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FULL PAPER

Synthesis of Novel Hybrids of Thymoquinone and Artemisinin with High Activity and Selectivity Against Colon Cancer

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Abstract: Colorectal cancer causes 0.5 million deaths each year. To combat this type of cancer the development of new specific drug candidates is urgently needed. In the present work seven novel thymoquinone-artemisinin hybrids with different linkers were synthesized for the first time and tested for their *in vitro* anticancer activity in a panel of different tumor cell lines. The thymoquinone-artesunic acid hybrid **7a** was found to be the most active compound and selectively reduced the viability of colorectal cancer cells with an IC_{50} of 2.4 μM (HCT116) and 2.8 μM (HT29). Remarkably, hybrid **7a** was up to 20-fold more active than its parental compounds (thymoquinone and artesunic acid), while not affecting non-malignant colon epithelial HCEC cells (IC_{50} >100 μM). Moreover, the activity of hybrid **7a** was superior to that of different 1:1 mixtures of thymoquinone and artesunic acid. Furthermore, hybrid **7a** was even more potent against both colon cancer cell lines than the clinically used drug 5-fluorouracil. These results are another excellent proof of the hybridization concept and confirm that the type and the length of the linker play a crucial role for the biological activity of a hybrid drug. Besides an increase in ROS, elevated levels of DNA-damage marker γ-H2AX, were observed. Both effects seem to be involved in the molecular mechanism of action for hybrid **7a** in colorectal cancer cells.

Introduction

Colorectal cancer is the third most common cancer with more than 1.3 million new cases reported annually and nearly 0.5 million deaths each year.[1] To control this type of cancer the development of new drug candidates is urgently needed, as most of the currently available drugs are no longer effective due to enhanced drug resistance in tumor cells or undesirable side effects because of their unselectivity for tumor versus normal cells.[2] One of the most promising and fundamentally novel approaches in order to obtain new specific anticancer active compounds with improved pharmacological properties is the hybridization of bioactive natural products: Two or more natural product fragments are combined and linked with each other via covalent bonds forming new hybrid molecules.[3] These synthetic hybrids containing partial structures of natural compounds are in many cases more active than their parental compounds.[3b,4] In addition, they are able to overcome drug resistance[5] and open up the possibility of combining positive properties of different natural products in one single structure, thus leading to lower toxicity. Many examples presented in literature have already documented the high potential of the hybridization concept.[4,6] In the search for new drug candidates that specifically target colon cancer cells, we focused on the concept of hybridization, encouraged also by our previous results and experiences with artemisinin based hybrids.[5a,7]

We hypothesized that linking the natural products artemisinin (**1**) and/or artesunic acid (Art, **2**) with thymoquinone (TQ, **3**) (Figure 1) might yield highly effective anticancer agents having low toxicity towards normal cells. To our knowledge, no thymoquinone-artemisinin hybrids have been reported yet.

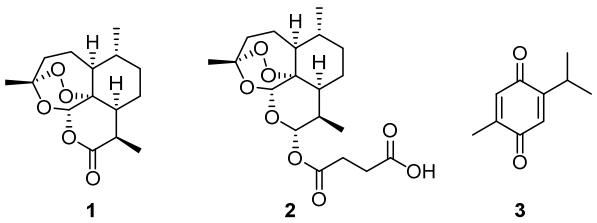

Figure 1. Structures of artemisinin (**1**), artesunic acid (Art, **2**) and thymoquinone (TQ, **3**).

Artemisinin (**1**) is an enantiomerically pure sesquiterpene containing a 1,2,4-trioxane ring, which was extracted from the Chinese medicinal plant *Artemisia annua* L. in 1972 by Youyou Tu (Nobel Prize 2015).[8] It is widely known as a traditional antimalarial drug,[9] but also possesses great anticancer potential.[10] Its semisynthetic derivative, Art (**2**), also demonstrated to be a very effective anticancer agent and exhibited remarkable cytotoxic activities against a wide range of cancer cell lines including prostate, ovarian, leukemia, melanoma,

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breast, colorectal and renal cancer cells.[11] Furthermore, *in vivo* experiments revealed its anticancer activity in pancreatic cancer, liver cancer, lung cancer and Kaposi sarcoma.[12]

Although the mechanism of action of artemisinin is still not completely understood, it is generally accepted that the endoperoxide moiety within the 1,2,4-trioxane system is essential for its anticancer activity.[13] It is assumed that the peroxide bridge is cleaved by intracellular Fe(II) leading to the formation of reactive oxygen species (ROS) and carbon-centered free radicals. These species induce oxidative stress, alkylation of target proteins, DNA damage, and apoptosis.[14] TQ (**3**) (Figure 1), a naturally occurring phytochemical compound, is the main constituent of the volatile oil of *Nigella sativa* (black seed) and was first extracted in 1963 by El-Dakhakhany.[15] This monoterpene was found to have strong anticancer effects both *in vitro* and *in vivo*.[16] Recent reports have shown that TQ induces apoptosis *in vitro* by p53-dependent and independent pathways, while exhibiting no toxicity in normal cells.[17] Being a short-chain ubiquinone-derivative, TQ is able to act as a pro-oxidant and consequently induce oxidative stress by triggering ROS production,[18] which is revealed to be directly linked to its pro-apoptotic effect in colon cancer and leukemia cells.[19] Recently, it has been shown that TQ suppresses metastasis through NF-kB inhibition and activation of JNK and p38 in CPT-11-R LoVo colon cancer cells, and to abrogate epithelial–mesenchymal transition in cancer cells mainly through the inhibition of PI3K/AKT signaling axis.[20]

In addition, *in vivo* studies showed that the general toxicity of TQ is relatively low,[21] making the compound worth considering for clinical applications.[22] Interestingly, it was shown that TQ enhances *in vitro* and *in vivo* the efficacy of many chemotherapeutic agents - even in resistant types of cancer - such as cisplatin in lung cancer and many solid tumors.[16b,23]

Thus, both natural products, Art and TQ, can be regarded as very promising anticancer active compounds, and, therefore are perfectly suitable for applying the hybridization concept in order to obtain new potent anticancer agents.

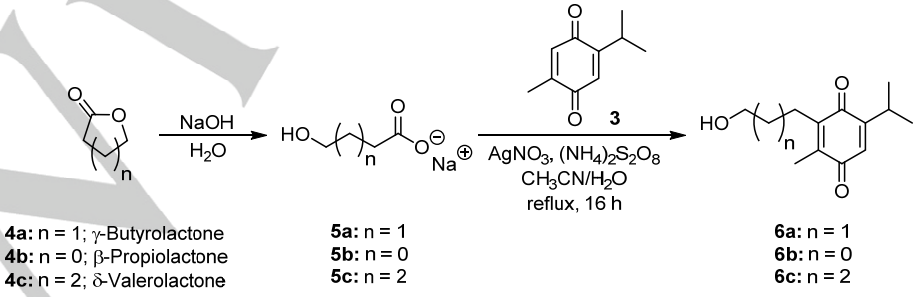
In this article, we report on seven novel TQ-Art hybrids (compounds **7a-c**, **9a/b** and **11a/b**, Scheme 2) whose synthesis consists of only a few steps. Their biological activity against various colon cancer cell lines (HCT116, HT29, Caco-2, DLD-1) and other cancer cell lines, such as breast cancer (MCF-7), prostate cancer (PC-3), liver cancer (HEPG2) and leukemia (JKT, HUT102) have been evaluated. The hybrid compounds exhibited potent and specific cytotoxic activity against colon cancer cell lines without toxic effects on normal colon cells. Further evaluation of the molecular mechanism of action of hybrid **7a** showed no direct interaction with DNA, but ROS-induced DNA damage and apoptosis.

Results and Discussion

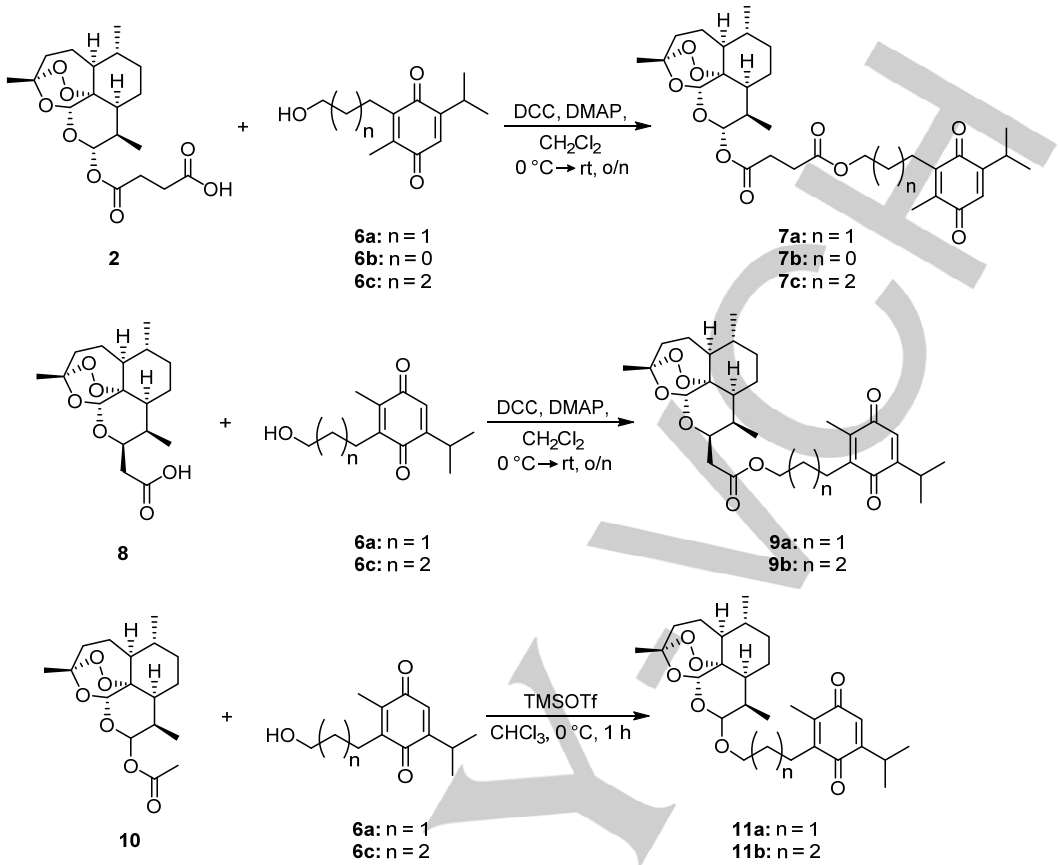
Chemistry: To be able to synthesize the novel thymoquinone-artemisinin hybrids **7a-c**, **9a/b** and **11a/b** (Scheme 2), first TQ (**3**) has to be converted into the corresponding alcohol derivatives **6a-c** (Scheme 1).

This was achieved by treating TQ and different sodium hydroxyl carboxylate salts **5a-c** (which were obtained in quantitative yield by simple ester hydrolysis of commercially available lactones **4a-c**) with $(NH_4)_2S_2O_8$ and catalytic amounts of $AgNO_3$ in a mixture of water and acetonitrile. This procedure, which is already reported in literature in the context of synthesis of other derivatives of TQ,[24] afforded alcohols **6a-c**. Subsequently, these alcohols were reacted with either Art (**2**) or the artemisinin-derived carboxylic acid **8**[25] under Steglich esterification conditions to furnish ester hybrids **7a-c** in 80-86% yield and **9a/b** in 32/39% yield. Hybrids **9a/b** belong to the group of C-10 non-acetals, which are known to be more hydrolytically stable than common artemisinin derivatives such as Art.[26]

In order to investigate the effect of the type of linkage between the artemisinin and the TQ subunit on biological activity, two ether hybrids **11a/b** were prepared, as ethers are known to be more stable than esters, using a documented procedure:[27] Dihydroartemisinin acetate **10** and alcohols **6b/c** were stirred at 0 °C for 1 h in the presence of catalytic amounts of TMSOTf and thereby ethers **11a/b** could be isolated in 43/52% yield. It has to be mentioned that both products occurred as a mixture of 10α- and 10β-diastereomers. In both cases the 10β-isomer ($J_{H\text{-}9;H\text{-}10}$ = 3.3/3.4 Hz) was predominantly formed (ratio 10β/10α-isomers = 8:1). The ratio between both isomers was determined by comparing the integrals of corresponding protons at C-10 and C-9 in the recorded ^{1}H-NMR spectra. This was possible, because a large coupling constant (7-10 Hz) is generally found for the 10α-isomer, indicating a relative *trans*-configuration, whereas a small coupling constant (3-5 Hz) appears for the 10β-isomer, indicating a *cis*-configuration.[28]



Scheme 1. Synthesis of TQ derivatives **6a-c**.



Scheme 2. Synthesis of thymoquinone-artemisinin hybrids **7**, **9** and **11**.

Biological evaluation: To study the cytotoxic effect of the novel seven hybrids we first investigated cell viability by crystal violet assay in colon (HCT116) and prostate (PC-3) cancer cell lines as well as in a normal colon epithelial cell line (HCEC), Table 1. We compared different TQ-Art hybrids **7a-c**, **9a/b** and **11a/b** harbouring different linkers.

The most effective hybrid on the colorectal HCT116 cancer cells was **7a** with IC_{50} of 2.4 µM. Hybrids **9b** and **11a** revealed IC_{50} values of 9.1 and 12.7 µM, respectively. The least active compounds were hybrids **11b** (IC_{50} = 18 µM) and **7c** (IC_{50} = 26.2 µM). Intriguingly, one additional CH_2-group or less between the natural product subunits is enough to considerably decrease anticancer activity. These results demonstrate that a linker in a hybrid drug is important and in fact its length and nature is relevant for the observed activities. Notably, the lowest IC_{50} value in PC-3 cells was found for hybrid **11b** (51.7 µM) and the highest one for hybrids **7c** and **9a** (IC_{50} values >100 µM). Interestingly, the activity of all hybrids was significantly lower on normal HCEC cells when compared to the other two tumor cell lines, suggesting a tumor specific effect (Table 1).

To study the effect of the most potent hybrid **7a** in comparison to the parental compounds Art (**2**) and TQ (**3**), we investigated cell viability of a panel of cancer cell lines in response to these three drugs (Table 2, Suppl. Figure 1). TQ showed comparable IC_{50} values in the different tumor cell lines (approx. 40-60 µM), reflecting its strong universal anticancer effect.

Table 1. IC_{50} values (in µM) of thymoquinone-artemisinin hybrids **7**, **9** and **11** in two cancer and a normal cell line after 24 h of incubation

Compound	HCT116	PC-3	HCEC
7a	**2.4 ± 0.19**[a]	93.0 ± 18.5	>100
7b	3.7 ± 0.24	84.6 ± 8.3	>100
7c	26.2 ± 7.3	>100	>100
9a	15.5 ± 6.3	>100	>100
9b	9.1 ± 2.7	96.8 ± 10.7	>100
11a	12.7 ± 0.8	71.3 ± 3.4	72.4 ± 1.8
11b	18.0 ± 2.7	51.7 ± 2.5	36.7 ± 1

[a] the corrisponding viability graphs for hybrid **7a** are given in Suppl. Figure 1 as an example.

Interestingly, Art was significantly more effective in HCT116 cells (5.3 µM) whereas the other cancer types required higher concentrations of Art (20 µM to higher than 500 µM) to induce

50% of cell death. Similarly, the IC_{50} values of hybrid **7a** in HCT116 and HT29 cells were remarkably lower (2.4 µM and 2.8 µM respectively) than in all the other cancer cell lines (60 µM to higher than 1 mM), which suggests a specific drug action in colorectal cancer cells (Tables 1-3, Figure 2 and Suppl. Fig. 1 and 2).

In addition, we evaluated the cytotoxicity of the synthetic TQ derivative **6a** (a subunit of hybrid **7a**) in HCT116 cells. Notably, **6a** showed an IC_{50} of >100 µM (Suppl. Figure 3) indicating that that the observed superior performance and reduced toxicity of **7a** (Table 1) is the result of covalent linking of TQ derivative **6a** with Art (**2**) to a hybrid molecule and that the hybridization concept is a powerful strategy to develop new effective anticancer drug candidates.

Table 2. IC_{50} values (in µM) of Art, TQ and hybrid **7a** in different cancer cell lines after 24 h of incubation*

Comp.	HCT 116	Hep G2	PC-3	MCF-7	JKT	HUT 102
Art	5.3 ± 0.1	21.9 ± 5.4	39.5 ± 1.5	25.0 ± 2.5	>500	>250
TQ	50.1 ± 6.1	37.6 ± 9.7	59.0 ± 7.7	42.0 ± 8.5	43.1 ± 3.6	40.3 ± 4.2
7a	**2.4 ± 0.19**	63.0 ± 8.4	93.0 ± 18.5	78.4 ± 14.1	>1000	>300

* IC_{50} value of clinically used anticancer drug 5-fluorouracil: IC_{50} (HCT116) = 10 ± 1.6 µM.

We next aimed to investigate the effect of hybrid **7a** in two other colorectal cancer cells (DLD-1 and Caco-2) to confirm our assumption that **7a** could act as a colon-specific drug (Table 3). Indeed, the IC_{50} values of hybrid **7a** in Caco-2 and DLD-1 cells were 15.3 and 8.2 µM, respectively, thus significantly lower than for hepatic, prostate, breast and blood cancer cells (Table 2, 3). Notably, hybrid **7a** again selectively affected cancer cells and not normal cells (Table 3). Moreover, all compounds showed solid tumor selectivity, as no effect was observed in leukemia cells (JKT and HUT102) even at very high doses (> 250 µM).

Table 3. IC_{50} values (in µM) of Art, TQ and hybrid **7a** in different colorectal cancer cells and a normal colon epithelial cell line after 24 h of incubation*

Comp.	HT29	Caco-2	DLD-1	HCEC
Art	19.0 ± 5.4	25.5 ± 7.2	13.0 ± 2.9	84.8 ± 18.3
TQ	60.3 ± 4.1	7.8 ± 0.8	35.0 ± 1.8	141 ± 30.7
7a	**2.8 ± 0.2**	15.3 ± 5.5	8.2 ± 2.4	>100

* IC_{50} value of clinically used anticancer drug 5-fluorouracil: IC_{50} (HT29) = 31.4 ± 5.2 µM.

For the next experiments we selected HCT116 and HT29 colorectal cancer cells that differ in their p53 status (HCT116: wildtype, HT29: mutant p53-R273H). We studied the effects of hybrid **7a** versus combination treatment with 1:1 mixture of both single drugs TQ and Art (1:1, 2.5:2.5, 5:5 mixtures, Figure 3 A-D, Suppl. Figure 2). Interestingly, hybrid **7a** was always more effective than the three different combination treatments in HCT116 cells after 24 h and 48 h of and in HT29 cells after 48 h of incubation.

In a next step, we determined the protein level of poly (ADP-ribose) polymerase (PARP) in HCT116 and HT29 cells which plays a critical role in the maintenance of DNA integrity. Its main role is to detect and report single-strand DNA breaks (SSB) to the enzymatic machinery involved in the SSB repair. Cleaved PARP is indicating a loss of repair signaling and the induction of apoptotic cell death.[29] For this, the cells were treated with 5 µM of each single drug (Art and TQ), the combination of both (5:5 µM) and the hybrid **7a** (5 µM). Treatment with Art, the combination, and **7a** led to PARP cleavage in HCT116 cells after 24 and 48 h, whereas PARP cleavage was detectable for TQ only after 48 h (Figure 3 E). In contrast, HT29 cells were more resistant to Art, the combination, and hybrid **7a** treatment and showed PARP cleavage only after 48 h (Figure 3 F). TQ did not induce cell death at either one of the analyzed time points in HT29 cells. Furthermore, TQ/ART combination treatment, and hybrid **7a** induced caspase 9 cleavage but only after 48h in both cell lines (Suppl. Figure 4 A, B) suggesting the involvement of the intrinsic apoptotic pathway.

To examine the drug-induced DNA damage we treated HCT116 and HT29 cells with the single drugs, the combination of both, and hybrid **7a**. Art, the combination treatment and **7a**, but not TQ induced strong DNA damage as determined by an increased level of γ-H2AX representing a marker of DNA double-strand breaks (DSBs), an ultimate result of missing SSB repair.[30] In HT29 cells this effect was less pronounced, which possibly explains the higher general drug resistance observed in the cytotoxicity assays (Figure 4 A, B).

To assess if this DNA damage might be caused by a direct interaction with DNA, pBR322 plasmid DNA was incubated with increasing concentrations of hybrid **7a** or the single drugs (Art and TQ). Afterwards, the electrophoretic mobility of the plasmid DNA was analyzed by agarose gel electrophoresis (Figure 4 C). Doxorubicin (Dox), which is known to intercalate into the DNA double helical structure,[31] was used as a positive control. Due to its interaction with double-stranded pBR322 DNA and the hereby caused extensive topological changes, treatment with Dox led to a strong shift of the plasmid DNA bands in a concentration-dependent manner. Retardation of the front most DNA band, which corresponds to the covalently closed circular (ccc) form of the plasmid, results from an unwinding of this supercoiled DNA to an open circular form (oc). In contrast to the positive Dox control, no band shift and hence no interaction with DNA could be observed for hybrid **7a** or the single drugs, Art and TQ. These results suggest that a direct interaction with DNA cannot be the cause of drug-induced DNA damage.

In a second ethidium bromide fluorescence-based (EtBr) assay we analyzed the potential interaction of the single drugs (Art & TQ) and hybrid **7a** with linear double-stranded DNA (linearized pBR322 plasmid), again applying Dox as a positive control. None of the three tested drugs showed any interaction with the linearized plasmid.

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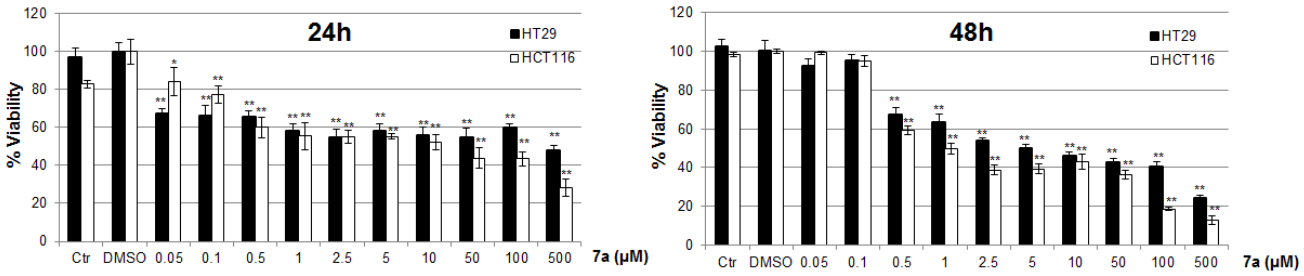

Figure 2. Viability of HCT116 and HT29 cells after treatment with hybrid **7a** in different concentrations (0.05 – 500 μM). Cell viability was assessed after 24 h and 48 h by crystal violet assay and is expressed as percentage of DMSO control. Significance between hybrid treatment and the DMSO control is indicated by * ($p<0.05$) and ** ($p<0.001$) as determined by one-way Anova analysis (SPSS, version 24). Error bars denote standard deviation of means calculated from six technical replicates. Note: viability measurements for hybrid **7a** have been done several times during a period of 12 months and a range of IC_{50} between 2.3 μM and 11.2 μM have been detected.

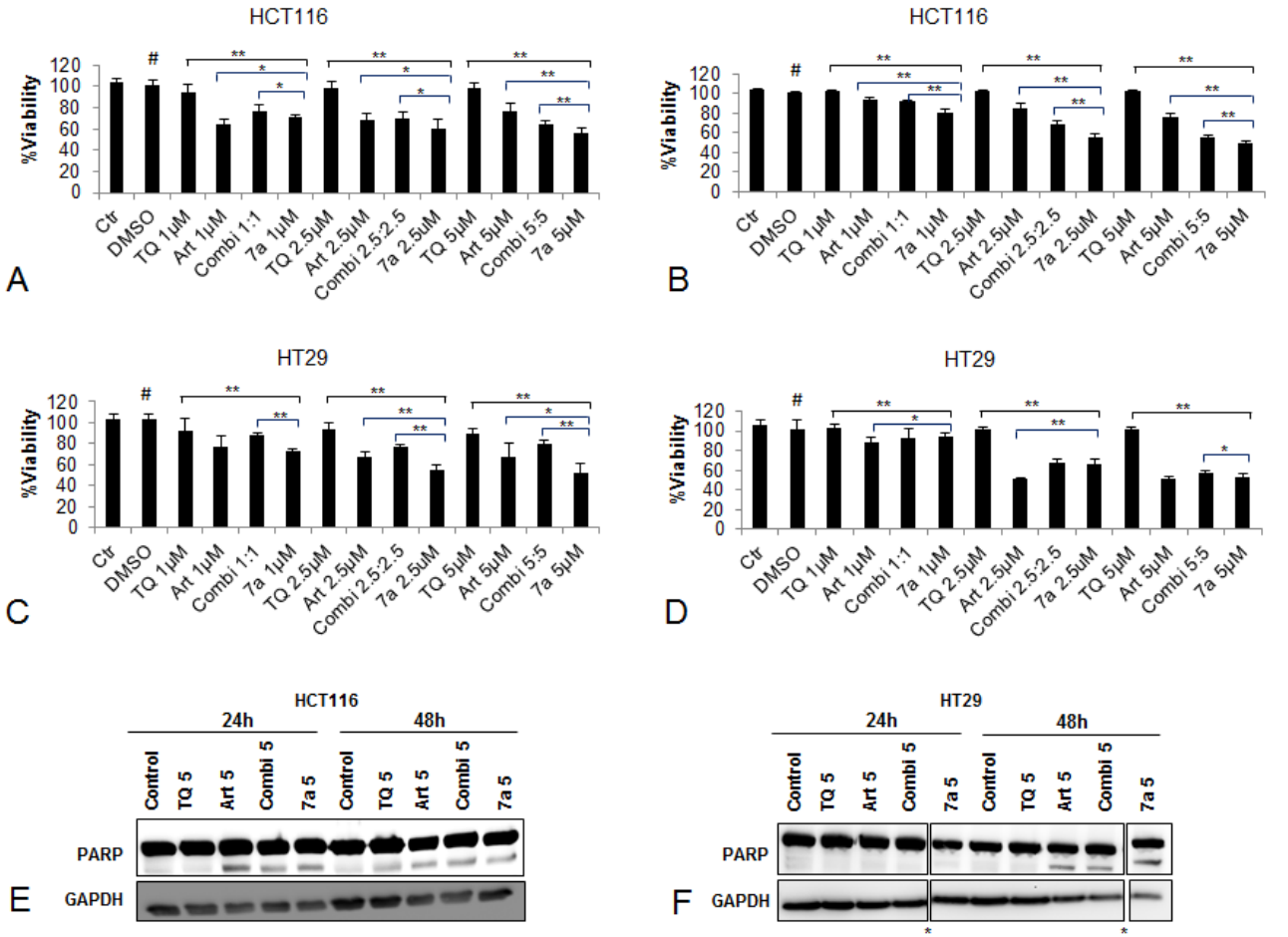

Figure 3. Viabiltiy of HCT116 (A, B) and HT29 (C, D) cells after treatment with TQ (1, 2.5, 5 μM), Art (1, 2.5, 5 μM), the combination of TQ and Art (1:1; 2.5:2.5, 5:5 μM), or hybrid **7a** (1, 2.5, 5 μM) for 24 h (A, C), and 48 h (B, D). The viability was assessed by crystal violet assay and is expressed as percentage of DMSO control. Significances between hybrid and other treatments are indicated by * ($p<0.05$) and ** ($p<0.001$), while all treatments that significantly differed from the DMSO group are marked with # ($p<0.001$). Error bars denote standard deviations of eight technical replicates. Levels of active and cleaved PARP as assessed by western blot analysis. The effects are shown for TQ, Art, the combination of the single drugs, and hybrid **7a** in HCT116 (E) and HT29 (F) cells after 24 and 48 h. GAPDH was used as a loading control. In Figure 3F two bands have been spliced out (*).

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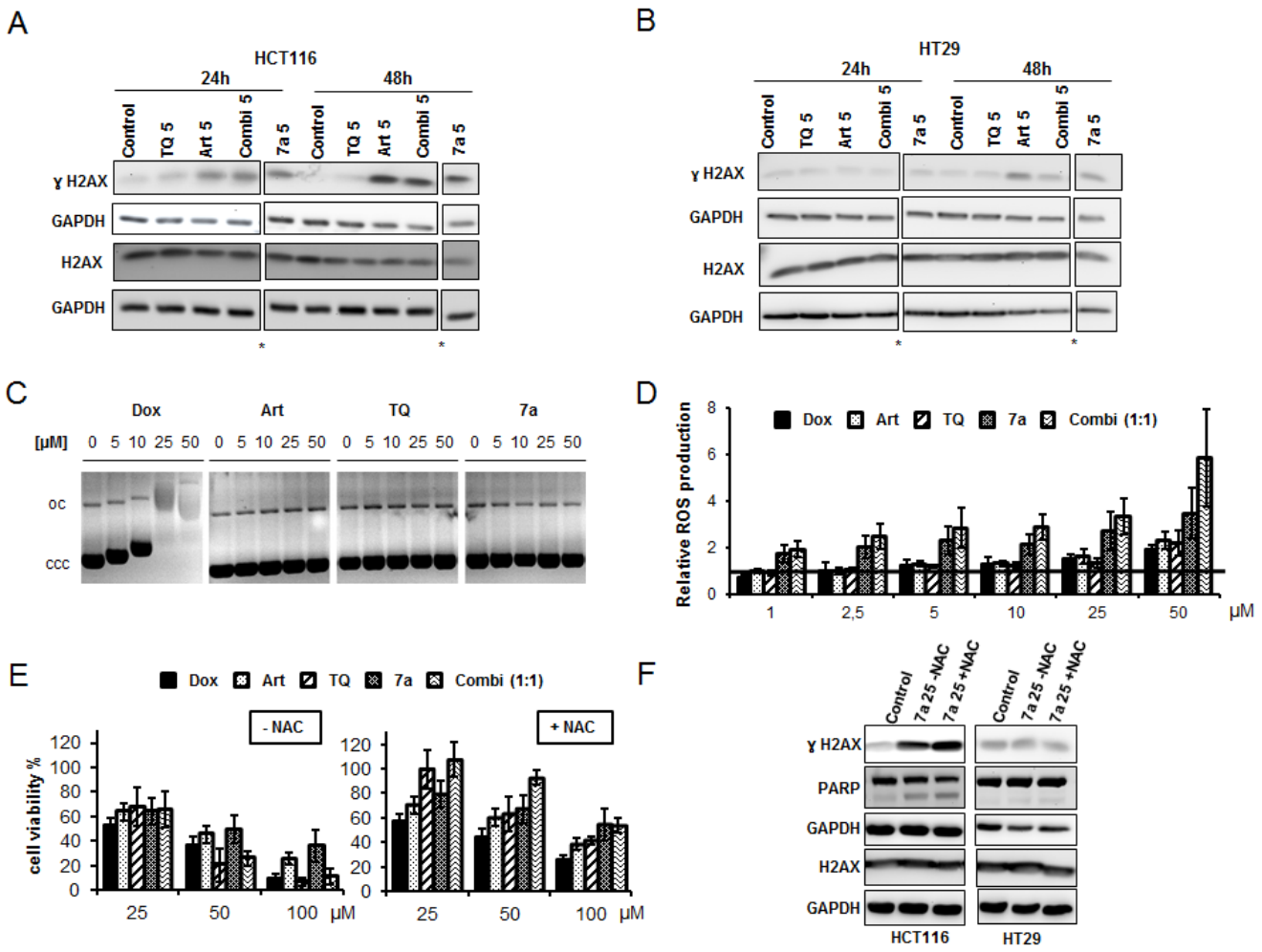

Figure 4. (A, B) Detection of γ-H2AX by western blot analysis in HCT116 (A) and HT29 (B) cells after treatment with 5 μM of Art, TQ, the combination of the single drugs or the hybrid **7a**. The cells were harvested after different time points (24, 48 h). The specific primary antibodies used are γ-H2AX and GAPDH was used as a loading control. (C) Interaction of doxorubicin (Dox), Art, TQ or hybrid **7a** with circular pBR322 plasmid DNA as determined by electrophoretic mobility shift assays (EMSAs) after 24 h of incubation. Pictures are representative of at least two independent experiments (oc: open circular; ccc: covalently closed circular). (D) Relative ROS levels in HCT116 cells after treatment with increasing concentrations of Dox, Art, TQ or hybrid 7a (1 – 50 μM) as assessed in NBT assays. Values represent means ± SD of two independent experiments in duplicate. (E) ROS dependent cytotoxicity of Dox, Art, TQ, hybrid **7a** and the combination of the single drugs (Art and TQ) in HCT116 and HT29 cells as determined in MTT assays after 24 h of incubation with different concentrations of the test compounds (25 μM, 50 μM and 100 μM) in the absence or presence of 10 mM NAC. Values represent means ± standard deviation (SD) of two independent experiments in quadruplicate. (F) Detection of γ-H2AX, PARP, H2AX by western blot analysis in HCT116 and HT29 cells after 48 h treatment with 25 μM of hybrid **7a** in the absence or presence of 10 mM NAC. GAPDH was used as a loading control. In figure 4 A, B two bands have been spliced out (*).

Only Dox strongly reduced EtBr fluorescence in the applied lower concentrations (5 and 10 μM) due to an inhibition of intercalation sites in the DNA (Suppl. Figure 5). However, Dox is a fluorochrome itself emitting light in the same wavelength range as EtBr and its fluorescence intensity is increased strongly upon intercalation into DNA just as it is the case for EtBr.[32] Thus, in the samples of Dox treated DNA a stronger fluorescence intensity was detected at the applied higher concentrations (25 and 50 μM), which is most likely only the result of a great amount of Dox molecules interacting with the DNA (= higher fluorescence intensity).

Another possibility for DNA damage induction might be drug-associated intracellular reactive oxygen species (ROS) production. For Art and TQ ROS-dependent mechanisms of action have already been reported.[19,33] Thus we evaluated the intracellular ROS levels in HCT116 cells after treatment with different doses of Dox, Art, TQ, hybrid **7a** or the combination of the single drugs (Art and TQ) after 24 h of incubation with the test compounds (Figure 4 D). We saw that ROS induction was clearly dose-dependent in all treatment settings (Figure 4 D). In a next step, HCT116 cells were incubated with 25, 50, and 100 μM Dox, Art, TQ, hybrid **7a** or the combination of the single drugs, in the absence or presence of the ROS scavenger N-acetyl-cysteine (NAC) in order to evaluate whether the cytotoxicity of the test compounds is dependent on ROS (Figure 4 E). Although the toxicity of all tested compounds measured by MTT-assay was

considerably decreased by the addition of NAC in HCT116 cells, the generation of γ–H2AX protein and PARP cleavage after hybrid treatment were not affected in both, HCT116 and HT29 cells (Figure 4 F). Thus, we suggest other ROS species that are not scavenged by NAC to be responsible for γ–H2AX formation and apoptosis induction by the hybrid **7a**. Otherwise, Lambert et al.[34] reported that ROS might interact with a quinone-derived drug (epigallocatechin-3-gallate) to produce an intracellular active compound, which finally could form toxic mediators with NAC.

Conclusions

In conclusion, in our study seven thymoquinone-artemisinin hybrids **7a-c**, **9a/b** and **11a/b** were successfully synthesized and investigated for the first time for their anticancer activity in different cancer cell lines (colon, breast, prostate, liver and leukemia). Remarkably, all novel thymoquinone-artemisinin hybrids showed a high specificity for colon cancer cells. In particular, hybrid **7a** was the most active compound against the tested colorectal cancer cell lines (with a IC_{50} of 2.4 μM in HCT116 cells and 2.8 μM in HT29 cells) and thus was more potent, with up to 20-fold higher activity, than the parental compounds Art and TQ, while being nontoxic to non-malignant colon epithelial cells (IC_{50} >100 μM). Moreover, selected hybrid **7a** was more effective than the combination treatment with different 1:1 mixtures of both single drugs. This is an encouraging result since the activity of hybrid **7a** against both colon cancer cell lines (HCT116 and less sensitive cells HT29) was even superior to that of a clinically used drug 5-FU. The molecular mechanism for the specific activity of hybrid **7a** in colorectal cancer cells seems to be accumulation of double strand breaks via specific ROS-subspecies that are not captured by NAC. Further *in vitro* and *in vivo* investigations will provide a deeper understanding of the involved signaling pathways and molecular targets of this promising novel drug candidate. Finally, these results are another excellent proof of the hybridization concept and also confirm that the type of linkage (ester or ether) and the linker length play a crucial role for the biological activity of a hybrid drug.

Experimental Section

Experimental Details. The purity of all hybrids was approved with Elemental Analysis and was >95%.

General procedure for hybrids 7a-c and 9a/b: A solution of Art (**2**) or artemisinin-derived acid **8**, the corresponding TQ alcohol **6a/b** or **c** and DMAP in dry CH_2Cl_2 was cooled to 0 °C. After addition of DCC the reaction mixture was slowly warmed to room temperature and stirred overnight. The precipitated dicyclohexylurea was removed by filtration and the solvent was removed under reduced pressure. The residue was purified by column chromatography and thereby hybrid **7a-c** or **9a/b** was obtained either as a yellow solid or a yellow gum.

Thymoquinone-artesunic acid hybrid 7a. Art (27.0 mg, 0.07 mmol, 1.0 eq), DMAP (2.60 mg, 0.02 mmol, 30 mol%), TQ alcohol **6a** (31.0 mg, 0.14 mmol, 2.0 eq), dry CH_2Cl_2 (2.0 mL), DCC (15.9 mg, 0.08 mmol, 1.1 eq). Column conditions: hexane/EtOAc 4:1. Yield: 34.0 mg, 0.06 mmol, 83%; regained starting material **6a**: 12.0 mg (0.05 mmol, 0.8 eq). R_f = 0.82 (hexane/EtOAc 1:1, UV and molybdato phosphate). Anal. calcd. for $C_{32}H_{44}O_{10}$: C, 65.29; H, 7.53; Found: C, 65.01; H, 7.32.

Thymoquinone-artesunic acid hybrid 7b. Art (119 mg, 0.31 mmol, 1.5 eq), DMAP (11.4 mg, 0.09 mmol, 30 mol%), TQ alcohol **6b** (43.0 mg, 0.21 mmol, 1.0 eq), dry CH_2Cl_2 (3.0 mL), DCC (63.9 mg, 0.31 mmol, 1.5 eq). Column conditions: hexane/EtOAc 4:1. Yield: 97.7 mg, 0.17 mmol, 80%. R_f = 0.81 (hexane/EtOAc 1:1, UV and molybdato phosphate). Anal. calcd. for $C_{31}H_{42}O_{10}$: C, 64.79; H, 7.37; Found: C, 64.62; H, 7.53.

Thymoquinone-artesunic acid hybrid 7c. Art (146 mg, 0.38 mmol, 1.5 eq), DMAP (13.9 mg, 0.11 mmol, 30 mol%), TQ alcohol **6c** (60.0 mg, 0.25 mmol, 1.0 eq), dry CH_2Cl_2 (3.5 mL), DCC (78.6 mg, 0.38 mmol, 1.5 eq). Column conditions: hexane/EtOAc 4:1. Yield: 131 mg, 0.22 mmol, 86%. R_f = 0.22 (hexane/EtOAc 4:1, UV and molybdato phosphate). Anal. calcd. for $C_{33}H_{46}O_{10}$: C, 65.76; H, 7.69; Found: C, 65.65; H, 7.81.

Thymoquinone-artemisinin-derived acid hybrid 9a. Acid **8** (41.0 mg, 0.13 mmol, 1.0 eq), DMAP (46.2 mg, 0.38 mmmol, 3.0 eq), TQ alcohol **6a** (28.0 mg, 0.13 mmol, 1.0 eq), dry CH_2Cl_2 (4.2 mL), DCC (77.9 mg, 0.38 mmol, 3.0 eq). Column conditions: hexane/Et_2O 1:1. Yield: 26.0 mg, 0.05 mmol, 39%. R_f = 0.49 (hexane/Et_2O 1:1, UV and molybdato phosphate). Anal. calcd. for $C_{30}H_{42}O_8$: C, 67.90; H, 7.98; Found: C, 68.18; H, 8.11.

Thymoquinone-artemisinin-derived hybrid 9b. Acid **8** (59.4 mg, 0.18 mmol, 1.0 eq), DMAP (27.8 mg, 0.23 mmmol, 1.3 eq), TQ alcohol **6c** (43.0 mg, 0.18 mmol, 1.0 eq), dry CH_2Cl_2 (3.0 mL), DCC (46.9 mg, 0.23 mmol, 1.3 eq). Column conditions: hexane/EtOAc 1:1 and hexane/EtOAc 2:1. Yield: 31.5 mg, 0.06 mmol, 32% yield. R_f = 0.67 (hexane/EtOAc 1:1, UV and molybdato phosphate). Anal. calcd. for $C_{31}H_{44}O_8$: C, 68.36; H, 8.14; Found: C, 68.37; H, 8.04.

General procedure for hybrids 11a/b: Dihydroartemisinin acetate **10** (1.0 eq) and the corresponding TQ alcohol **6a/c** (1.1 eq) were dissolved under N_2 in dry $CHCl_3$ (1.4 mL) and cooled to 0 ° C. Then TMSOTf (0.1 eq) was added under N_2 at 0 ° C. The resulting reaction mixture was stirred at 0 ° C for 1 h and afterwards quenched with sat. Na_2CO_3 (1.5 mL). Additional H_2O was added and the aqueous phase extracted with either $CHCl_3$ (3 x 2 mL) in the case of ether hybrid **11a** or with EtOAc (3 x 20 mL) in the case of ether hybrid **11b**. The combined organic layers were washed with H_2O, dried over $NaSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography in order to obtain hybrid **11a/b** as an orange to yellow gum.

Thymoquinone-artemisinin hybrid 11a (configuration: 10β/10α 8:1). Dihydroartemisinin acetate **10** (62.5 mg, 0.19 mmol 1.0 eq), TQ alcohol **6a** (46.8 mg, 0.21 mmol, 1.1 eq), TMSOTf (3.47 μl, 4.26 mg, 0.02 mmol, 0.1 eq). Column conditions: hexane/EtOAc 3:1, 2:1. Yield: 39.8 mg, 0.08 mmol, 43%. R_f = 0.34 (hexane/Et_2O 1:1, UV and molybdato phosphate). Anal. calcd. for $C_{28}H_{40}O_7$: C, 68.83; H, 8.25; Found: C, 69.29; H, 8.27.

Thymoquinone-artemisinin hybrid 11b (configuration: 10β/10α 8:1). Dihydroartemisinin acetate **10** (45.1 mg, 0.14 mmol, 1.0 eq), TQ alcohol **6c** (36.0 mg, 0.15 mmol, 1.1 eq), TMSOTf (2.50 μL, 3.07 mg, 0.01 mmol, 0.1 eq). Column conditions: hexane/EtOAc 9:1. Yield: 36.0 mg, 0.07 mmol, 52%. R_f = 0.19 (hexane/EtOAc 9:1, UV and molybdato phosphate). Anal. calcd. for $C_{29}H_{42}O_7$: C, 69.30; H, 8.42; Found: C, 69.19; H, 8.79.

Supporting Information

Experimental conditions and procedures for intermediates **4a-c** and **6a-c**; spectral data of intermediates **4a-c**, **6a-c** and target compounds **7a-c**, **9a/b** and **11a/b**; recorded spectra of target compounds; details of cell lines and reagents as well as cell viability assay for biological evaluation. This material is available free of charge via the Internet at http://.

ABBREVIATIONS

Art, artesunic acid; DCC, *N*,*N'*-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)-pyridine; eq, equivalent; EtOAc, ethyl acetate; HCEC, human colon epithelial cells; ROS, reactive oxygen species; TMSOTf, trimethylsilyl triflate; TQ, thymoquinone; Dox, doxorubicin; NAC, *N*-acetyl-cysteine.

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Keywords: Artemisinin • Artesunic acid • Thymoquinone • Natural product hybrid • Anticancer activity

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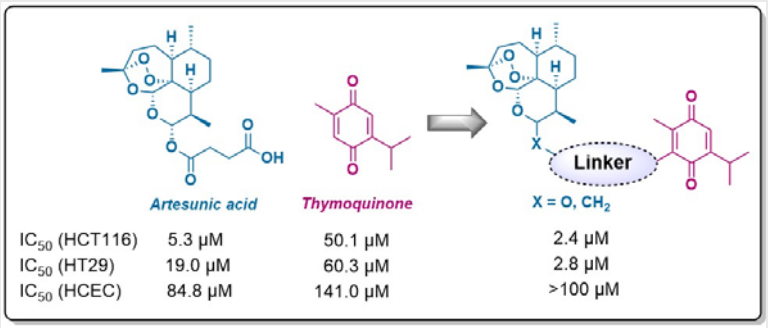

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Synthesis of Novel Hybrids of Thymoquinone and Artemisinin with High Activity and Selectivity Against Colon Cancer

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