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Synthesis and Biological Characterization of Argyrin F

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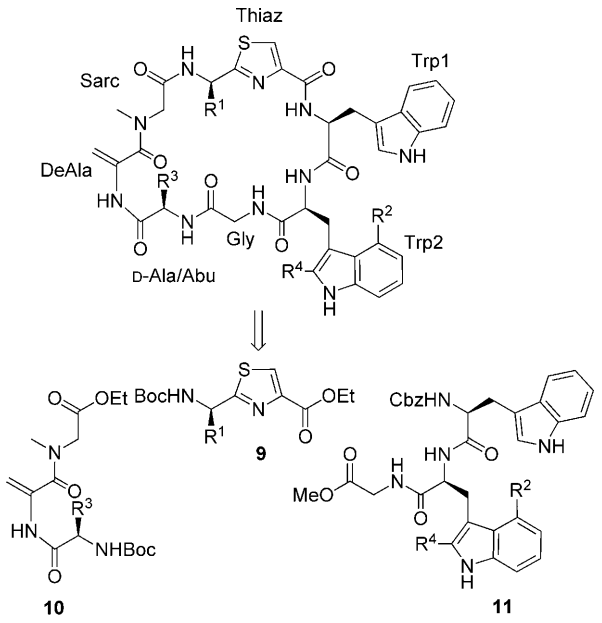
In the quest for new targets for antitumor therapy, the proteasome and its inhibitors have been the focus of academic and industrial research.[1] In this context, we recently reported the p27 stabilization effect of argyrin A[2] through selective inhibition of the proteasome.[3] Consequently, increased levels of p27, one of the pivotal tumor suppressor proteins, stop proliferation of tumor cells. Additionally, argyrin A effects neovascularization and therefore inhibits tumor progression through a second mode-of-action. At the same time, argyrin A exhibits biological activity at remarkably low concentration at which no cytotoxic effects were observed. Even though these data already support the high potential of argyrin A as a potential drug candidate, detailed structure–activity relationship studies, and a molecular understanding of its mode-of-action are a prerequisite for the further optimization of its medicinal potential. Since only limited structural modifications can be introduced starting from the natural product, and the marginal amounts of the minor metabolites derived from fermentation do not allow for detailed biological investigations, we aimed at establishing a synthetic access to argyrin A and its analogues. Here, we took advantage of the elegant construction of argyrin B put forward by the Ley group, which dissected argyrin in three equally complex segments (**9**, **10**, **11**) as delineated in Figure 1.[4] Nevertheless, the modifications we sought to introduce and the requirements imposed by practical considerations necessitated modification in the overall synthesis and of the three building blocks. At the outset of our synthetic endeavors, we identified functional groups and structural elements as potential characteristics that are likely to be essential for the biological activity or might be omitted in order to simplify the synthesis of lead structures.



Argyrin A, **1**: $R^1 = CH_3$, $R^2 = OCH_3$, $R^3 = CH_3$, $R^4 = H$
Argyrin B, **2**: $R^1 = CH_3$, $R^2 = OCH_3$, $R^3 = C_2H_5$, $R^4 = H$
Argyrin C, **3**: $R^1 = CH_3$, $R^2 = OCH_3$, $R^3 = CH_3$, $R^4 = CH_3$
Argyrin D, **4**: $R^1 = CH_3$, $R^2 = OCH_3$, $R^3 = C_2H_5$, $R^4 = CH_3$
Argyrin E, **5**: $R^1 = CH_3$, $R^2 = H$, $R^3 = CH_3$, $R^4 = H$
Argyrin F, **6**: $R^1 = CH_2OH$, $R^2 = OCH_3$, $R^3 = CH_3$, $R^4 = H$
Argyrin G, **7**: $R^1 = CH_2OH$, $R^2 = OCH_3$, $R^3 = C_2H_5$, $R^4 = H$
Argyrin H, **8**: $R^1 = H$, $R^2 = OCH_3$, $R^3 = CH_3$, $R^4 = H$

Figure 1. Natural occurring argyrins and their synthetic assembly based on three segments **9**, **10** and **11**.

The unusual 4-methoxy tryptophan moiety attracted our attention immediately since the site of the methoxy substitution is biosynthetically the least accessed position.[5] Additionally, it was reported that classic strategies used for amino acids syntheses failed for the construction of this unusual amino acid.[4] As a solution to this problem, Ley et al. reported an enzymatic kinetic resolution of the racemic precursor. However, efforts to carry out the synthesis as described by Ley et al. were hampered as the immobilized enzyme used in their synthesis was no longer available, and other enzymes resulted in changing yields in the range of 10–25%. In order to provide an efficient access to this uncommon amino acid, we carefully analyzed different chiral auxiliaries for their use in catalytic hydrogenations and identified the DuanPhos ligand, in combination with $Rh(cod)_2BF_4$, to provide the required amino acid in quantitative yield (99%) and 99% *ee* (Scheme 1).[6]

Next, we turned our attention to the synthesis of the dehydroalanine moiety. In order to circumvent the use of selenium

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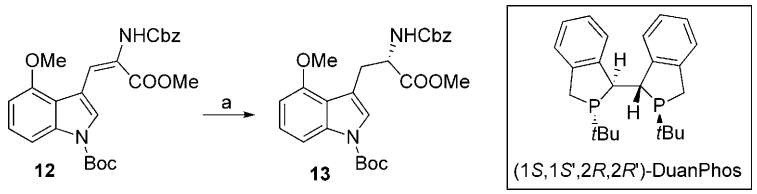
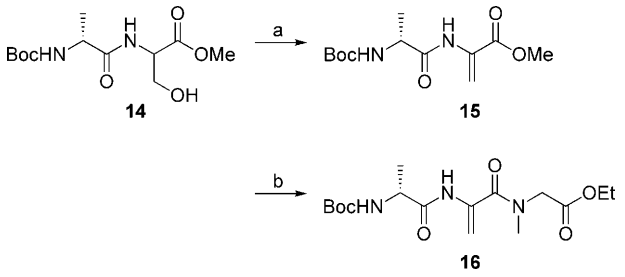


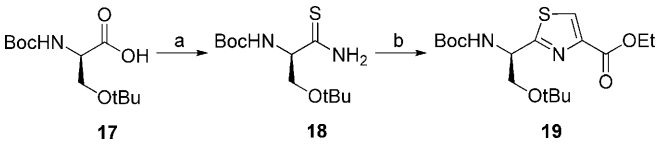
Scheme 1. Enantioselective hydrogenation of olefin **13**. *Reagents and conditions*: a) $Rh(cod)_2BF_4$/DuanPhos, H_2 (10 bar), MeOH, RT, 24 h, 99% (99% *ee*).

compounds for the introduction of the *exo*-methylene moiety, we reasoned that the dehydroalanine moiety would be chemically stable as long as the amino group was substituted by an electron-withdrawing group.[7] Consequently, we performed a copper(I)-catalyzed elimination of the serine hydroxy group from dipeptide **14**, which was generated under standard peptide-coupling conditions (Scheme 2). The next peptide coupling was performed after hydrolysis of the ester group with the ammonium salt of the sarcosin ester and with PyBroP as the coupling reagent to provide building block **16**.



Scheme 2. Synthesis of dehydroalanine segment **16**. *Reagents and conditions*: a) CuCl, EDC, CH_2Cl_2, 96%; b) 1. LiOH,THF/MeOH/H_2O; 2. HCl-Sar-OEt, PyBroP, DIPEA, CH_2Cl_2, 0 °C→RT, 74%.

Finally, we had to provide a route that would allow for the incorporation of a hydroxymethyl group in the thiazole dipeptide. Based on our analysis of potential protecting groups, we used *tert*-butyl protected serine (**17**), which was converted into the corresponding thioamide (**18**) using DCC, NH_3 and Belleau's reagent. Treatment with ethyl bromo pyruvate provided building block **19** necessary for introducing a hydroxy moiety as observed for argyrin F (Scheme 3).

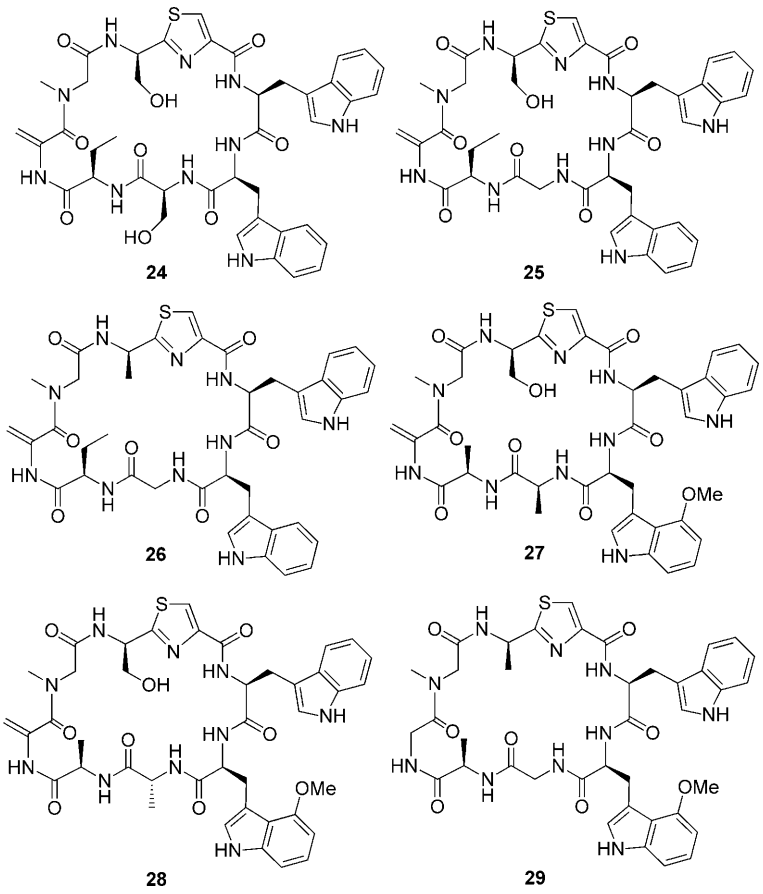


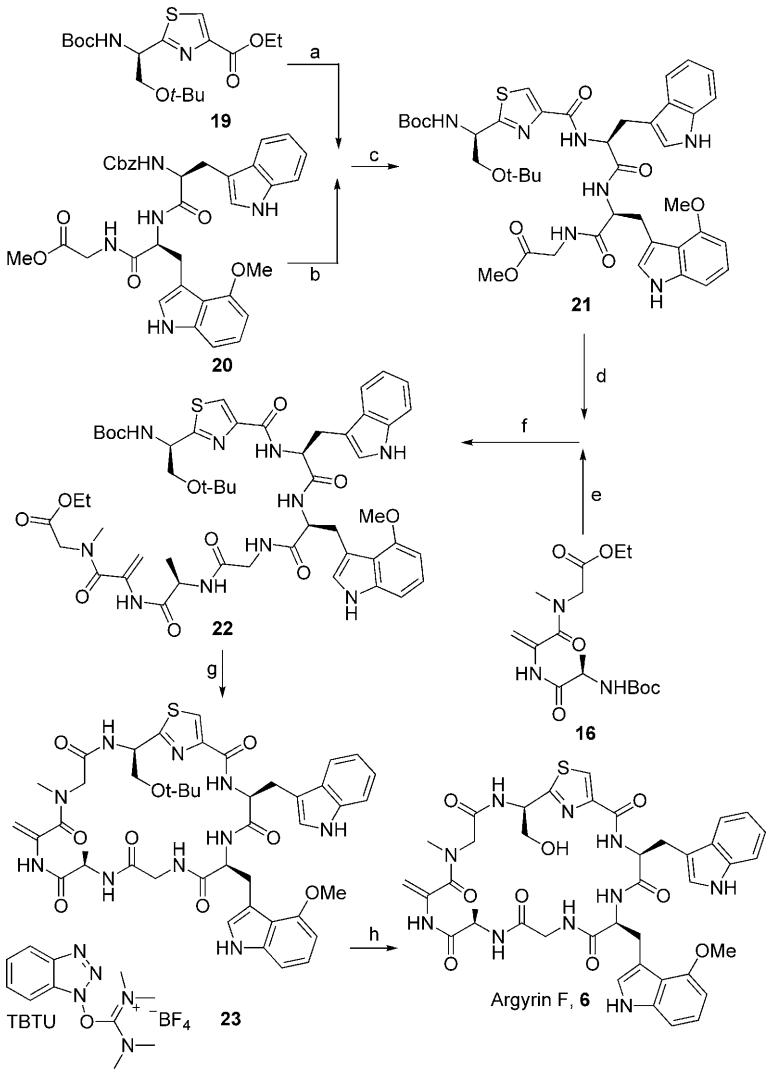
Scheme 3. Synthesis of thiazole moiety **19**. *Reagents and conditions*: a) 1. DCC, HOBT, NH_3; 2. Belleau's reagent, 70%; b) Ethyl bromo pyruvate, 78%.

Representative for all argyrin derivatives that are described in the context of their biological activity, the synthesis of argyrin F (**6**) is outlined in Scheme 4. The synthesis starts with the coupling of thiazole **19** to the N terminus of the tryptophan-containing building block **20**, which was obtained by standard peptide-coupling conditions.[3] Subsequent hydrolysis of the ester moiety and coupling with dehydroalanine segment **16** provides the open-chain precursor of argyrin F. Ring closure was achieved by liberation of both the C and the N terminus of the linear peptide and condensation with TBTU and HOBt. Finally, the *tert*-butyl protecting group was selectively removed without affecting the *exo*-methylene group or peptidic linkages through treatment with trifluoroacetic acid (TFA) at 0 °C. Based on the building blocks and protecting group strategies described above, several argyrin analogues were synthesized.

In order to provide derivatives for structure–activity relationship (SAR) studies, argyrins A (**1**), F (**6**) and G (**7**), and derivatives lacking the methoxy group at the tryptophan moiety (**24**–**26**) were synthesized.[8] Additionally, analogues lacking the *exo*-methylene group (**29**) and containing an extra methyl group provided by replacing the glycine residue to alanine (**27**, **28**) were generated.

Initially, the proteasome inhibition efficacy of the different argyrins and related analogues were investigated, in addition to measurements of their antiproliferative activity against SW-480 colon carcinoma cells (Table 1). It became apparent that





Scheme 4. Synthesis of argyrin F. *Reagents and conditions*: a) LiOH, THF/MeOH/H_2O; b) H_2, Pd/C; c) EDC, HOBt, DIPEA, 82% (two steps); d) LiOH, THF, MeOH/H_2O; e) TFA, CH_2Cl_2; f) EDC, HOBt, DIPEA, 85% (two steps); g) 1. LiOH, THF/MeOH/H_2O; 2. TFA, CH_2Cl_2; 3. TBTU, HOBt, DIPEA, 88% (three steps); h) TFA, CH_2Cl_2, 83%.

Table 1. Inhibition of proteasome activity.

Compound	Remaining proteasome activity			IC_{50} [nM]
	Trypsin-like	Caspase-like	Chymotrypsin-like	SW-480
Argyrin A (**1**)	45±4.5	29±5.2	30±6.0	3.8±0.3
Argyrin B (**2**)	55±8.5	52±3.0	58±6.5	4.6±0.6
Argyrin C (**3**)	40±7.0	38±8.3	43±5.5	1.5±1.1
Argyrin D (**4**)	62±8.0	50±6.0	64±5.2	3.6±2.0
Argyrin E (**5**)	70±3.0	65±4.0	70±5.5	520±270
Argyrin F (**6**)	38±6.5	35±7.0	28±2.5	4.2±0.4
Argyrin G (**7**)	75±3.0	60±6.5	65±4.5	63±55
Argyrin H (**8**)	60±7.0	52±5.5	51±8.0	30±2
Argyrin **24**	70±7.5	65±7.0	55±3.5	1050±180
Argyrin **25**	75±2.7	68±4.6	62±19	3800±43
Argyrin **26**	74±2.9	72±13.3	92±3.0	>4000
Argyrin **27**	72±4.5	54±2.5	50±3.0	90±0.2
Argyrin **28**	89±2.5	65±3.0	87±2.5	2300±180
Argyrin **29**	85±6.5	100±6.5	87±6.5	3600±400

the methoxy group of tryptophan Trp 2 and the *exo*-methylene group are essential for biological activity. An exchange of the glycine by alanine residues reduced the observed activity. This was especially severe in the case of D-alanine.

The additional hydroxy group in the thiazole segment in argyrin F (**6**) on the other side does not seem to influence the pharmacodynamics. However, this moiety might be a beneficial derivatization in order to increase the water solubility of the argyrin analogues and to improve their pharmacokinetic parameters. Remarkably, the presence of several hydroxy groups only partially restored the proteasome inhibition activity in analogues lacking the methoxy indole moiety. This supports the hypothesis that the methoxy group at tryptophan Trp 2 contributes to specific interactions with the target rather than by changing the hydrophobic character of the molecule. Surprisingly, the presence of an ethyl group at position R^3 (argyrin E and G) decreases the activity significantly, which can be seen by comparing the activities of argyrin F and argyrins E or G. These data demonstrate that argyrin F has approximately the same activity as argyrin A but with a higher solubility, and therefore it exhibits possible advantages in its pharmacokinetic behavior (water solubility: argyrin A, 5 μg mL^{-1}; argyrin F, 24 μg mL^{-1}).

In order to further characterize the biochemical interactions of argyrin F with the proteasome, Michaelis–Menten kinetics were generated (see Supporting Information). The results indicated that argyrin F acts as a competitive, reversible inhibitor (trypsin-like, K_i = 112 nM; chymotrypsin-like, K_i = 76 nM; caspase-like, K_i = 81 nM).

Taking all the biochemical data together, it became apparent that argyrin A and F are the most potent derivatives among the analogues tested so far. Given the comparable activities of argyrins A and F as inhibitors of the 20S proteasome, we next asked whether both compounds would also exhibit similar in vitro and in vivo activities. We had previously shown that the biological activities of argyrin A depend on the stabilization of the tumor suppressor protein p27kip1. Therefore, we investigated whether argyrin F would show a similar dependency on p27 stabilization. First, we determined the kinetics of p27 induction in cells treated with argyrin A or F. As shown in Figure 2, treatment with argyrin F resulted in a dose-dependent expression of p27 in SW-480 colon carcinoma cells. Next, we suppressed p27 expression using specific siRNAs in HeLa cells and measured the number of apoptotic cells after incubation with argyrin F. As shown in Figure 2, argyrin F induced apoptotic cell death in HeLa cells after 48 h incubation. Conversely, cells in which p27 expression was suppressed due to siRNA treatment did not respond to argyrin F, a result that is in agreement with our previous observations using argyrin A. Together, these experiments suggested that argyrin F, like argyrin A, requires p27 expression for the induction of its biological phenotype.

Next, we tested the ability of argyrin A, argyrin F, and argyrin **29**, a derivative without the *exo*-methylene group, to suppress the growth of colon carcinoma xenotransplant tumors derived from SW-480 cells in nude mice. We found that argyrin **29** (no *exo*-methylene group) was unable to inhibit the proteasome in vitro and shows no detectable antitumor activity in vivo. These data provide direct evidence that the proteasome inhibitory activity of the argyrins is required for their antitumor activities. Next, we compared the activities of argyrin A and F against colon carcinoma xenotransplants. We found that both compounds lead to a clear reduction in tumor volume when compared with untreated mice. Strikingly, however, argyrin F-treated tumors remained a very small size even after treatment was stopped at day 21. This remarkable activity was detected in more than 50% of all tumors in mice treated with argyrin F. These tumors did not regrow after treatment was stopped. Conversely, most of the argyrin A-treated tumors reappeared after discontinuation of the treatment (Figure 3).

To understand this phenotype in more detail, we studied the effect of argyrin F on endothelial cells. Our previous studies suggested that the antitumor activity of argyrin A in vivo was in large part caused by antiangiogenic and direct vessel-damaging activities of the compound. We therefore tested whether argyrin F would have similar activities against endothelial cells. As shown in Figure 4a, we found that at identical concentrations argyrin F had an even more pronounced effect on human umbilical vein endothelial cells (HUVECs) in that it induced apoptosis in the vast majority of cells. We also observed an almost complete inhibition of tube formation in cells treated with argyrin F as compared with argyrin A. Next we assayed the effects of argyrin F on xenotransplanted tumors. For this we stained the tumor tissue with CD31 antibody to detect endothelial cells and determined the microvessel density after treatment. As shown in Figure 4, we found that argyrin F leads to an even faster destruction of blood vessels in vivo compared with argyrin A.

In conclusion, we have identified argyrin F (**6**) as the most promising antitumor compound, exerting the same biological effects as argyrin A. Besides its potential to stabilize p27, due to a competitive, reversible inhibition of the proteasome, argyrin F shows vascular-damaging effects, which increases the ability of argyrin F to reduce the size of solid tumors in mice models. These two biological effects exerted by argyrin F make this natural product a promising compound that has the potential to become a unique and unprecedented drug in antitumor therapy. Additionally, we were able to identify the pivotal structural requirements, such as the methoxy group in the tryptophan Trp2, the *exo*-methylene group and the alanine building block, necessary for proteasome inhibition. The SAR studies presented here will not only help to understand the molecular interaction of argyrin F with the proteasome, but they open the door for further optimization of its biological profile and pharmacological parameters.

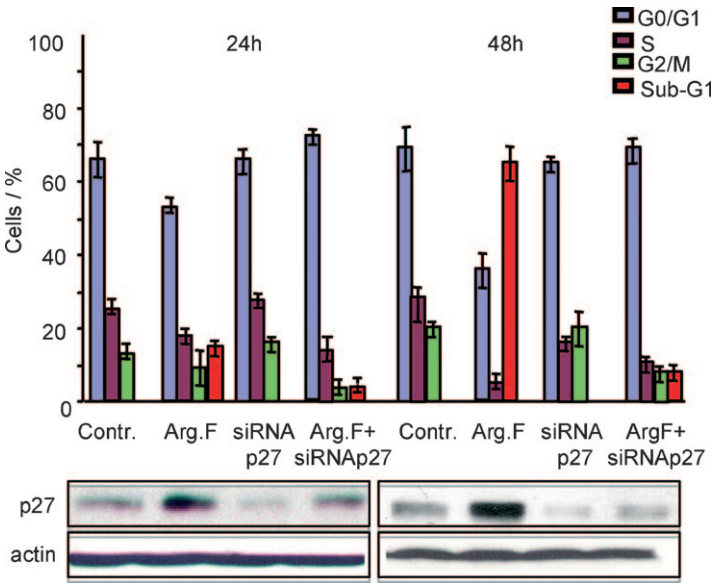


Figure 2. Argyrin F activity depends on the induction of p27 expression. SW-480 cells were treated with argyrin F, p27 siRNA or both for either 24 hr (left) or 48 hr (right). The sub-G_1 fraction of apoptotic cells was determined by flow cytometric analysis and p27 expression by immunoblotting using a p27 specific antibody.

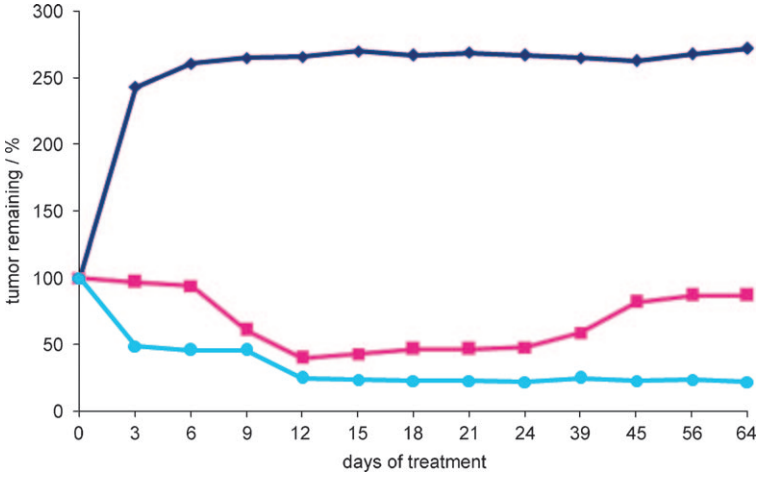


Figure 3. The proteasome inhibitory activity of argyrin F is required for its antitumor activities. SW-480 colon car/|cinoma cells were mixed with Matrigel and injected under the skin of nu/nu-mice to establish xenotransplant tumors. Treatment with argyrin A, or argyrin F was started when the tumors reached a volume of 150 mm^3. The graphs show quantification of tumor volumes at the indicated time points compared with the starting size, which was set as 100, after treatment with argyrin A (■) and F (●) or a control (◆). $n = 3$ (0.066 mg kg^{-1} argyrin A and F); $n = 3$ (control: PBS/DMSO).

Experimental Section

Experimental details and full analytic characterization of all argyrins reported here are provided in the Supporting Information.

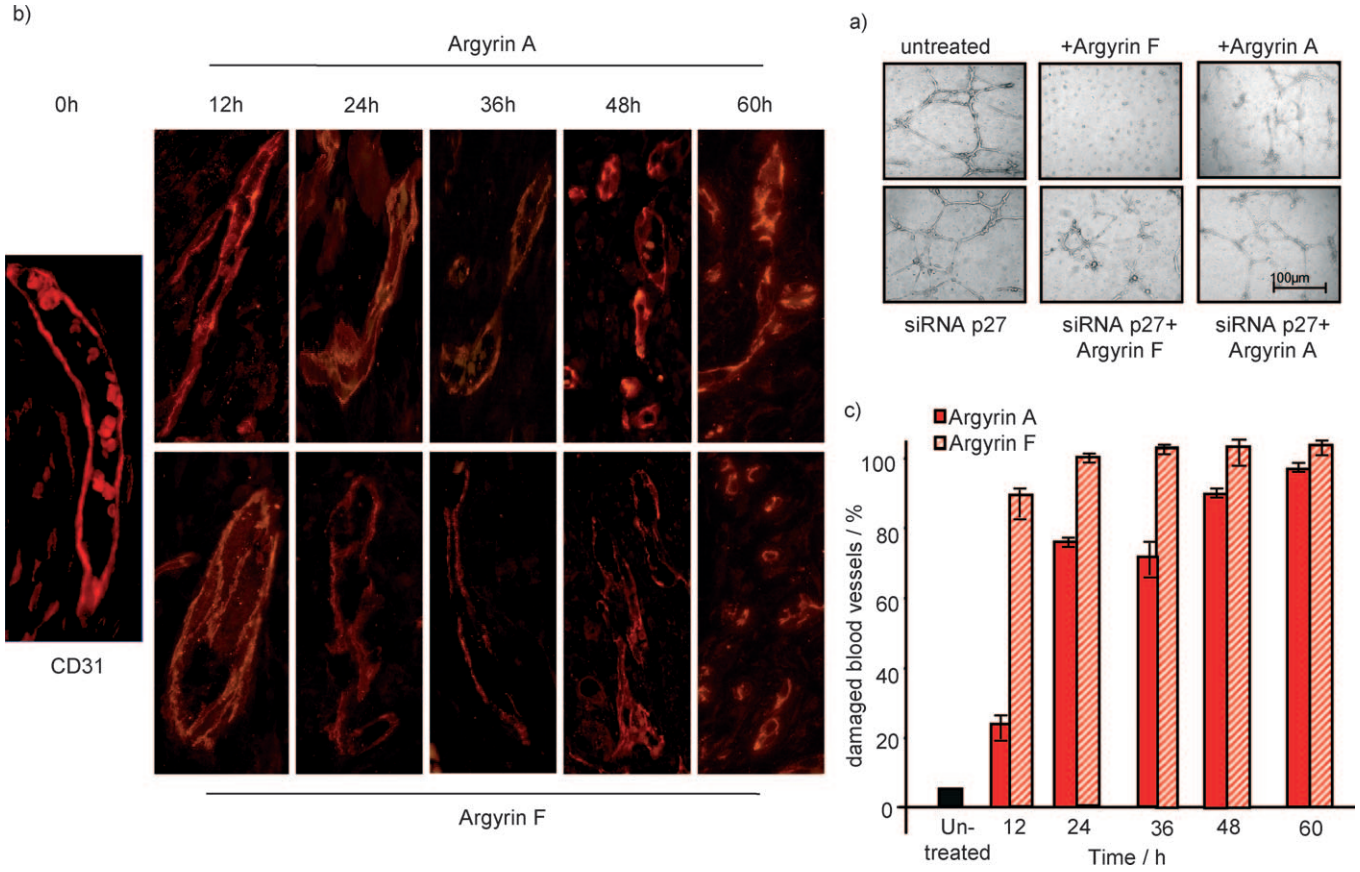


Figure 4. Argyrin A and argyrin F damage existing tumor blood vessels. a) Human umbilical vein endothelial cells (HUVECs) were incubated with argyrin A or argyrin F for 24 h. The enclosed network of tubular structures were photographed and quantified. Scale bars = 100 µm; b) After a single injection of argyrin A and argyrin F (0.066 mg kg^{-1}) tumors were explanted at the indicated time points and blood vessels in tumor tissue were stained for CD31 (red). Scale bars = 25 µm; c) Quantification of data in panel b.

All experiments were performed after review by and in accordance with the animal rights and protection agencies of Lower Saxony, Germany.

Keywords: antitumor agents · drug design · inhibitors · natural products · structure–activity relationships

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[8] Argyrins B, C, D, and H were obtained by fermentation at the Helmholtz Center for Infection Research (HZI), Braunschweig, Germany.

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