditritylspermine **(20)**[46] were orthogonally protected at their secondary amino functions with the methoxycarbonyl group, upon treatment with methyl chloroformate in the presence of Et_3N. From the thus obtained fully protected PA derivatives **21**

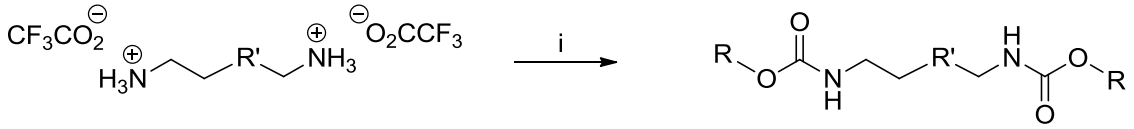
and **22**, respectively, the anticipated compounds **23** and **24** were obtained, in the form of their corresponding bistrifluoroacetate salts, upon TFA-mediated deprotection of the former (Scheme 4).



Scheme 4. Reagents and conditions: (i) methyl chloroformate, Et_3N, CH_2Cl_2, 0 to 25°C, 24 h, 98% (for **21**) or 85% (for **22**); (ii) 45% TFA, CF_3CH_2OH, CH_2Cl_2, 0 to 25°C, 1.5 h, 98% (for **23** and **24**).

*2.1.5. Synthesis of ART-PA conjugates **5-13***

Finally, the assembly of the projected ART-PA conjugates **5-13** was successfully accomplished upon coupling of active esters **14**, **17** and **18** with the PA put, and the spd and spm derivatives **23** and **24**, respectively, in the presence of $^{i}Pr_2NEt$ in yields ranging from 20 to 56% (Scheme 5).



put: R' = CH_2
23: R' = $CH_2N(CO_2Me)(CH_2)_3$
24: R' = $CH_2N(CO_2Me)(CH_2)_4N(CO_2Me)(CH_2)_2$

5-13: R = A/B/C

Scheme 5. Reagents and conditions: (i) active ester **14** or **17** or **18**, $^{i}Pr_2NEt$, CH_2Cl_2, 0 to 25°C, overnight, 20-56% yield.

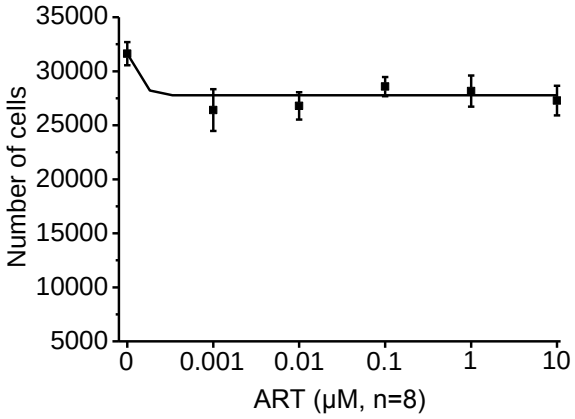
2.2. Pharmacology

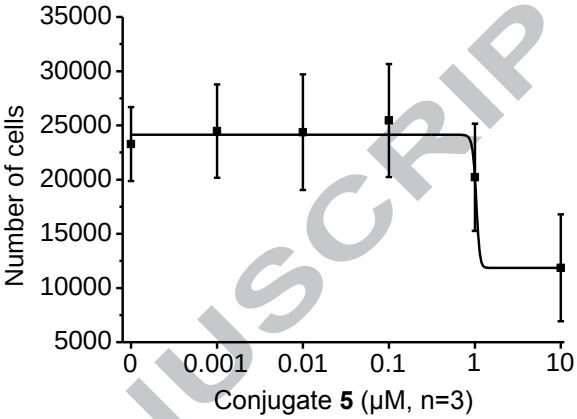
2.2.1. Effect of ART and its conjugates on the number of MCF7 breast cancer cells

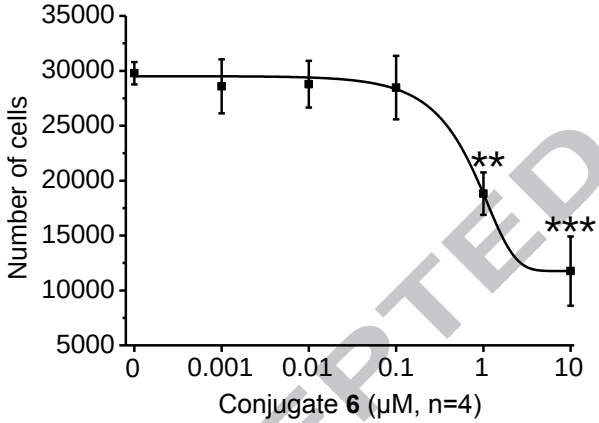
We first investigated the effect of different concentrations of ART (**1**) and ART-PA conjugates **5-13** on MCF7 cell number, 48 h after the addition of the tested agents into the cell culture medium. As shown in Fig. 2 and Table 1, all conjugates decreased the number of MCF7 cells in a concentration-dependent manner, with the maximum effect being observed at the concentration of 10 μM. Higher concentrations could not be used because of solubility issues. ART itself had a minor effect on the number of MCF7 cells. The IC_{50} values and the maximum effect (efficacy) of the tested agents, as interpolated from graphical data, are shown in Table 1. All the tested conjugates had similar IC_{50} values but based on their efficacy and the concentration-dependency, the most interesting seem to be the conjugates **6**, **7**, **9** and **12**.

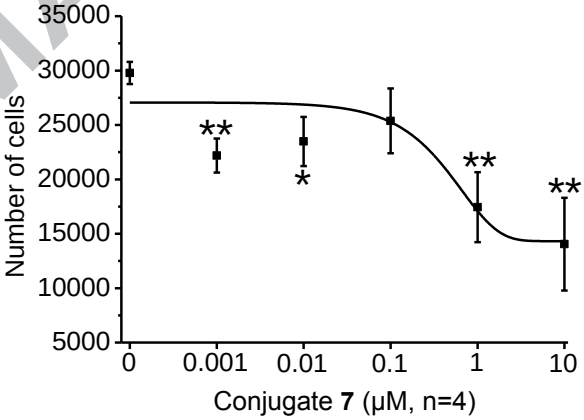
These results suggest that conjugation of ART with PAs leads to a significant enhancement of the anticancer efficacy of the parent compound ART and that the most effective combinations of PA chain, modified ART and linker are the longer spd or spm chains, containing additional amino

functions compared to the put chain, the 10-carba-ART or the 11-aza-ART nucleus and the hydroxypropyl or the diethylene glycol linker attached at position 11 of 11-aza-ART.









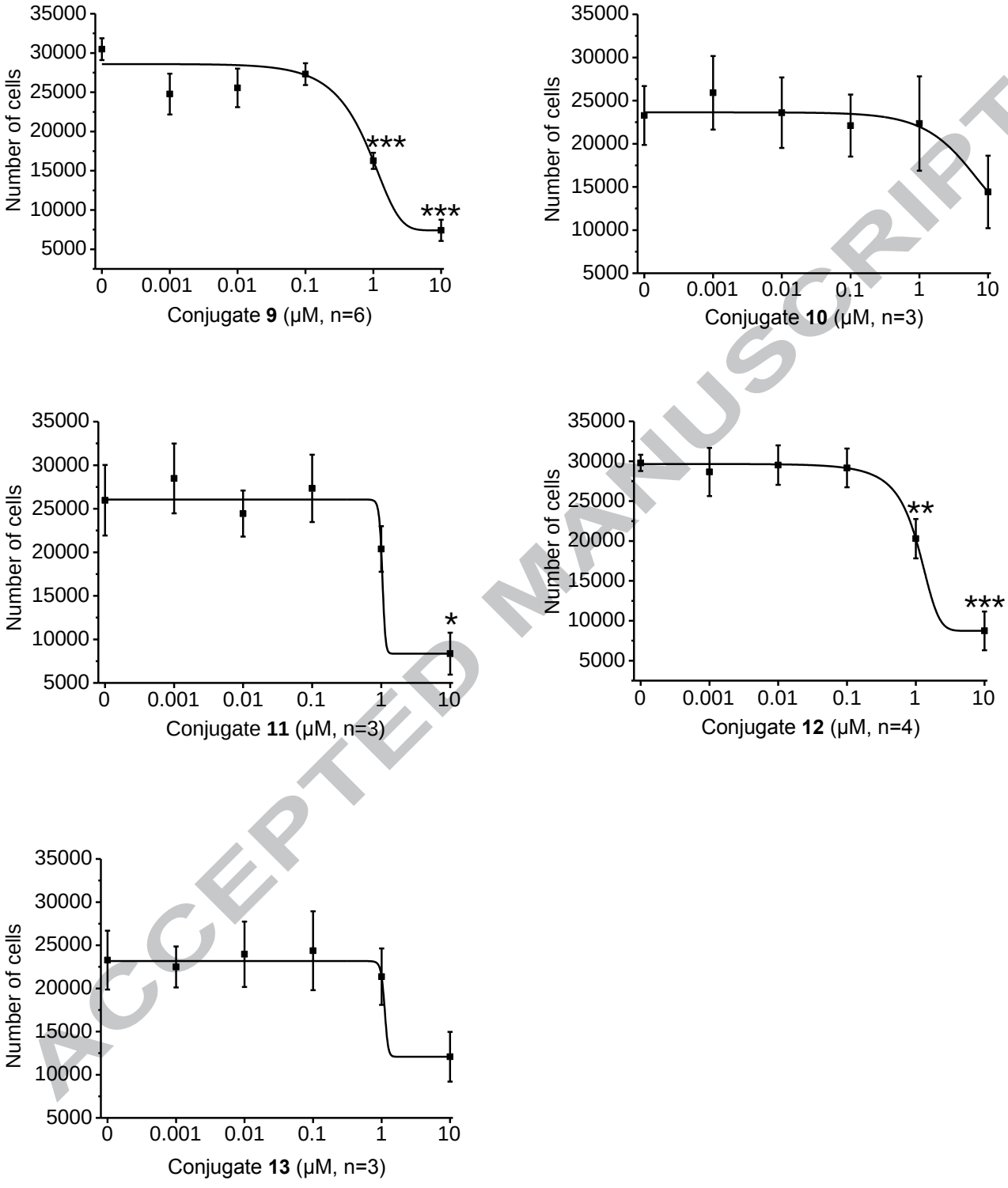


Fig. 2. Effect of ART and its conjugates on the number of MCF7 cells 48 h after addition of the tested agents into the cell culture medium. Results are expressed as mean ± S.E.M. of the number

of cells in treated compared with untreated cells. Asterisks denote statistical significance from untreated cells. *P<0.05, **P<0.01, ***P<0.001.

Table 1. IC_{50} values and efficacy (maximum percent decrease in the number of cells) of ART and its conjugates in decreasing the number of MCF7 cells. IC_{50} values were calculated from interpolations of the graphical data presented in Fig. 2.

	IC_{50} (μM)	**Efficacy (%)**
ART	$1.4x10^{-4}$ ± 0.0164	14 ± 0.7
Conjugate **5**	1 ± 0.097	50 ± 20.5
Conjugate **6**	1 ± 0.000006	61 ± 16.35
Conjugate **7**	0.999 ± 006	52 ± 15.6
Conjugate **8**	n.d.	11.2 ± 13
Conjugate **9**	1 ± 000018	76 ± 13.7
Conjugate **10**	0.999 ± 0.164	39 ± 11.3
Conjugate **11**	1 ± 0.046	64 ± 17.9*
Conjugate **12**	1 ± 0.000006	70 ±18.9
Conjugate **13**	1 ± 0.013	48 ± 11.0

n.d.: not determined; due to the very low efficacy, a concentration-dependent assay was not performed for this analogue.

*Although efficacy of this conjugate is high, there is no concentration-dependency of its effect; therefore, it is not considered physiologically significant.

Interestingly, the mere combination of ART derivative **3** and spd, co-administered at similar concentrations, did not mimic the effect of conjugate **9** on the number of MCF7 cells (Fig. 3), suggesting that conjugation plays a decisive role in the observed efficacy of this compound. If conjugate **9** were considered as ART dimer, our results parallel the data from other research groups showing that dimeric ART is more effective than ART itself in various biological systems.[23]

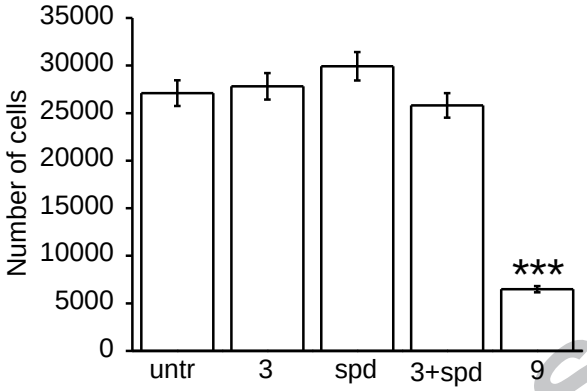


Fig. 3. MCF7 cells were treated with conjugate **9** or the unconjugated ART derivative **3**, spd or their combination (**3**+spd), all used at 10 μM. Results are expressed as mean ± S.E.M. of the number of cells compared with the untreated cells (untr). Asterisks denote a statistically significant difference from untreated cells. ***P<0.001.

2.2.2. *Efforts to elucidate the mechanism through which ART and its conjugates **6**, **7**, **9**, and **12** affect the number MCF7 breast cancer cell*

It is well known that the antimalarial action of ART and analogs thereof implicates the formation of free radicals, which mediate eradication of the Plasmodium species and the same has been also suggested as a possible mechanism for its anticancer activity.[7,11] In the present study, we investigated the effect of ART and its most interesting PA conjugates on ROS production by MCF7 breast cancer cells. As shown in Supplementary Fig. S1, ART and conjugates **6** and **7**, and to a lesser extend conjugate **12**, increased ROS production by MCF7 cells, while conjugate **9**, which is the most effective in inhibiting MCF7 cell growth, had no effect, suggesting that the observed inhibitory effect of the tested conjugates on the number of MCF7 cells is unrelated to a

possible increased ROS production and thus, to oxidative stress caused by ART conjugates. This is in line with observations by other groups,[7,11] adding to the notion that the mechanism of action of ART and derivatives is multivariate and may be cell type-specific.

ART and derivatives have been previously reported to induce apoptosis of several types of cancer cells.[11] In the present study, we investigated whether the decrease in the number of MCF7 cells caused by conjugates **6**, **7**, **9** and **12** was, at least partly, due to induction of apoptosis. As shown in Supplementary Fig. S2, neither ART nor its conjugates had a significant effect on apoptosis or necrosis of MCF7 cells, as estimated by the relative amount of the annexin V and propidium iodide-stained cells in the population. These data suggest that the observed decrease in the number of MCF7 cells may be due to inhibition of cancer cell proliferation and are in line with previously published data showing that the anticancer effects of artesunate on MCF7 and MDA-MB-231 breast cancer cells were exerted through cell cycle arrest or an autophagy pathway.[47,48]

Since neither ROS production nor apoptosis or necrosis induction seems to be the mechanism, through which the tested ART conjugates affect the growth of MCF7 breast cancer cells, we investigated whether ART or its conjugates **6**, **7**, **9** and **12** had any effect on the expression and secretion of the growth factor pleiotrophin (PTN). We chose PTN because it has been implicated in cancer cell growth[49] and has been previously shown to be expressed by MCF7 breast cancer cells and act in an autocrine way to confer a growth advantage on MCF7 tumors[50] As shown in Fig. 4, all tested conjugates significantly decreased expression and secretion of PTN, while the effect of ART was marginal and not statistically significant. Conjugate **9** seems to be the most effective in decreasing PTN expression and secretion.

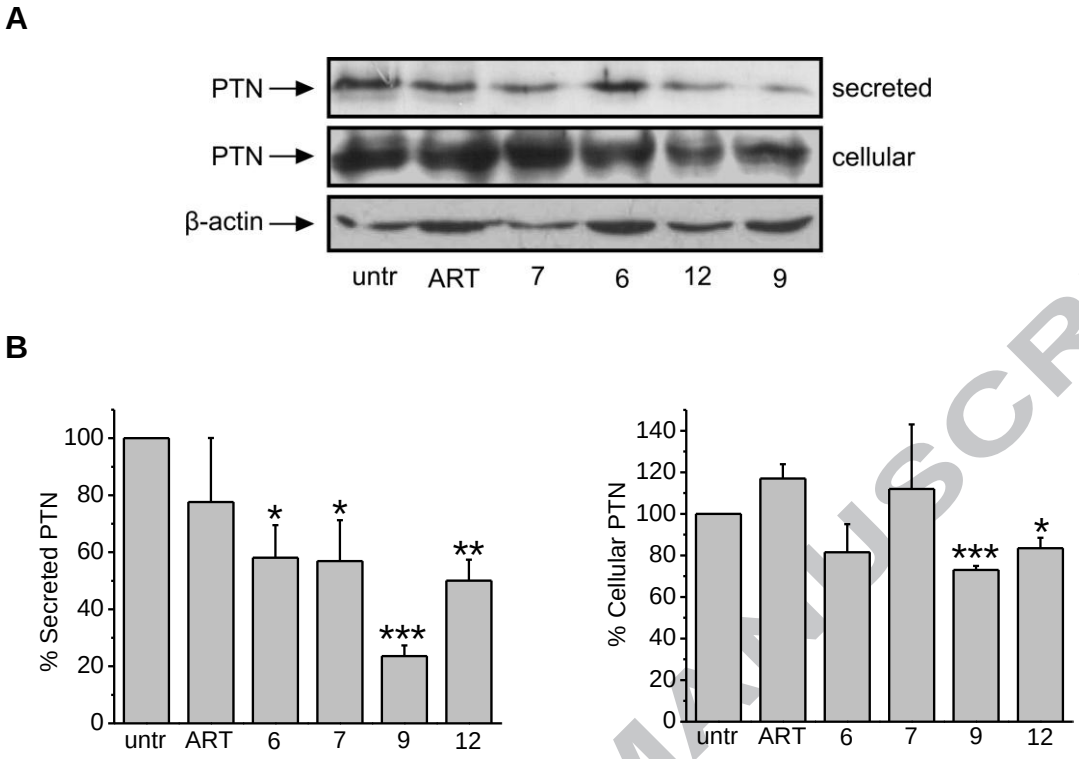
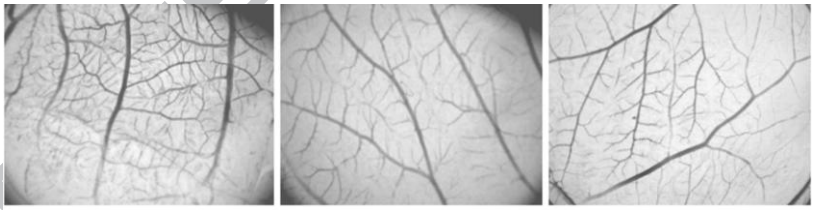


Fig. 4. Effect of ART and its conjugates **6**, **7**, **9** and **12** on PTN expression and secretion by MCF7 breast cancer cells. (**A**) Representative picture of Western blot analysis of PTN protein levels in the conditioned media (secreted) or the cell lysates (cellular) of MCF7 cells following treatment with the tested conjugates at the concentration of 10 μM for 24 h. (**B**) PTN protein levels were quantified by densitometric analysis of the corresponding band in each lane. Results in all cases are expressed as mean ± S.E.M. (n=3) of the percent change of the amounts of secreted PTN protein in treated compared with the untreated cells (untr, set as default =100). The content of β-actin in the corresponding cells at the time that the conditioned medium was collected is shown as a measure of normalization for PTN secretion. Asterisks denote a statistically significant difference from untreated cells. *P<0.05, **P<0.01, ***P<0.001.

2.2.3. *Effect of ART, DHA and ART conjugates **6**, **9** and **12** on angiogenesis in vivo*

Besides a direct effect on cancer cells, ART and DHA also inhibit angiogenesis, thus indirectly inhibiting cancer growth.[7] In the present work, we studied the effect of ART, DHA and its conjugates that were more effective in inhibiting MCF7 cancer cells growth on angiogenesis in vivo, using the chick embryo chorioallantoic membrane (CAM) assay. As shown in Fig. 5, conjugates **9** and **12** showed a small but statistically significant anti-angiogenic effect in the chicken embryo CAM model. The decrease in the total vessel length at the dose of 10 nmol/egg reached 12 ± 3.7% for conjugate **9** and 22 ± 2.4% for conjugate **12**. Conjugate **6** at the dose of 10 nmol/egg also caused a statistically significant 14.3 ± 2.6% decrease in the total CAM vessel length ($P<0.01$), while neither ART not DHA have any significant effect (6.3 ± 7.9% and 2.8 ± 9.3% decrease compared with the control respectively).



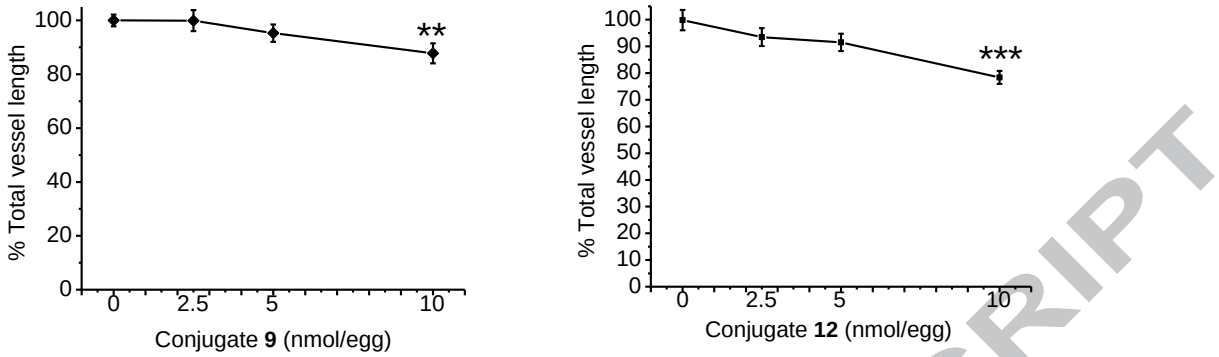
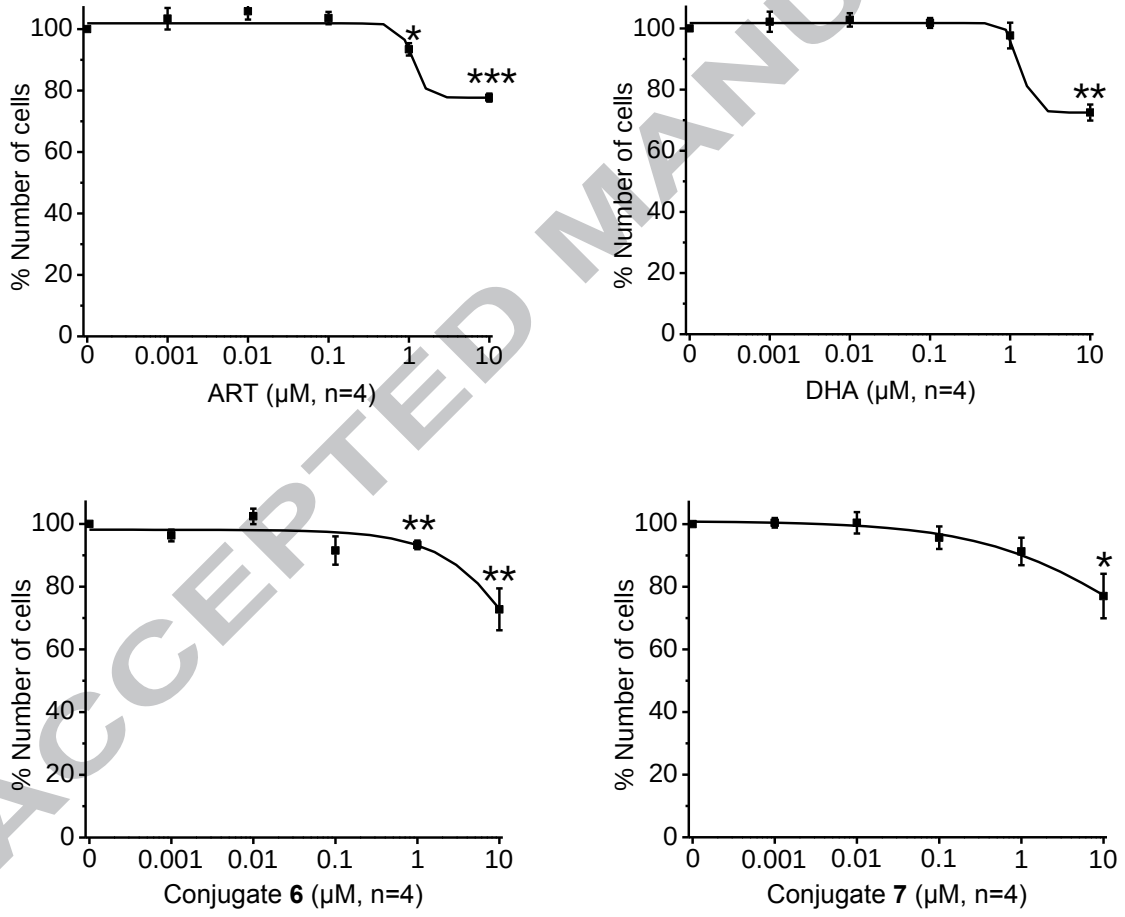


Fig. 5. Effect of ART conjugates **9** and **12** on angiogenesis in the chick embryo CAM. Different amounts of the tested compounds were applied on the CAM and 48 h later, the total vessel length was estimated using image analysis software. Results are expressed as mean ± S.E.M. of the percent change of the total vessel length in treated compared with the untreated tissue (control) from at least four independent experiments. Asterisks denote a statistically significant difference from control. **P<0.01, ***P<0.001. The pictures are representative, showing the vessel network of the chicken embryo chorioallantoic membrane after treatment with the tested conjugates (10 nmoles/egg).

2.2.4. *Effect of ART, DHA and ART conjugates **6**, **7**, **9** and **12** on the number of human endothelial cells*

Previous studies have shown that ART and DHA have a direct effect on endothelial cells, decreasing their proliferation.[51-53] In the present work, we studied whether the tested ART conjugates also had a direct effect on endothelial cells, by measuring their effect on the number of human umbilical vein endothelial cells (HUVEC), as described above for MCF7 breast cancer cells. As shown in Fig. 6, ART and DHA significantly decreased HUVEC proliferation in a similar manner, in line with published data.[51-53] All tested conjugates also decreased the number of human endothelial cells in a concentration-dependent manner, with the maximum effect being observed at the concentration of 10 μM. Higher concentrations could not be used because of

solubility issues. The IC_{50} values and the efficacy of the tested agents, as interpolated from graphical data, are shown in Table 2. All tested agents had similar efficacy and IC_{50} values, with conjugate **9** being slightly more effective. It is interesting to note that although ART and DHA significantly affected HUVEC proliferation, they had no effect on CAM angiogenesis, at least at the concentrations used, while the ART-PA conjugates were effective in vivo, suggesting that they are either more effective than the parent compounds or that they also affect other pathways yet to be identified.



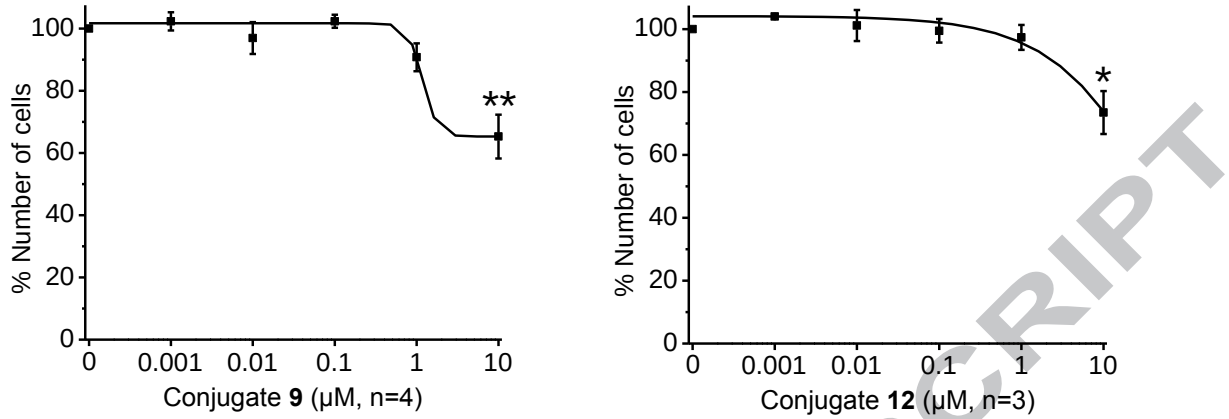


Fig. 6. Effect of ART, DHA and conjugates **6**, **7**, **9** and **12** on the number of human endothelial cells 48 h after addition of the tested agents into the cell culture medium. Results are expressed as mean ± S.E.M. of the percent number of cells in treated compared with untreated cells (set as default 100%). Asterisks denote statistical significance from untreated cells. *P<0.05, **P<0.01, ***P<0.001.

Table 2. IC_{50} values and efficacy (maximum percent decrease in the number of cells) of ART, DHA and conjugates **6**, **7**, **9** and **12** in decreasing the number of human endothelial cells. IC_{50} values were calculated from interpolations of the graphical data presented in Fig. 6.

Conjugate	IC_{50} (μM)	Efficacy (%)
ART	1.1. ± 0.0006	22 ± 1.3
DHA	1.4 ± 0.0035	28 ± 2.6
6	3.6 ± 0.0030	27± 2.5
7	2.3 ± 0.00015	23± 2.3
9	1.2± 0.0003	35± 3.7
12	7.5± 0.0020	27± 2.5

3. Conclusion

A series of new conjugates of the urethane type of ART with the natural PAs put, spd and spm, have been readily and efficiently synthesized by employing ART derivatives incorporating hydroxypropyl or diethylene glycol linkers. The biological evaluation of these compounds revealed that: 1) ART-PA conjugates are more effective than ART alone in inhibiting breast cancer cell growth; 2) Among all the tested agents, conjugates **6**, **7**, **9** and **12** were the most effective in inhibiting human breast cancer cell growth in a dose dependent manner; 3) This effect of the ART conjugates could not be correlated to ROS production or apoptosis/necrosis induction; 4) ART conjugates **6**, **7**, **9** and **12** decreased expression and secretion of the angiogenic growth factor PTN, inhibited angiogenesis in vivo and decreased endothelial cells growth in vitro. As concerns their chemical structure characteristics, the most interesting ART conjugates (**9** and **12**) appear to combine the longer PA chains of spd or spm with the 10-carba-ART derivative and the hydroxypropyl spacer at position 10 of the ART nucleus.

4. Experimental section

4.1. Chemistry

4.1.1. General chemistry methods

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 400.13MHz and ^{13}CNMR spectra at 100.62 MHz on a Bruker DPX spectrometer. Tetramethylsilane (TMS) was used as internal standard. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. Electro-spray ionization (ESI) mass spectra were recorded on a Micromass-Platform LC spectrometer using MeOH as solvent. Flash column chromatography (FCC) was performed on Merck silica gel 60 (230-400 mesh) and TLC on 60 Merck 60 F254 films (0.2 mm) precoated on aluminium foil. Spots were visualized

with UV light at 254 nm and the ninhydrin or charring agent. All solvents were dried and/or purified according to standard procedures prior to use. Anhydrous Na_2SO_4 was used for drying organic solvents and subsequently solvents were routinely removed at *ca.* 40 ^oC under reduced pressure (water aspirator). All reagents employed in the present work were purchased from commercial suppliers and used without further purification. Reactions were run in flame-dried glassware under an atmosphere of argon with the exception of those involving aqueous solutions.

*4.1.2. Synthesis of alcohol **2***

To an ice-cold solution of DHA **1a** (1.0 g, 3.52 mmol) and diethylene glycol (1.68 mL,17.6 mmol), in DCM (30 mL), catalytic amount of $BF_3{\cdot}Et_2O$ (0.2 mL) was added dropwise. The reaction mixture was stirred at RT for 1.5 h. Then it was diluted with DCM and washed once with a saturated $NaHCO_3$ solution and brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness. Alcohol **2** was obtained in pure form after FCC purification. Yield: 0.97 g (74%); White solid; m.p: 94-96 ^oC; R_f (PhMe/EtOAc 1:1): 0.21;MS (ESI, 30eV): *m/z* 411.38 [M+K], 395.45 [M+Na]; ^1H NMR: ($CDCl_3$, δ) 5.40 (1H, s), 4.85 (1H, d, J = 3.2 Hz), 3.98-3.90 (1H, m), 3.75-3.60 (7H, m), 2.67-2.60 (1H, m), 2.39 (1H, td, J =3.6 and 13.6 Hz), 2.04 (1H, dq, J = 4.5 and 14.0 Hz), 2.08-1.22 (10H, m), 1.45 (3H, s), 0.96 (3H, d, J = 6 Hz), 0.91 (3H, d, J = 7.2 Hz) ppm. ^{13}C-NMR: ($CDCl_3$, δ)104.4, 96.4, 87.8, 81.1, 72.2, 70.7, 68.0, 61.8, 52.5, 44.3, 37.5, 36.4, 34.7, 30.8, 26.0, 24.7, 24.1, 20.4, 13.2 ppm.

*4.1.3. Synthesis of alcohol **3***

To a cold solution (-20 ^oC) of 10β-allyldeoxoartemisinin **16** (1.54 g, 5.0 mmol) in THF (200 mL), a 1 M solution of $BH_3{\cdot}SMe_2$ in THF (5.0 mL) was added dropwise. The reaction mixture was stirred at RT for 2 h. Upon completion, the mixture was quenched with a saturated Na_2CO_3 solution (10 mL), a 30% H_2O_2 solution (5.0 mL) was added and the resulting mixture was stirred for further 30 min. Then, it was evaporated to dryness. The residue was diluted with DCM, washed once with water and once with brine. The organic phase was dried over Na_2SO_4 and

evaporated to dryness to afford pure compound **3** after FCC purification. Yield: 1.39 g (85%); colorless oil; R_f (PhMe/EtOAc 7:3): 0.17;MS (ESI, 30eV): *m/z*675.45 [2M+Na], 349.55 [M+Na], 327.57 [M+H]; ^{1}H NMR: ($CDCl_3$, δ) 5.32 (1H, s), 4.25-4.21 (1H, m), 3.74-3.65 (2H, m), 2.65 (1H, sextet, J = 7.2 Hz),2.32 (1H, td, J = 3.6 and 13.6 Hz), 2.05-2.00 (1H, m), 1.93-1.88 (1H, m), 1.82-1.22 (12H, m), 1.41 (3H, s), 0.96 (3H, d, J = 6.0 Hz), 0.87 (3H, d, J = 7.2 Hz) ppm. ^{13}C-NMR: ($CDCl_3$, δ) 103.3, 89.4, 81.3, 75.6, 62.9, 52.4, 44.4, 37.6, 36.7, 34.6, 31.3, 30.7, 26.6, 26.1, 25.0, 24.9, 20.3, 13.0 ppm.

*4.1.4. Synthesis of alcohol **4***

To an ice-cold solution of 2-(2-aminoethoxy)ethanol (14.04 mL, 140.0 mmol) in $CHCl_3$/MeOH (40 mL, 3:7), a solution of ART (2.0 g, 7.10 mmol) in $CHCl_3$ (10 mL) was added dropwise over 5 min. The reaction mixture was stirred at 0 ^{o}C for 2 h, diluted with water and it was extracted thrice with $CHCl_3$. Then, 2,4-bis-*tert*-butylphenol (130 mg), H_2SO_4 (130 mL), silica gel (130 g) were added to the organic phase and the resulting suspension was stirred at RT for 12 h. Upon completion, the mixture was filtered under vacuo, silica gel was washed thrice with $CHCl_3$ and the filtrate washed once with water and once with brine. The organic phase was dried over Na_2SO_4 and evaporated to dryness to afford pure compound **4** after FCC purification. Yield: 1.32 g (50%); yellow oil; R_f (PhMe/EtOAc 1:1): 0.18;MS (ESI, 30eV): *m/z* 408.29 [M+K], 392.36 [M+Na]; ^{1}H NMR ($CDCl_3$, δ):9.35 (1H, s), 5.43 (1H, s), 4.03-3.86 (2H, m), 3.74-3.64 (4H, m), 3.63-3.55 (2H, m), 3.48-3.37 (1H, m), 2.73-2.62 (1H, m), 2.59-2.47 (1H, m), 2.46-2.31 (2H, m), 1.90-1.69 (2H, m), 1.66-1.58 (1H, m), 1.38 (3H, s), 1.33-1.22 (1H, m), 1.16 (3H, d, J = 7.2 Hz), 1.07-0.94 (3H, m), 0.97 (3H, d, J = 5.4 Hz); ^{13}C NMR ($CDCl_3$, δ): 172.1, 104.9, 80.1, 79.0, 72.1, 69.0, 68.3, 51.3, 45.7, 40.6, 37.1, 36.6, 34.5, 33.1, 25.3, 25.1, 22.7, 20.1, 12.9 ppm.

*4.1.5. General procedure for the synthesis of activated compounds **14**, **17**, **18***

To a solution of **2, 3** or **4** (2.5 mmol) and *p*-nitrophenyl chloroformate (0.6 g, 3.0 mmol,) in DCM (18 mL), Et_3N (0.7 mL, 5.0 mmol) was added dropwise. The reaction mixture was stirred at RT overnight and then diluted with DCM. The organic phase was washed once with an ice-cold 5%

aq. solution citric acid and once with brine, dried over Na_2SO_4 and evaporated to dryness. The residues were subjected to FCC to afford pure compounds **14**, **17** and **18** respectively.

Compound **14**: Yield: 1.21 g (90%); yellow oil; R_f (PhMe/EtOAc 9:1): 0.15;MS (ESI, 30eV): *m/z* 576.33 [M+K], 560.27 [M+Na];^{1}H NMR ($CDCl_3$, δ):8.22 (2H, d, *J* = 9.2 Hz), 7.32 (2H, d, *J* = 9.2 Hz), 5.38 (1H, s),4.79 (1H, d, *J* = 3.6 Hz), 4.36 (2H, t, *J* = 4.4 Hz), 3.78-3.65 (2H, m), 3.64-3.55 (4H, m), 2.61-2.53 (1H, m), 2.35-2.24 (1H, m), 1.99-1.91 (1H, m), 1.84-1.76 (1H, m), 1.75 (1H, d, *J* = 3.6 Hz), 1.72-1.64 (1H, m), 1.59-1.51 (1H, m), 1.49-1.34 (2H, m), 1.37 (3H, s), 0.90-0.78 (2H, m), 0.87 (3H, d, *J* = 5.6 Hz), 0.85 (3H, d, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, δ): 155.5, 152.5, 145.4, 125.3 (2C), 121.8 (2C), 104.1, 102.2, 87.9, 81.1, 70.7, 68.4, 68.3, 67.3, 52.6, 44.5, 37.5, 36.4, 34.7, 30.9, 26.2, 24.7, 24.4, 20.4, 13.0 ppm.

Compound **17**: Yield: 1.03 g (84%); yellow oil; R_f (PhMe/EtOAc 9:1): 0.12;MS (ESI, 30eV): *m/z* 530.16 [M+K], 514.29 [M+Na];^{1}H NMR ($CDCl_3$, δ): 8.21 (2H, d, *J* = 9.2 Hz), 7.32 (2H, d, *J* = 9.2 Hz), 5.25 (1H, s), 4.36-4.24 (2H, m), 4.22-4.15 (1H, m), 2.64-2.54 (1H, m), 2.32-2.24 (1H, m), 2.06-1.93 (2H, m), 1.90-1.82 (1H, m), 1.81-1.70 (2H, m), 1.65-1.54 (3H, m), 1.54-1.44 (2H, m), 1.41-1.28 (2H, m), 1.36 (3H, s), 1.23-1.17 (2H, m), 0.92-0.88 (1H, m), 0.90 (3H, d, *J* = 6.0 Hz), 0.82 (3H, d, *J* = 7.6 Hz);^{13}C NMR ($CDCl_3$, δ):155.6, 152.5, 145.3, 125.3 (2C), 121.8 (2C), 103.1, 89.4, 81.1, 74.2, 69.4, 52.2, 44.1, 37.5, 36.6, 34.4, 30.4, 26.6, 26.0, 25.8, 24.9, 24.7, 20.2, 12.8 ppm.

Compound **18**: Yield: 1.14 g (85%); yellow oil; R_f (PhMe/EtOAc 2:8): 0.21;MS (ESI, 30eV): *m/z* 573.24 [M+K], 557.18 [M+Na], 535.20 [M+Na]; ^{1}H NMR ($CDCl_3$, δ):8.27 (2H, d, *J* = 8.8 Hz), 7.38 (2H, d, *J* = 8.8 Hz), 5.44 (1H, s), 4.43-4.36 (2H, m), 3.84-3.79 (2H, m), 3.78-3.73 (2H, m), 3.72-3.66 (2H, m), 3.33-3.25 (1H, m), 2.46-2.36 (1H, m), 2.04-1.94 (2H, m), 1.83-1.76 (1H, m), 1.75-1.69 (1H, m), 1.67-1.62 (1H, m), 1.38 (3H, s), 1.36-1.27 (2H, m), 1.14 (3H, d, *J* = 7.2 Hz), 1.07-0.94 (3H, m), 0.96 (3H, d, *J* = 5.4 Hz); ^{13}C NMR ($CDCl_3$, δ):171.9, 155.6, 152.6, 145.5, 125.3 (2C), 121.8 (2C), 104.8, 80.2, 79.3, 69.3, 68.4, 68.2, 51.4, 45.8, 40.8, 37.5, 36.7, 33.8, 33.2, 25.5, 25.1, 22.7, 19.9, 12.9 ppm.

*4.1.6. General procedure for the synthesis of fully protected polyamines **21** and **22***

To an ice-cold solution of N^1,N^8-ditritylspermidine (**19**) or N^1,N^{12}-ditritylspermine (**20**) (2.0 mmol) and Et_3N (0.36 mL, 2.6 mmol) in DCM (8.0 mL) methyl chloroformate (1.1 eq per amino function) was added. The resulting mixture was left at 0°C for 10 min and for further 1 h at RT. Then, it was diluted with DCM, washed once with an aqueous solution of 5% $NaHCO_3$, once with brine, dried over Na_2SO_4 and evaporated to dryness. Compounds **21** and **22** were obtained pure after FCC purification.

Compound **21**: Yield: 1.69 g (98%);white solid; m.p. 100-103°C; R_f ($CHCl_3$/MeOH 95:5): 0.50;MS (ESI, 30eV): *m/z* 710.41 [M+Na], 688.41 [M+H], 243.14 [Trt^+];^{1}H NMR ($CDCl_3$, δ): 7.40-7.34, 7.21-7.14 and 7.12-7.06 (30H, three m), 3.51 (3H, s), 3.19 (2H, unresolved t), 3.01 (2H, unresolved t), 2.03 (4H, q, *J* = 7.2 Hz), 1.60 (2H, m), 1.42 (4H, m); ^{13}C NMR ($CDCl_3$, δ):156.8, 146.2 (6C), 128.6 (12C), 127.7 (12C), 126.3 (6C), 70.8 (2C), 52.3, 46.7, 45.4, 43.3, 40.8, 28.0, 26.9, 26.3 ppm.

Compound **22**: Yield: 1.36 g (85%); white solid; m.p. 105-108°C; R_f ($CHCl_3$/MeOH 9:1): 0.18; IR (KBr, cm^{-1}): 2936, 1708, 1218, 704; MS (ESI, 30eV): *m/z* 804.37 [M+H], 243.14 [Trt^+];^{1}H NMR ($CDCl_3$, δ):7.50-7.42, 7.31-7.22 and 7.21-7.13 (30H, three m), 3.60 (6H, s), 3.35-3.20 (4H, m), 3.19-3.05 (4H, m), 2.14-2.04 (4H, m), 1.75-1.64 (4H, m), 1.49-1.36 (4H, m); ^{13}C NMR ($CDCl_3$, δ):156.7 (2C), 146.2 (6C), 128.6 (12C), 127.8 (12C), 126.2 (6C), 70.9 (2C), 52.4 (2C), 46.8 (2C), 45.0 (2C), 40.9 (2C), 29.9 (2C), 25.4 (2C) ppm.

*4.1.7. General procedure for the selective deprotection of compounds **21** and **22***

To a round-bottom flask containing compound **21** or **22** (2.0 mmol) an ice-cold solution of 45% TFA in anhydrous DCM (6.0 mL) and CF_3CH_2OH (0.29 mL, 4.0 mmol) were added. The resulting mixture was stirred at 0 °C for 20 min and at RT for further 1 h. Then, it was evaporated to dryness. A mixture of Et_2O and hexane (1:2) was added to the residue and it was refrigerated overnight. The supernatant liquid was decanted to leave pure compounds **23** or **24**, respectively, as oily residues.

Compound **23**: Yield: 0.85 g (98%);colorless oil; R_f ($CHCl_3$/MeOH/NH_3 9:1:0.1): 0.14; MS (ESI, 30eV): *m/z* 227.39 [M+Na], 204.42 [M+H].

Compound **24**: Yield: 1.07 g (98%);colorless oil; R_f ($CHCl_3$/MeOH/NH_3 9:1:0.1): 0.28; MS (ESI, 30eV): *m/z* 342.42 [M+Na], 319.44 [M+H].

*4.1.8. General procedure for the synthesis of ART-PA conjugates **5-13***

To an ice-cold solution of put or **23** or **24** (1.0 mmol) in DCM (3.0 mL) activated carbonates **14**, **17** or **18** (2.0 mmol) and $^{i}Pr_2NEt$ (5.0 mmol, 0.87 mL) were added. The reaction mixture was stirred at ambient temperature overnight. Upon completion of the reaction, the mixture was diluted with DCM and the organic phase was washed once with 5% aqueous citric acid solution, once with water, once with 5% $NaHCO_3$ solution, once with brine, dried over Na_2SO_4 and evaporated to dryness. The projected compounds were obtained in pure form after FCC purification.

For compound **5**: Yield: 0.48 g (54%); colourless oil; R_f (PhMe/EtOAc 3:7): 0.20; MS (ESI, 30eV): *m/z* 923.25 [M+K], 907.20 [M+Na]; ^{1}H NMR ($CDCl_3$, δ): 5.45 (2H, s), 4.83 (2H, d, *J* = 3.6 Hz), 4.25-4.12 (4H, m), 3.96-3.88 (2H, m), 3.69-3.58 (10H, m), 3.18 (4H, unresolved q), 2.66-2.57 (2H, m), 2.41-2.29 (4H, m), 2.06-1.98 (2H, m), 1.92-1.83 (3H, m), 1.80 (1H, d, *J* = 3.6 Hz), 1.78-1.70 (2H, m), 1.66-1.58 (2H, m), 1.55-1.50 (4H, m), 1.48-1.40 (4H, m), 1.42 (6H, s), 1.34-1.18 (4H, m), 0.94 (6H, d, *J* = 7.2 Hz), 0.92-0.85 (2H, m), 0.90 (6H, d, *J* = 6.0 Hz); ^{13}C NMR ($CDCl_3$, δ):156.6 (2C), 104.2 (2C), 102.1 (2C), 87.9 (2C), 81.2 (2C), 70.5 (2C), 69.4 (2C), 67.3 (2C), 64.1 (2C), 52.6 (2C), 44.6 (2C), 40.6 (2C), 37.5 (2C), 36.4 (2C), 34.7 (2C), 30.9 (2C), 27.2 (2C), 26.2 (2C), 24.7 (2C), 24.4 (2C), 20.3 (2C), 13.0 (2C) ppm.

For compound **6**: Yield: 0.25 g (32%); colourless oil; R_f (PhMe/EtOAc 1:1): 0.22;MS (ESI, 30eV): *m/z* 831.27 [M+K], 815.34 [M+Na], 793.17 [M+H]; ^{1}H NMR ($CDCl_3$, δ):5.32 (2H, s), 4.24-4.16 (2H, m), 4.15-4.08 (4H, unresolved t), 3.24-3.14 (4H, unresolved m), 2.73-2.61 (2H, m), 2.35-2.29 (2H, m), 2.08-2.00 (2H, m), 1.98-1.89 (4H, m), 1.84-1.77 (2H, m), 1.71-1.59 (8H, m), 1.56-1.52 (4H, m), 1.50-1.42 (4H, m), 1.42 (6H, s), 1.36-1.22 (8H, m), 1.02-0.91 (2H, m), 0.97 (6H, d, *J* = 5.6 Hz), 0.87 (6H, d, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, δ): 157.0 (2C), 103.2 (2C), 89.2 (2C), 81.2 (2C), 74.9 (2C), 65.0 (2C), 52.3 (2C), 44.3 (2C), 40.6 (2C), 37.5 (2C), 36.6 (2C), 34.4 (2C), 30.3 (2C), 27.3 (2C), 27.0 (2C), 26.1 (2C), 24.9 (2C), 24.7 (2C), 21.5 (2C), 20.2 (2C), 12.9 (2C) ppm.

For compound **7**: Yield: 0.18 g (21%); colorless oil; R_f (PhMe/EtOAc 2:8): 0.14; MS (ESI, 30eV): *m/z* 917.31 [M+K], 901.37 [M+Na], 879.27 [M+H];^{1}H NMR ($CDCl_3$, δ): 5.10 (2H, s), 4.11-4.05 (4H, m), 3.93-3.80 (4H, m), 3.59-3.48 (8H, m), 3.15-3.06 (4H, m), 2.66-2.58 (2H, m), 2.52-2.42 (2H, m), 2.35-2.27 (6H, m), 2.04-2.00 (6H, m), 1.80-1.68 (4H, m), 1.49-1.41 (6H, m), 1.43 (6H, s), 1.15 (6H, d, *J* = 6.8 Hz), 1.08-0.92 (4H, m), 1.00 (6H, d, *J* = 6.0 Hz);^{13}C NMR ($CDCl_3$, δ):162.4 (2C), 156.8 (2C), 105.7 (2C), 80.1 (2C), 78.0 (2C), 70.8 (2C), 69.1 (2C), 67.9 (2C), 41.3 (2C), 40.8 (2C), 37.3 (2C), 34.5 (2C), 33.9 (2C), 31.9 (2C), 29.9 (2C), 29.7 (2C), 27.1 (2C), 26.2 (2C), 22.7 (2C), 20.5 (2C), 20.1 (2C), 16.9 (2C) ppm.

For compound **8**: Yield: 0.38 g (38%); colorless oil; R_f (PhMe/EtOAc 1:9): 0.22; MS (ESI, 30eV): *m/z* 1038.35 [M+K], 1022.38 [M+Na];^{1}H NMR ($CDCl_3$, δ):5.38 (2H, d, *J* = 3.6 Hz), 4.77 (2H, d, *J* = 3.6 Hz), 4.20-4.15 (2H, m), 4.15-4.07 (4H, m), 3.90-3.82 (2H, m), 3.70-3.51 (12H, m), 3.62 (3H, s), 3.18-3.07 (4H, m), 2.60-2.51 (2H, m), 2.38-2.24 (4H, m), 2.01-1.92 (4H, m), 1.86-1.77 (4H, m), 1.74 (1H, d, *J* = 3.6 Hz), 1.72-1.67 (2H, m), 1.67-1.60 (4H, m), 1.59-1.50 (5H, m), 1.44-1.34 (2H, m), 1.36 (6H, s), 1.23-1.09 (2H, m), 0.89 (6H, d, *J* = 7.2 Hz), 0.90-0.82 (2H, m), 0.84 (6H, d, *J* = 6.0 Hz);^{13}C NMR ($CDCl_3$, δ): 156.5 (3C), 104.1 (2C), 102.1 (2C), 87.9 (2C), 81.1 (2C), 70.5 (2C), 69.5 (2C), 67.3 (2C), 64.1 (2C), 52.6, 44.5 (2C), 44.1, 40.6, 37.5 (3C), 36.5 (2C), 34.7 (3C), 30.9 (3C), 29.7, 27.3, 26.2 (3C), 24.4 (2C), 21.4 (2C), 20.4 (3C), 13.0 (2C) ppm.

For compound **9**: Yield: 0.39 g (43%); colorless oil; R_f (PhMe/EtOAc 1:1): 0.14; MS (ESI, 30eV): *m/z* 946.38 [M+K], 930.27 [M+Na];^{1}H NMR ($CDCl_3$, δ): 5.23 (2H, s), 4.16-4.10 (2H, m), 4.06-3.97 (4H, m), 3.63 (3H, s), 3.29-3.18 (2H, m), 3.15-3.06 (6H, m), 2.63-2.53 (2H, m), 2.30-2.19 (2H, m), 2.00-1.91 (2H, m), 1.90-1.79 (4H, m), 1.75-1.68 (2H, m), 1.63-1.48 (10H, m), 1.43-1.35 (4H, m), 1.33 (6H, s), 1.36-1.22 (12H, m), 0.93-0.81 (2H, m), 0.89 (6H, d, *J* = 5.6 Hz), 0.79 (6H, d, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, δ):162.9 (2C), 156.9,103.2 (2C), 89.2 (2C), 81.2 (2C), 77.2 (2C), 64.9 (2C), 52.7, 52.3 (2C), 44.3 (2C), 40.5 (2C), 37.4 (2C), 36.6 (2C), 34.4 (2C), 31.6, 30.3 (2C), 29.7, 27.2, 27.1, 27.0, 26.1 (2C), 26.0, 25.9, 24.9 (2C), 24.7 (2C), 22.6 (2C), 20.2 (2C), 14.1 (2C) ppm.

For compound **10**: Yield: 0.26 g (26%); colorless oil; R_f (EtOAc): 0.12; MS (ESI, 30eV): *m/z* 1031.96 [M+K], 1016.11 [M+Na], 994.34 [M+H];^{1}H NMR ($CDCl_3$, δ):5.32 (2H, s), 4.10-4.05 (4H, m), 3.72-3.65 (4H, m), 3.63-3.54 (8H, m), 3.62 (3H, s), 3.26-3.17 (4H, m), 3.15-3.06 (6H,

m), 2.39-2.27 (4H, m), 2.00-1.88 (6H, m), 1.73-1.54 (10H, m), 1.37-1.25 (6H, m), 1.29 (6H, s), 1.06 (6H, d, *J* = 7.2 Hz), 1.01-0.88 (4H, m), 0.92 (6H, d, *J* = 6.0 Hz); ^{13}C NMR ($CDCl_3$, δ):171.9, 171.1, 162.7, 162.6, 155.4, 107.1, 104.7, 80.2, 79.3, 69.4, 69.3, 69.2, 69.1, 64.2, 64.1, 54.9, 45.7, 44.2, 43.2 (2C), 41.3 (2C), 40.8, 40.6, 39.7, 37.5, 37.3, 36.6 (2C), 31.1 (2C), 29.9, 29.7, 27.2, 26.7, 25.4 (2C), 25.1 (2C), 24.3 (2C), 21.3, 21.1, 20.5 (2C), 20.1 (2C), 14.2 (2C) ppm.

For compound **11**: Yield: 0.41 g (37%); colorless oil; R_f (EtOAc): 0.14;MS (ESI, 30eV): *m/z* 1153.23 [M+K], 1138.30 [M+Na];^{1}H NMR ($CDCl_3$, δ): 5.43 (2H, s), 4.83 (2H, d, *J* = 3.6 Hz), 4.22-4.14 (4H, m), 3.96-3.90 (2H, m), 3.68 (6H, s), 3.68-3.59 (12H, m), 3.33-3.19 (4H, m), 3.17-3.11 (8H, m), 2.65-2.57 (2H, m), 2.41-2.28 (2H, m), 2.07-1.98 (4H, m), 1.92-1.82 (3H, m), 1.80 (1H, d, *J* = 3.6 Hz), 1.78-1.58 (10H, m), 1.51-1.40 (2H, m), 1.42 (6H, s), 1.25-1.18 (6H, m), 0.94 (6H, d, *J* = 6.0 Hz), 0.92-0.82 (2H, m), 0.90 (6H, d, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, δ):156.5 (4C), 104.1 (2C), 102.1 (2C), 87.8 (2C), 81.3 (2C), 70.4 (2C), 69.6 (2C), 64.0 (2C), 61.7 (2C), 52.5 (2C), 51.4 (2C), 44.5 (4C), 44.1 (2C), 37.4 (2C), 36.4 (2C), 34.7 (2C), 34.2 (2C), 30.9 (2C), 30.1 (2C), 29.7 (2C), 26.1 (2C), 24.7 (2C), 24.4 (2C), 20.5 (2C), 12.9 (2C) ppm.

For compound **12**: Yield: 0.20 g (20%); colorless oil; R_f (PhMe/EtOAc 3:7): 0.17;MS (ESI, 30eV): *m/z* 1061.53 [M+K], 1045.40 [M+Na];^{1}H NMR ($CDCl_3$, δ):5.23 (2H, s), 4.15-4.08 (2H, m), 4.06-3.99 (4H, m), 3.62 (6H, s), 3.27-3.20 (4H, m), 3.16-3.04 (8H, m), 2.63-2.54 (2H, m), 2.27-2.20 (2H, m), 1.99-1.93 (2H, m), 1.90-1.80 (4H, m), 1.76-1.67 (4H, m), 1.67-1.50 (10H, m), 1.45-1.32 (4H, m), 1.34 (6H, s), 1.27-1.20 (12H, m), 0.96-0.83 (2H, m), 0.89 (6H, d, *J* = 6.0 Hz), 0.79 (6H, d, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, δ):156.9 (4C), 103.2 (2C), 89.1 (2C), 81.1 (4C), 64.8 (2C), 52.7 (2C), 52.3 (2C), 46.5 (2C), 44.3 (2C), 44.1 (2C), 37.5 (2C), 36.6 (2C), 34.5 (2C), 30.3 (2C), 29.7 (2C), 28.2 (2C), 27.1 (2C), 26.1 (2C), 26.0 (2C), 24.9 (2C), 24.7 (2C), 21.5 (2C), 20.2 (2C), 12.9 (2C) ppm.

For compound **13**: Yield: 0.62 g (56%); colorless oil; R_f (EtOAc): 0.11; MS (ESI, 30eV): *m/z* 1147.31 [M+K], 1131.18 [M+Na], 1109.27 [M+H];^{1}H NMR ($CDCl_3$, δ): 5.39 (2H, s), 4.16-4.09 (4H, m), 3.77-3.68 (4H, m), 3.66 (3H, s), 3.65-3.58 (8H, m), 3.31-3.20 (6H, m), 3.17-3.07 (7H, m), 3.04-2.98 (1H, m), 2.43-2.33 (4H, m), 2.02-1.92 (4H, m), 1.78-1.57 (12H, m), 1.50-1.41 (9H, m), 1.34 (6H, s), 1.11 (6H, d, *J* = 7.2 Hz), 1.00-0.89 (4H, m), 0.96 (6H, d, *J* = 6.0 Hz); ^{13}C NMR ($CDCl_3$, δ):171.8 (2C), 157.0 (2C), 156.4 (2C), 104.7 (2C), 80.1 (2C), 79.3 (2C), 69.3 (2C), 69.1 (2C), 64.2 (2C), 51.3 (2C), 46.4 (2C), 45.8 (2C), 44.1 (2C), 40.9 (2C), 37.5 (2C), 36.6 (2C), 29.8

(2C), 28.1 (2C), 25.4 (2C), 25.1 (2C), 24.3 (2C), 22.6 (2C), 21.4 (2C), 21.2 (2C), 21.0 (2C), 19.8 (2C), 12.9 (2C) ppm.

4.2. Biology

4.2.1. Cell culture

Human MCF7 breast cancer cells (from ATCC) were cultured in MEM Earle's supplemented with 10% fetal bovine serum (FBS), 0.01 mg/ml human insulin, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B. HUVEC were isolated from human umbilical cords, grown as monolayers in medium M199 that was supplemented with 15% FBS, 150 mg/ml endothelial cell growth supplement, 5 U/ml heparin sodium, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 2.5 µg/ml amphotericin B and used at passages 2-3.[36] All cultures were maintained at 37^{o}C, 5% CO_2 and 100% humidity.

4.2.2. MTT assay

To measure the number of cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described.[36] MCF7 cells were seeded at $10X10^3$ and HUVEC at $15X10^3$ cells/well, in 48-well tissue culture plates. The number of cells was determined 48 h after addition of the tested agents at different concentrations. MTT stock (5 mg/ml in phosphate buffered saline pH 7.4, PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37^{o}C for 2 h. The medium was removed, the cells were washed with PBS and 100 µl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) were added to all wells and agitated thoroughly to solubilise the dark blue formazan crystals. The solution was transferred to a 96-well plate and immediately read on a microplate reader at a

wavelength of 490 nm. Results were always confirmed by direct counting of cells under the microscope, using a standard haemocytometer.

4.2.3. ROS measurement

ROS were assayed using the ROS-sensitive fluorescent dye carboxy-H_2DCFDA. Briefly, MCF7 cells were seeded in 96-well plates specific for fluorescence at a concentration of $15X10^3$ cells/well at a final volume of 100 µl culture medium. Twenty-four hours after seeding, cells were incubated in medium containing the tested conjugates for 4 h. At the end of the incubation period, cells were washed with PBS pH 7.4 twice, and incubated in the dark for 15 min in PBS, containing 50 µM carboxy-H_2DCFDA. The fluorescence intensity was determined in a fluorescence plate reader, by using excitation and emission wavelengths 492–495 and 517–527 nm respectively.

4.2.4. Western blot analysis

Secreted PTN in the cell culture medium of MCF7 breast cancer cells was detected as previously described.[54] Briefly, 24 h after cell stimulation with the tested agents, the entire cell conditioned medium from each plate was collected and incubated for 16 h with 100 µl of heparin-Sepharose (GE Healthcare, UK) at 4 °C under continuous agitation. Bound proteins were eluted with 50 µl of Laemmli sample buffer under reducing conditions by SDS-PAGE and transferred to Immobilon P membranes. The corresponding cell lysates were analysed in parallel. As a measure of normalization for PTN, the levels of β-actin were determined in each sample. Blocking was performed by incubating the membranes with Tris-buffered saline (TBS), pH 7.4, with 0.05%

Tween (TBS-T), containing 5% non-fat dry milk. Membranes were incubated with mouse anti-PTN (1:1,000; Abnova, Heidelberg, Germany) or mouse anti-β-actin (Santa Cruz Biotechnology Inc, Heidelberg, Germany) for 16 h at 4°C under continuous agitation, washed 3 times with TBS-T, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Fab-specific; 1: 5,000; Sigma or Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Detection of immunoreactive bands was performed using the enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL, USA). The protein levels that corresponded to the immunoreactive PTN bands were quantified using the ImagePC image analysis software (Scion Corp., Frederick, MD, USA).

4.2.5. Apoptosis assay

MCF7 cells were seeded at $15X10^4$ cells/well in 6-well plates at a final volume of 2 ml culture medium. Twenty-four hours later the medium was replaced with fresh containing the tested conjugates and cells were incubated for 48 h. At the end of the incubation period with the tested agents, the supernatants were collected and centrifuged for 4 min at 1660 rpm. Cells were trypsinized, washed and resuspended in 100 µl binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). The cell suspension was incubated with 2.5 µl Annexin V-FITC in the dark at 25^{o}C, for 15 min. Then, 5µl of propidium iodide stock solution were added followed by 200 ml of binding buffer and the cells were analyzed by flow cytometry (BD FacsCalibur), per the kit manufacturer's instructions (Annexin V/FITC kit, Bender Med Systems).

4.2.6. Chick embryo CAM assay

The *in vivo* chick embryo CAM angiogenesis model was used, as previously described.[55] Leghorn fertilized eggs (Pindos, Greece) were incubated for 4 days at 37ºC, when a window was opened on the egg shell, exposing the CAM. The window was covered with tape and the eggs were returned to the incubator. Different amounts of the tested agents were diluted in a final volume of 20 μl of PBS and were applied at the 9th day of embryo development on an area of 1 cm^2 of the CAM, restricted by a plastic ring. Forty-eight hours after treatment and subsequent incubation at 37^{o}C, chorioallantoic membranes were fixed *in situ*, excised from the eggs, placed on slides and left to air-dry. Pictures were taken through a stereoscope equipped with a digital camera and the total length of the vessels was measured using ImagePC image analysis software. Briefly, vessel length was measured by adjusting all vessels of a picture of defined dimensions, to one pixel thickness. The vessel area was then expressed as the percentage of pixels occupied by the 1-pixel-thick vessels in the picture and was quantified using ImagePC image analysis software. Assays were carried out at least three times and each experiment contained 10–20 eggs per data point.

4.2.7. Statistical Analysis

The significance of variability between groups was determined by one-way ANOVA or unpaired t-test. Each experiment included triplicate measurements for each condition tested, unless otherwise indicated.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/.....

References

1. Visser BJ, Wieten RW, Kroon D, Nagel IM, Bélard S, van Vugt M, Grobusch MP. *Malaria J.* 2014; 13: 463.

2. Wells TN, Hooft van Huijsduijnen R, Van Voorhis WC. *Nat Rev Drug Discov.* 2015; 14: 424-442.

3. Muangphrom P, Seki H, Fukushima EO, Muranaka T. *J. Nat. Med.* 2016; 70:318-334.

4. Tilley L, Straimer J, Gnädig NF, Ralph SA, Fidock DA. *Trends Parasitol.* 2016; 32:682-696.

5. Hindley S, Ward SA, Storr RC, Searle NL, Bray PG, Park BK, Davies J, O'Neill PM. *J. Med. Chem.* 2002; 45:1052-1063.

6. O'Neill PM, Barton VE, Ward SA. *Molecules* 2010; 15:1705-1721.

7. Efferth T. *Semin. Cancer Biol*. 2017; doi: 10.1016/j.semcancer.2017.02.009.

8. Lai HC, Singh NP, Sasaki T. *Invest. New Drugs* 2013; 31:230-246.

9. Ho WE, Peh HY, Chan TK, Wong WS. *Pharmacol. Ther.* 2014; 142:126-139.

10. Goodrich SK, Schlegel CR, Wang G, Belinson JL. *Future Oncol.* 2014; 10: 647-654.

11. Das AK. *Ann. Med. Health Sci. Res.* 2015; 5: 93-102.

12. Singh NP, Lai H. *Life Sci.* 2001; 70:49-56.

13. Yan X, Yu Y, Ji P, He H, Qiao C. *Eur. J. Med. Chem.* 2015; 102:180-187.

14. Xu CC, Wu JJ, Xu T, Yao CH, Yu BY, Liu J.H. *Eur. J. Med. Chem.* 2016; 123:763-768.

15. Reungpatthanaphong P, Mankhetkorn S. *Biol. Pharm. Bull.* 2002; 25:1555-1561.

16. Reiter C, Capci-Karagöz A, Fröhlich T, Klein V, Zeino M, Viertel K, Held J, Mordmüller B, Emirdağ-Öztürk S, Anıl H, Efferth T, Tsogoeva SB. *Eur. J. Med. Chem.* 2014; 75: 403-412.

17. Luo J, Zhu W, Tang Y, Cao H, Zhou Y, Ji R, Zhou X, Lu Z, Yang H, Zhang S, Cao J. *Radiat. Oncol.* 2014; 9: 84.

18. Zhao Y, Jiang W, Li B, Yao Q, Dong J, Cen Y, Pan X, Li J, Zheng J, Pang X, Zhou H. *Int. Immunopharmacol.* 2011; 11:2039-2046.

19. Kim SJ, Kim MS, Lee JW, Lee CH, Yoo H, Shin SH, Park MJ, Lee SH. *J. Cancer Res. Clin. Oncol.* 2006; 132:129-135.

20. Huang XJ, Li CT, Zhang WP, Lu YB, Fang SH, Wei EQ. *Pharmacology* 2008; 82: 1-9.

21. Chen T, Li M, Zhang R, Wang H. *J. Cell Mol. Med.* 2009; 13:1358-1370.

22. Gaur R, Pathania AS, Malik FA, Bhakuni RS, Verma RK. *Eur. J. Med. Chem.* 2016; 122: 232-246.

23. Fröhlich T, ÇapcıKaragöz A, Reiter C, Tsogoeva SB. *J. Med. Chem.* 2016; 59:7360-7388.

24. Xie S, Wang J, Zhang Y, Wang C. *Expert Opin. Drug Deliv.* 2010; 7:1049-1061.

25. Nowotarski SL, Woster PM, Casero Jr. RA. *Expert Rev. Mol. Med.* 2013; 15: e3.

26. Karigiannis G, Papaioannou D. *Eur. J. Org. Chem.* 2000; 1841-1863.

27. Barret JM, Kruczynski A, Vispé S, Annereau JP, Brel V, Guminski Y, Delcros JG, Lansiaux A, Guilbaud N, Imbert T, Bailly C. *Cancer Res.* 2008; 28: 9845-9853.

28. Thibault B, Clement E, Zorza G, Meignan S, Delord JP, Couderc B, Bailly C, Narducci F, Vandenberghe I, Kruczynski A, Guilbaud N, Ferré P, Annereau JP. *Cancer Lett.* 2016; 370: 10-18.

29. Leblond P, Boulet E, Bal-Mahieu C, Pillon A, Kruczynski A, Guilbaud N, Bailly C, Sarrazin T, Lartigau E, Lansiaux A, Meignan S. *Invest. New Drugs* 2014; 32: 883-892.

30. Mouawad F, Gros A, Rysman B, Bal-Mahieu C, Bertheau C, Horn S, Sarrazin T, Lartigau E, Chevalier D, Bailly C, Lansiaux A, Meignan S. *Oral Oncol.* 2014; 50:113-119.

31. Brel V, Annereau JP, Vispé S, Kruczynski A, Bailly C, Guilbaud N. *Biochem. Pharmacol.* 2011; 82:1843-1852.

32. Kruczynski A, Pillon A, Créancier L, Vandenberghe I, Gomes B, Brel V, Fournier E, Annereau JP, Currie E, Guminski Y, Bonnet D, Bailly C, Guilbaud N. *Leukemia* 2013; 27: 2139-2148.

33. Tian ZY, Xie SQ, Mei ZH, Zhao J, Gao WY, Wang CJ. *Org. Biomol. Chem.* 2009; 7: 4651-4660.

34. Li M, Li Q, Zhang YH, Tian ZY, Ma HX, Zhao J, Xie SQ, Wang CJ. *Anticancer Drugs* 2013; 24: 32-42.

35. Szumilak M, Szulawska-Mroczek A, Koprowska K, Stasiak M, Lewgowd W, Stanczak A, Czyz M. *Eur. J. Med. Chem.* 2010; 45:5744-5751.

36. Vourtsis D, Lamprou M, Sadikoglou E, Giannou A, Theodorakopoulou O, Sarrou E, Magoulas GE, Bariamis SE, Athanassopoulos CM, Drainas D, Papaioannou D, Papadimitriou E. *Eur. J. Pharmacol.* 2013; 698:122-130.

37. Kostopoulou ON, Kouvela EC, Magoulas GE, Garnelis T, Panagoulias I, Rodi M, Papadopoulos G, Mouzaki A, Dinos GP, Papaioannou D, Kalpaxis DL. *Nucleic Acids Res.* 2014; 42: 8621-8634.

38. Stoica S, Magoulas GE, Antoniou AI, Suleiman S, Cassar A, Gatt L, Papaioannou D, Athanassopoulos CM, Schembri-Wismayer P. *Bioorg. Med. Chem. Lett.* 2016; 26: 1145-1150.

39. Chadwick J, Jones M, Mercer AE, Stocks PA, Ward SA, Kevin Park B, O'Neill PM. *Bioorg. Med. Chem.* 2010; 18: 2586-2597.

40. Kokkinogouli V. MSc Thesis, Department of Chemistry, University of Patras, 2006; Unpublished results; Free secondary amino functions of PA conjugates with ART, modified at position 10 as an acetal with the glycolic acid, caused decomposition of the ART-PA conjugates on standing even at 0 ^{o}C through elimination of the ART nucleus in the form of anhydrodihydroartemisinin (see Supplementary data).

41. Lin AJ, Lee M, Klayman D.L. *J. Med. Chem.* 1989; 32:1249-1252.

42. Liu Y, Lok CN, Ko BCB, Shum TYT, Wong MK, Che CM. *Org. Lett.* 2010; 12:1420-1423.

43. Singh AS, Verma VP, Hassam M, Krishna NN, Puri SK, Singh C. *Org. Lett.* 2008; 10: 5461-5464.

44. Torok DS, Ziffer H. *Tetrahedron Lett.* 1995; 36:829-832.

45. Magoulas GE, Bariamis SE, Athanassopoulos CM, Papaioannou D. *Tetrahedron Lett.* 2010; 51:1989-1993.

46. Mamos P, Karigiannis G, Athanassopoulos C, Bichta S, Kalpaxis D, Papaioannou D, Sindona G. *Tetrahedron Lett.* 1995; 36:5187-5190.

47. Liu WM, Gravett AM, Dalgleish AG. *Int. J. Cancer* 2011; 128: 1471-1480.

48. Chen K, Shou LM, Lin F, Duan WM, Wu MY, Xie X, Xie F, Li W, Tao M. *Anticancer Drugs* 2014; 25:652-662.

49. Papadimitriou E, Pantazaka E, Castana P, Tsalios T, Polyzos A, Beis D. *Biochim. Biophys. Acta.* 2016; 1866: 252-265.

50. Chang Y, Zuka M, Perez-Pinera P, Astudillo A, Mortimer J, Berenson JR, Deuel TF. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104: 10888-10893.

51. Chen HH, Zhou HJ, Fang X. *Pharmacol. Res*. 2003; 48: 231-236.

52. Chen HH, Zhou HJ, Wang WQ, Wu GD. *Cancer Chemother. Pharmacol*. 2004; 53: 423-432.

53. Dong F, Tian H, Yan S, Li L, Dong X, Wang F, Li J, Li C, Cao Z, Liu X, Liu J. *Int. J. Mol. Med*. 2015; 35: 1381-1387.

54. Poimenidi E, Theodoropoulou C, Koutsioumpa M, Skondra L, Droggiti E, van den Broek M, Koolwijk P, Papadimitriou E. *Vascul. Pharmacol.* 2016; 80: 11-19.

55. Lampropoulou E, Manioudaki M, Fousteris M, Koutsourea A, Nikolaropoulos S, Papadimitriou E. *Biomed. Pharmacother.* 2011; 65: 142-150.

Highlights

- Artemisinin was modified at position 10 or 11 with linkers bearing a hydroxyl group
- Urethane-type conjugates of modified artemisinins with polyamines were synthesized
- Conjugation resulted in more efficient compounds in inhibiting MCF7 cell growth
- The most active compounds (e.g. **9, 12**) did not induce ROS production or apoptosis
- They inhibited angiogenesis in vivo and in vitro