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Original article

Synthesis and study of cytotoxic activity of 1,2,4-trioxane- and egonol-derived hybrid molecules against *Plasmodium falciparum* and multidrug-resistant human leukemia cells

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ABSTRACT

Malaria and cancer cause the death of millions of people every year. To combat these two diseases, it is important that new pharmaceutically active compounds have the ability to overcome multidrug resistance in cancer and *Plasmodium falciparum* strains. In search of effective anti-cancer and anti-malaria hybrids that possess improved properties compared to their parent compounds, a series of novel 1,2,4-trioxane-based hybrids incorporating egonol and/or ferrocene fragments were synthesized and tested *in vitro* against *P. falciparum* strains, CCRF–CEM cells and the multidrug-resistant P-glycoprotein-over-expressing CEM/ADR5000 cells. The most active compounds against *P. falciparum* strains were artesunic acid homodimers **12** and **13** (IC_{50} of 0.32 and 0.30 nM, respectively), whereas novel hybrids **7** (1,2,4-trioxane–ferrocene–egonol), **9** (1,2,4-trioxane–ferrocene) and **11** (artesunic acid–egonol) showed a remarkable cytotoxicity toward CCRF–CEM cells (IC_{50} of 0.07, 0.25 and 0.18 μM, respectively). A cooperative and synergistic effect of the three moieties 1,2,4-trioxane, ferrocene and egonol in hybrid molecule **7** is significant and is obviously stronger than in hybrids **9** (1,2,4-trioxane–ferrocene) and **11** (artesunic acid–egonol), which comprises of only two of the three considered parent compounds. Interestingly, hybrid **9** containing a 1,2,4-trioxane and a ferrocene fragment has shown to be the most effective among the studied hybrids against the tested multidrug-resistant leukemia CEM/ADR5000 cells (IC_{50} of 0.57 μM) and possesses a degree of cross-resistance of 2.34.

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1. Introduction

Malaria infections caused by *Plasmodium falciparum* annually lead to more than one million deaths, mainly in Africa, Asia and South America [1]. After the appearance of *P. falciparum* strains, which are resistant to well-established anti-malarial drugs (e.g. chloroquine (CQ)), new drug candidates capable to combat drug resistance are urgently needed [2].

In addition to malaria, another big challenge in human medicine is the fight against cancer, especially the overcoming of multidrug resistance. This disease also causes the death of millions of people every year [3].

A promising redox-directed, multidrug resistance overcoming anti-malarial [4] and anti-cancer [5] compound is artemisinin (**1**) (Fig. 1) – a natural product and well known representative of the 1,2,4-trioxane family from the plant *Artemisia annua* L. Artemisinin (**1**) contains an endoperoxide bridge, that can be activated and fragmented by intracellular Fe(II) leading to the formation of peroxyl free radicals and reactive oxygen species (ROS), which induce oxidative stress, DNA damage, alkylation of target proteins and apoptosis [6,7]. In case of malaria a disruption of the cellular redox cycling [8], an inhibition of a calcium-ATPase (SERCA, pFATP6) as

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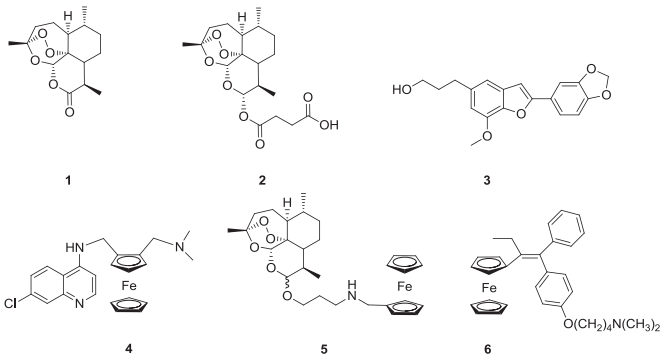

Fig. 1. Compounds with anti-malaria and/or anti-cancer activity.

well as a depolarization of the mitochondrial potential is discussed furthermore [9]. In artemisinin's mode of action against cancer also the inhibition of metastasis [10], cancer-related signaling pathways [11] and inhibition of angiogenesis occurs [5g,12].

A major remarkable advantage of artemisinin is its lack of side effects on normal cells [13]. The applicability of artemisinin (**1**) as drug is, nevertheless, limited by its low solubility and poor oral bioavailability [14]. A solution for these problems may be derivatives of artemisinin (**1**), such as artesunic acid (**2**) (Fig. 1). This compound has also been shown to induce apoptosis [5a,15] and inhibit angiogenesis [5g].

To develop even more effective drug candidates than artemisinin (**1**) and artesunic acid (**2**) against cancer and malaria chemical hybridization as a known and promising approach may be applied: Two or more natural product fragments are linked with each other and thereby new structures are obtained that possess improved properties compared to their parent compounds [16].

Several homodimer and hybrid molecules based on artemisinin (**1**) are described in literature [17], but homodimers containing artesunic acid (**2**) have been scarcely reported as yet [18], although they possess great therapeutic potential.

As a continuation of our interest in the exploration of the potential of 1,2,4-trioxanes (including artesunic acid) for the preparation of potent anti-cancer and anti-malaria homodimers and hybrid molecules [18], we envisioned the synthesis of novel target trioxane-based hybrids incorporating egonol (**3**) and/or ferrocene fragments (compounds **7**–**11**, Fig. 2).

Egonol (**3**) is a natural benzofuran that is widely distributed in *Styrax* species and possesses interesting biological properties such as insecticidal, fungicidal, anti-microbial, anti-proliferative, and cytotoxic activities toward cancer cell lines [19], as well as cyclooxygenase inhibitory [20] and anti-oxidant properties [21].

Since ferrocene derivatives have proven to be active anti-malaria (e.g. ferroquine (**4**) and hybrid **5**) [22] and anti-cancer (e.g. ferrocifen (**6**), Fig. 1) [23] agents, we selected ferrocene as a promising hybrid subunit and/or linker. In the presence of ferrocene-linker the oxidative cleavage of the endoperoxide bond of the trioxane fragment might be triggered more facially and consequently a higher amount of ROS with involvement of Fenton chemistry [6e] might be formed, leading to the intrinsic cell death pathway.

Here we wish to report the synthesis of the designed trioxane-derived hybrids **7**–**11** (Fig. 2) from easily accessible starting materials in a few chemical steps, as well as the results of their biological tests against drug-sensitive CCRF–CEM leukemia cells, their multidrug-resistant sub-line CEM/ADR5000 and against the *P. falciparum* 3D7 strain. The evaluation of the anti-malaria activity of our previously reported homodimers **12** and **13** (highly active against leukemia) [18b] will be also discussed in the present work.

2. Results and discussion

2.1. Chemistry

Artemisinin (**1**) and its derivatives are active anti-malarial compounds, especially since they can also successfully combat complicated and severe forms of malaria and overcome resistance of Plasmodia to other anti-malarial drugs [24]. Artemisinin-type drugs also inhibit cancer growth *in vitro* and *in vivo* [5g,5j,5k,25]. First clinical reports indicate that they are active in cancer patients [26].

One strategy to kill otherwise unresponsive *Plasmodia* or cancer cells is to use compounds with novel modes of action. The generation of hybrid molecules has been described during the past decade as an attractive approach in pharmaceutical and medicinal chemistry [16]. In the present investigation we synthesized and analyzed 1,2,4-trioxane and egonol-derived novel hybrid molecules (Fig. 2) for their activity toward malaria parasites (*P. falciparum* 3D7) and leukemia cancer cells (CCRF–CEM and multidrug-resistant CEM/ADR5000 cells).

All compounds of the present work (Fig. 2) were accessible starting from commercially available artesunic acid (**2**), from plants (egonol (**3**)) or easily obtained precursors (1,2,4-trioxane-derived alcohol **16**). The literature known alcohol **16** [17g,27] was chosen as a building block for some of our novel hybrid molecules (e.g. **7** and **9**) as it has been shown that C-10 non-acetal substances are 15- to 22-times more stable and reveal a greater bioavailability than recently established drugs like artemether or arteether [28]. Additionally, this precursor was already applied for the synthesis of very effective anti-cancer and anti-malaria agents [27e].

Based on 1,2,4-trioxane-derived alcohol **16** and natural product egonol (**3**), hybrids **7**–**10** were prepared. The approach of linking 1,2,4-trioxanes with ferrocene is rare, but does have precedent (e.g. anti-malaria hybrid **5**, Fig. 1) [22b], yet to our knowledge there are no reports on hybridization of egonol (**3**) with 1,2,4-trioxane and/or ferrocene compounds.

The egonol-derived hybrid **11** was synthesized for the first time starting from artesunic acid (**2**). The synthesis and studies of cytotoxic activity of artesunic acid homodimers **12** and **13** against human leukemia cells were recently reported by us [18b]. In the present work we studied the anti-malaria activity of these homodimers **12** and **13** for the first time.

Generally, the applied syntheses of this research work can be divided into three groups (Scheme 1): At first, attempts were made to synthesize the novel hybrids **7** and **8** using a Mitsunobu reaction. These approaches did not yield any of the desired products. Therefore, an alternative synthetic route had to be found. To get a very reactive precursor, ferrocene dicarboxylic acid dichloride **15** was synthesized in 85% yield using oxalyl chloride and pyridine in dry DCM. As a follow-up, dichloride **15** was reacted with the corresponding alcohols (1.0 eq of each alcohol for hybrid **7** and 2.0 eq of egonol (**3**) for hybrid **8**) in DCM using DMAP (4.0 eq) as a base. Applying these reaction conditions, 1,2,4-trioxane–ferrocene–egonol hybrid **7** was obtained in 30% yield and egonol–ferrocene–egonol hybrid **8** in 97% yield.

As the second reaction type a Mitsunobu reaction, to obtain 1,2,4-trioxane–ferrocene hybrid **9** and egonol–ferrocene hybrid **10** between the corresponding alcohol (1,2,4-trioxane-derived alcohol **16** or egonol (**3**), 1.0 eq) and ferrocene monocarboxylic acid **17** (1.1 eq with alcohol **16** and 1.0 eq with egonol (**3**)) in the presence of DIAD (1.0 eq) and PPh_3 (1.0 eq) in THF proved to be successful. The yields were moderate to good (68% for **10** and 77% for **9**) for the two compounds.

A Steglich esterification as the last of the three reaction types led to artesunic acid–egonol hybrid **11**. Artesunic acid (**2**) (1.0 eq) was

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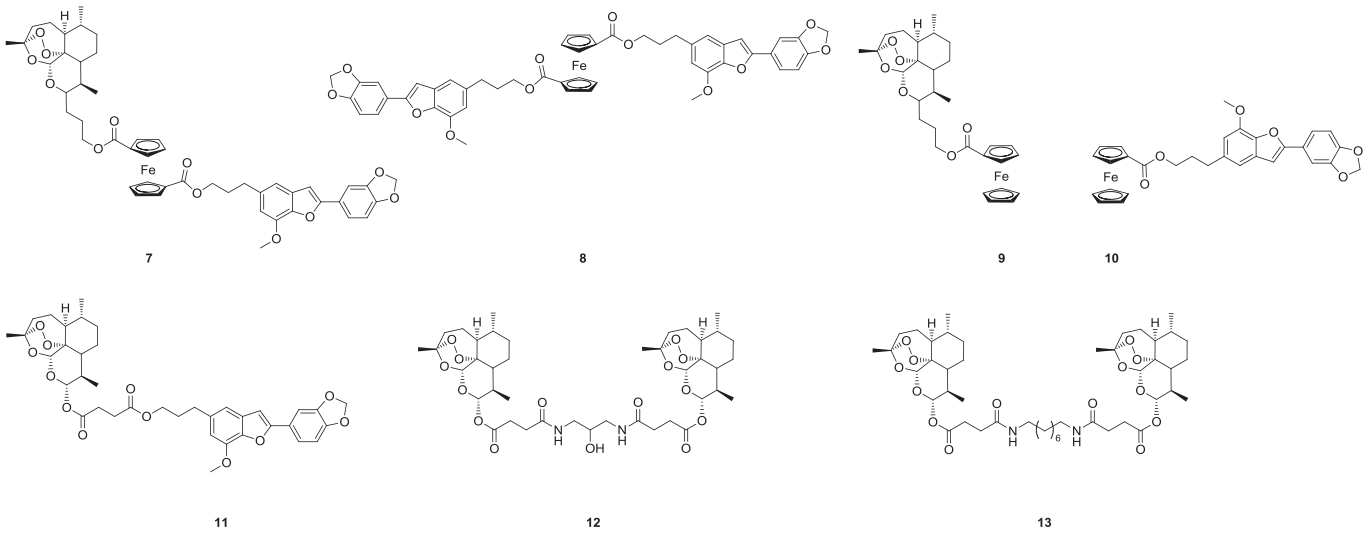
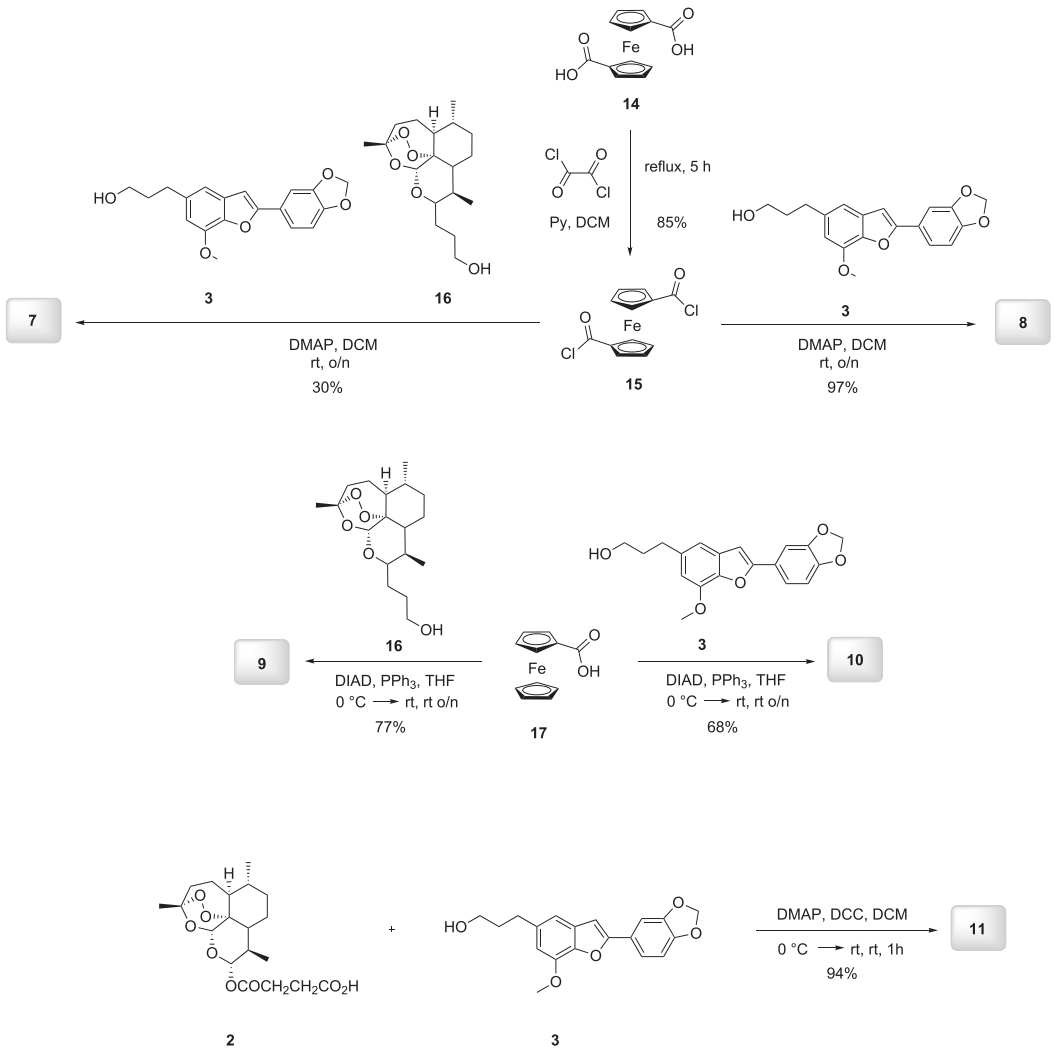


Fig. 2. Hybrids applied for biological tests against CCRF–CEM, CEM/ADR5000 cells and *P. falciparum* 3D7 parasites in this work: 1,2,4-trioxane–ferrocene–egonol hybrid **7**, egonol–ferrocene–egonol hybrid **8**, 1,2,4-trioxane–ferrocene hybrid **9**, egonol–ferrocene hybrid **10**, artesunic acid–egonol hybrid **11**, artesunic acid homodimer **12** and artesunic acid homodimer **13**.



Scheme 1. Synthesis route for 1,2,4-trioxane–ferrocene–egonol hybrid **7**, egonol–ferrocene–egonol hybrid **8**, 1,2,4-trioxane–ferrocene hybrid **9**, egonol–ferrocene hybrid **10** and artesunic acid–egonol hybrid **11**.

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coupled with egonol (**3**) (3.0 eq) employing DCC (1.1 eq) to form an ester bond. To facilitate the reaction the active ester formation was applied by the use of DMAP (10 mol%). After completion of the reaction, DCU was filtered off and hybrid **11** could be purified by column chromatography to obtain the desired product in 94% yield.

All of our new compounds (**7**–**11**) have proven to be stable under similar conditions introduced by Posner [17b] to test the stability of hybrids: After heating the compounds for 20 h at 60 °C, less than 5% decomposition was observed via ^{1}H NMR spectroscopy.

2.2. Biological evaluation and discussion

2.2.1. Cytotoxicity toward Plasmodium falciparum 3D7

The anti-malarial activity of the hybrids **7**, **8** and **10**–**13**, as well as of their parent compounds artesunic acid (**2**) and egonol (**3**) was evaluated *in vitro* against *P. falciparum* 3D7 (Table 1). Artesunic acid (**2**) revealed a 50% inhibition concentration (IC_{50}) of 0.82 nM and egonol (**3**) of 25436 nM. Ferrocene monocarboxylic acid (**17**) and ferrocene dicarboxylic acid (**14**) were not active in the tested dose range (inhibition can only be seen at the highest concentration test (112 μM), therefore no IC_{50} can be given).

Connecting one egonol moiety to ferrocene monocarboxylic acid (**17**) yields egonol–ferrocene hybrid **10** with a fivefold higher activity (IC_{50} of 5090 nM) against *P. falciparum* 3D7 than egonol (**3**) (IC_{50} of 25436 nM) itself. On the contrary, the egonol–ferrocene–egonol hybrid **8** possesses almost no activity. The IC_{50} of 1,2,4-trioxane–ferrocene–egonol hybrid **7** (88 nM) is 100-fold higher than the IC_{50} of artesunic acid (**2**), but almost 300-fold smaller than the one of egonol. These values indicate that a combination of 1,2,4-trioxane and egonol (**3**) in the same molecule result in a hybrid that is active against *P. falciparum* 3D7 (compared to egonol–ferrocene–egonol hybrid **8**), but does not deliver a more effective compound than artesunic acid (**2**). This observation is corroborated by the fact, that also artesunic acid–egonol hybrid **11** (IC_{50} of 2.30 nM) revealed a higher IC_{50} value than artesunic acid (**2**). By comparing the IC_{50} values of hybrids **7** (88 nM) and **11** (2.30 nM) it can be seen that a ferrocene moiety does not have a positive influence on the anti-malaria activity of our hybrids. Remarkably, artesunic acid homodimers **12** and **13** revealed increased cytotoxicity (>2.5-fold higher activity) compared to artesunic acid (**2**) alone with IC_{50} values of 0.32 and 0.30 nM, respectively, and therefore artesunic acid homodimers can be regarded as effective agents against the malaria parasite *P. falciparum* 3D7. The breakage of the endoperoxide bridge in artemisinin-type compounds could be facilitated by iron in the hemoglobin of *Plasmodia*-infected erythrocytes [30]. The large excess of iron in erythrocytes may explain the excellent activity of artesunic acid homodimers **12** and **13** in *P. falciparum* 3D7.

Table 1
IC_{50} values for artesunic acid (**2**), egonol (**3**) and new hybrids **7**, **8**, **10**–**13** against *P. falciparum* 3D7 parasites.

Compound	Molecular weight	3D7 IC_{50} (nM)
Artesunic acid (**2**)	384.42	0.82
Egonol (**3**)	326.34	25436
7	890.79	88
8[a]	890.71	n.a.
10	538.37	5090
11	692.75	2.30
12	822.94	**0.32**
13	877.07	**0.30**

The best results are highlighted in bold.
[a] n.a = Not active. Inhibition can only be seen at the highest concentration tested (1.2 μM) and therefore no IC_{50} can be given.

The investigation of anti-malarial activity of these homodimers against CQ-resistant (K1 and Dd2) strains is part of further studies. The results will be reported elsewhere.

2.2.2. Cytotoxicity toward sensitive CCRF–CEM and multidrug-resistant CEM/ADR5000 leukemia cells

The hybrids were further investigated for their cytotoxicity toward wild-type CCRF–CEM and multidrug-resistant P-glycoprotein over-expressing CEM/ADR5000 human leukemia cells (Table 2). The dose–response curves shown in Fig. 3 were used to calculate the 50% inhibition concentrations (IC_{50}). Artesunic acid (**2**) (IC_{50} of 1.80 ± (1.20) μM) was more cytotoxic toward CCRF–CEM cells than artemisinin (**1**) (36.90 ± (6.90) μM). Egonol (**3**) also showed considerable cytotoxicity (IC_{50} of 5.75 ± (0.41) μM), but 1,2,4-trioxane-derived alcohol **16** was hardly active (IC_{50} of 173.90 μM).

Ferrocene monocarboxylic acid (**17**) and ferrocene dicarboxylic acid (**14**) were not cytotoxic in the dose range tested. Notably, 1,2,4-trioxane–ferrocene–egonol hybrid **7** and 1,2,4-trioxane–ferrocene hybrid **9** were considerably more cytotoxic toward CCRF–CEM cells than their corresponding monomeric molecules (egonol (**3**), ferrocene dicarboxylic acid (**14**), 1,2,4-trioxane-derived alcohol **16** and ferrocene monocarboxylic acid (**17**)) with IC_{50} values of 0.07 ± (0.01) μM and 0.25 ± (0.14) μM, respectively (Table 2). As the IC_{50} value of the egonol–ferrocene–egonol hybrid **8** (3.17 μM) is 12-fold smaller than the one of the egonol–ferrocene hybrid **10** (36.15 μM), it seems that the integration of more egonol moieties reduces the IC_{50} to a great extent. Interestingly, hybrid **11**, consisting of an artesunic acid and an egonol fragment, showed an IC_{50} of 0.18 ± (0.01) μM, whereas the homodimers **12** and **13**, described previously by us [18b], revealed a slightly higher IC_{50} (2.16 ± (0.99) μM and 2.94 ± (0.33) μM, respectively) than artesunic acid (**2**). Making a comparison between the IC_{50} of artesunic acid (**2**) (1.80 ± (1.20) μM), artesunic acid homodimers **12** and **13** and artesunic acid–egonol hybrid **11** (0.18 ± (0.01) μM) it seems likely that the egonol moiety is suitable to obtain effective anti-cancer compounds. The IC_{50} of 1,2,4-trioxane–ferrocene–egonol hybrid **7** (0.07 ± (0.01) μM) and artesunic acid–egonol hybrid **11** (0.18 μM) demonstrate, that it is possible to decrease the IC_{50} even further by applying ~~a~~ ferrocene as a linker between the egonol- and the 1,2,4-trioxane-moiety. Another proof for the positive cooperative and synergistic effect of ferrocene, egonol and 1,2,4-trioxane moieties is, that the IC_{50} value of hybrid **7** (1,2,4-trioxane–ferrocene–egonol

Table 2
IC_{50} values for doxorubicin, artemisinin (**1**), artesunic acid (**2**), egonol (**3**), 1,2,4-trioxane-derived alcohol **16** and hybrids **7**–**13** in sensitive wild-type CCRF–CEM and multidrug-resistant P-glycoprotein-over-expressing CEM/ADR5000 cells.

Compound	Molecular Weight	CCRF–CEM IC_{50} (μM)	CEM/ADR5000 IC_{50} (μM)	Degree of cross-resistance
Doxorubicin[a]	579.98	0.009	23.27	2585
Artemisinin (**1**)	282.14	36.90 ± (6.90)	26.90 ± (4.40)	0.73
Artesunic acid (**2**)	384.42	1.80 ± (1.20)	1.20 ± (0.70)	0.67
Egonol (**3**)	326.34	5.75 ± (0.41)	9.78 ± (1.08)	1.70
16	326.43	173.90	n.a.[b]	–
7	890.79	**0.07 ± (0.01)**	147.27 ± (5.83)	1966
8	890.71	3.17	n.a.[c]	–
9	538.45	**0.25 ± (0.14)**	**0.57 ± (0.22)**	2.34
10	538.37	36.15	n.a.[c]	–
11	692.75	**0.18 ± (0.01)**	1.12	6.12
12[d]	822.94	2.16 ± (0.99)	1.21 ± (0.15)	0.56
13[d]	877.07	2.94 ± (0.33)	3.04 ± (0.44)	1.03

The best results are highlighted in bold.
[a] IC_{50} values for both cell lines have been previously reported [29].
[b] n.a. = Not active, even with a concentration of 400 μM.
[c] n.a = Not active, even with a concentration of 200 μM.
[d] IC_{50} values for both cell lines have been previously reported [18b].

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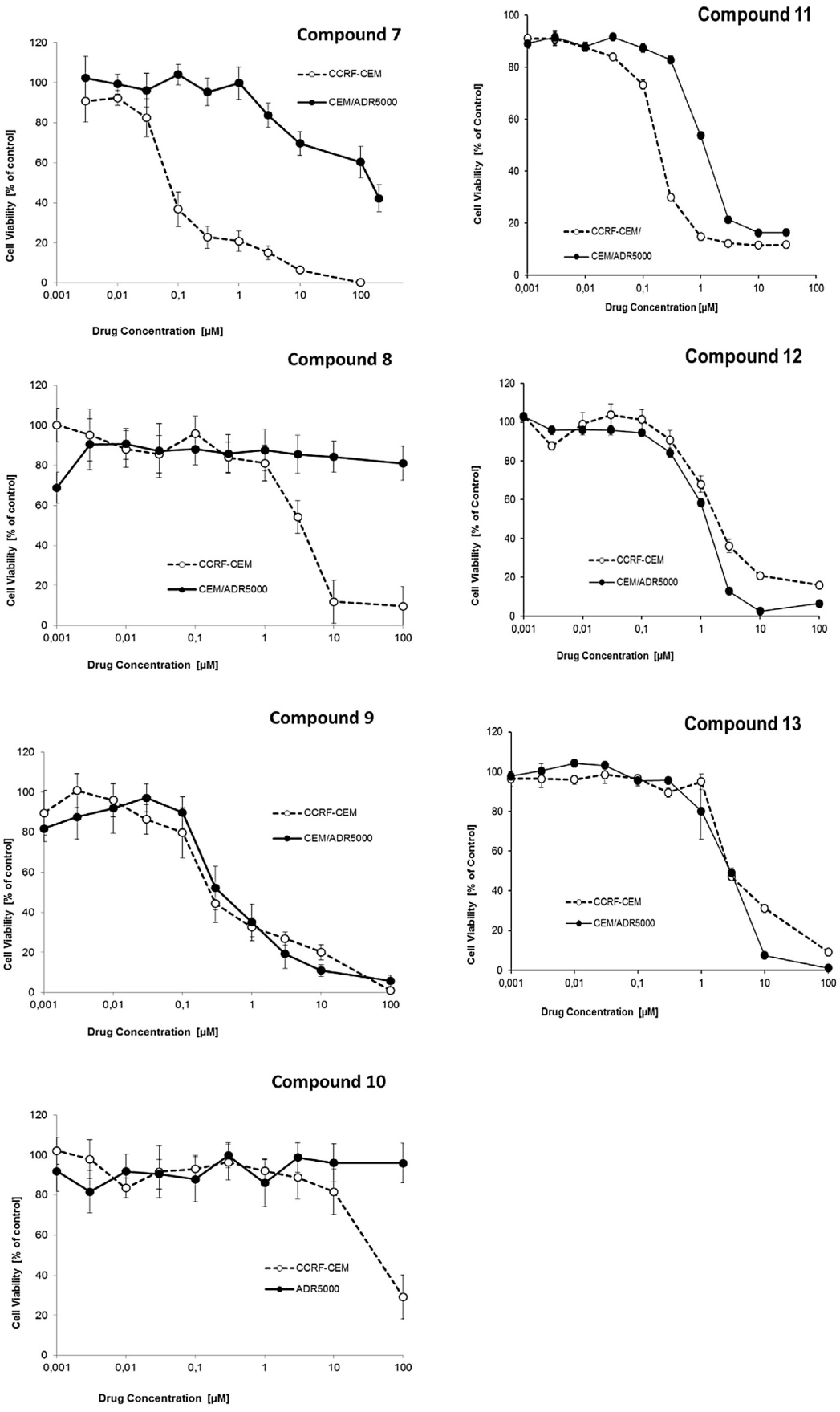

Fig. 3. Cytotoxicity of compounds **7**–**13** toward CCRF–CEM and multidrug-resistant CEM/ADR5000 human leukemia cells. Each experiment was independently performed at least two times with 6 parallel measurements each, leading to 12 values per concentration. Mean values (±SD) are plotted as a function for each compound for both cell lines.

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hybrid) is lower than the one of hybrid **9** (1,2,4-trioxane–ferrocene), which does not include an egonol moiety.

All monomeric and hybrid molecules were also tested for their activity in the CEM/ADR5000 cell line, a multidrug-resistant subline of CCRF–CEM parental cells (Table 2). For control purposes the obtained results were compared to the established anti-cancer drug doxorubicin. Multidrug-resistant CEM/ADR5000 cells displayed a 2585-fold resistance to doxorubicin compared to their drug-sensitive parental CCRF–CEM cells. Artemisinin (**1**) and artesunic acid (**2**) were slightly more cytotoxic in CEM/ADR5000 cells than in CCRF–CEM cells, resulting in degrees of resistance <1 (0.73 and 0.67, respectively). Egonol (**3**) revealed a 1.7-fold resistance in CEM/ADR5000 cells (IC_{50} of 9.78 ± (1.08) μM) and 1,2,4-trioxane-derived alcohol **16** was not cytotoxic at all in the tested dose range. Ferrocene monocarboxylic acid (**17**) and ferrocene dicarboxylic acid (**14**) were not cytotoxic in CEM/ADR5000 cells. 1,2,4-Trioxane–ferrocene hybrid **9** revealed an IC_{50} value of 0.57 ± (0.22) μM resulting in a 2.34-fold resistance of CEM/ADR5000 cells compared to their sensitive parental counterparts. As CEM/ADR5000 cells were hypersensitive to artesunic acid homodimer **12** (IC_{50} of 1.21 ± (0.15) μM) compared to CCRF–CEM cells (IC_{50} of 2.16 ± (0.99) μM), the degree of resistance was 0.56. Artesunic acid–egonol hybrid **11** (IC_{50} of 1.12 μM) and artesunic acid homodimer **13** (IC_{50} of 3.04 ± (0.44) μM) revealed degrees of resistance >1 (6.12 and 1.03, respectively). 1,2,4-Trioxane–ferrocene–egonol hybrid **7** showed a high degree of resistance (1966-fold) in CEM/ADR5000 cells. Egonol–ferrocene hybrid **10** and egonol–ferrocene–egonol hybrid **8** were not cytotoxic toward CEM/ADR5000 cells in the tested dose range. CEM/ADR5000 cells were thus resistant to these compounds compared to CCRF–CEM cells.

While 1,2,4-trioxane-derived hybrid **7** containing a ferrocene moiety was not more effective than artesunic acid (**2**) against *P. falciparum* 3D7 (Table 1), it has shown considerably higher activity against human leukemia cells (IC_{50} of 0.07 ± (0.01) μM) than the monomeric compounds or other hybrid molecules (Table 2). The active moiety of artesunic acid is an endoperoxide bridge, which may form reactive oxygen species and carbon-centered radical molecules upon breakage facilitated by iron (e.g. in the transferrin of cancer cells [30]). Bringing iron in the form of ferrocene in close proximity to artesunic acid may explain the improved activity of compounds **7** and **9** towards CCRF–CEM leukemia cells.

Since the main problem in chemotherapy is the development of drug resistance, the question arises also about resistance toward 1,2,4-trioxane hybrid molecules. While multidrug-resistant CEM/ADR5000 leukemia cells were not cross-resistant toward artemisinin (**1**) and artesunic acid (**2**), only very low cross-resistance was observed toward compound **9** (2.34-fold) but very high cross-resistance to compound **7** (1966-fold), whereas CEM/ADR5000 cells are not cross-resistant to artesunate [31]. This is not a general feature of all artemisinin derivatives. In a recent investigation it was found, that CEM/ADR5000 cells were cross-resistant to 7 out of 16 artemisinin derivatives, while the multidrug-resistant cells were sensitive or even hypersensitive (collateral sensitive) to 9 other artemisinin derivatives [12b]. CEM/ADR5000 cells overexpress the multidrug resistance conferring ATP-binding cassette (ABC) transporter, P-glycoprotein, which extrudes a wide range of xenobiotic compounds including many anticancer drugs out of tumor cells [31,32]. The results of monomeric artemisinin derivatives described in literature and the hybrid molecules of the present investigation indicate that P-glycoprotein may recognize some, but not all derivatives as substrates, which are transported out of multidrug-resistant cells. More detailed analyses are necessary in future to reveal the structure–activity relationships of artemisinin and other 1,2,4-trioxane derivatives as P-glycoprotein substrates.

3. Conclusion

A main challenge in fighting malaria as well as cancer is the development of resistance to clinically established drugs. The search for novel compounds for both diseases has to be continued, as long as a successful treatment is not realizable for all patients.

Here, a series of novel 1,2,4-trioxane-, egonol- and ferrocene-derived hybrids **7**–**11** have been successfully prepared for the first time and evaluated against drug-sensitive CCRF–CEM leukemia cells, their multidrug-resistant sub-line CEM/ADR5000 and against the *P. falciparum* 3D7 strain. While hybrid **11** (artesunic acid–egonol), which does not contain a ferrocene moiety, showed a remarkable cytotoxicity toward CCRF–CEM cells (IC_{50} of 0.18 ± (0.01) μM), hybrids **7** (1,2,4-trioxane–ferrocene–egonol) and **9** (1,2,4-trioxane–ferrocene), both comprising of a ferrocene fragment, were highly active (**7** is even most active) against CCRF–CEM cells (IC_{50} of 0.07 ± (0.01) μM and 0.25 ± (0.14) μM, respectively). As hybrid **7** is less effective against multidrug-resistant CEM/ADR5000 cells (147.27 ± (5.83) μM) and therefore possesses a degree of cross-resistance of 1966, compound **9** has to be regarded as the most powerful hybrid (0.57 ± (0.22) μM, cross-resistance of 2.34) against multidrug-resistant leukemia cells (CEM/ADR5000) of this investigation. Consequently, the investigated novel 1,2,4-trioxane–ferrocene-based hybrids **7** and **9**, which are accessible in few synthetic steps, can be regarded as effective agents against leukemia cells and their high activity could be explained by the cooperative and synergistic effect of the endoperoxide bridge and the ferrocene unit.

Interestingly, among the investigated hybrids, artesunic acid homodimers **12** and **13** were the most potent compounds against *P. falciparum* 3D7 strains (IC_{50} of 0.32 and 0.30 nM, respectively). The high activity of artesunic acid homodimers, obtainable in only one step, against malaria parasites suggests the assumption, that the number of endoperoxide bridges is responsible for the high anti-malaria activity.

Prospectively, the results obtained through these novel trioxane-based hybrids call for further investigations exploiting the potential of the hybridization concept.

4. Experimental section

4.1. Chemistry

4.1.1. Isolation of egonol

Fruits of *Styrax officinalis* L. were collected in September 2010 from Ephesus (Selçuk-İZMİR). The seeds were dried in the shade and pulverized. The shade dried and pulverized seeds (43.0 g) of *S. officinalis* L. were extracted with *n*-hexane at rt. Afterward, the solvent was removed in *vacuo*. A crude yellow oily extract was obtained. The hexane extract (20.0 g) was hydrolyzed with 33% KOH at 100 °C for 3 h. The reaction mixture was cooled to rt and extracted with DCM. The reaction mixture was purified by column chromatography using silica gel and *n*-hexane/EtOAc solvent mixture as eluent to afford 5-(3-hydroxypropyl)-7-methoxy-2-(3,4-methylendioxyphenyl)benzofuran (**3**) as a white powder (1.1 g).

4.1.2. Synthesis of hybrid molecules - general

All reactions were performed in flame-dried glassware under a nitrogen atmosphere. After column chromatography all hybrids, besides 1,2,4-trioxane–ferrocene–egonol hybrid **7**, were re-precipitated from DCM in *n*-hexane to yield a pure compound for Elemental Analysis and biological tests. DCM was dried initially over $CaCl_2$ and then distilled from P_2O_5. THF was dried initially over KOH. Afterward, THF was distilled from sodium/benzophenone. All

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other solvents were purified by distillation using rotary evaporation or were purchased in HPLC-quality. Reagents obtained from commercial sources were used without further purification. TLC chromatography was performed on precoated aluminum silica gel SIL G/UV254 plates (Macherey–Nagel & Co.). The detection occurred via fluorescence quenching or development in a phosphomolybdic acid solution (10% in EtOH) or 10% sulfuric acid. All products were dried in high-vacuum (10^{-3} mbar). ^{1}H NMR and ^{13}C NMR spectra were recorded at rt on a Bruker Avance or JEOL JNM GX 400 spectrometer operating at 400 MHz. ESI Mass spectra were recorded on a Bruker Daltonik maXis 4G or a Bruker Daltonik micrOTOF II focus. IR spectra were recorded on a Varian IR-660 apparatus. The Absorption is indicated in wave numbers [cm^{-1}]. Elemental Analysis (C, H, N), carried out with an Euro EA 3000 (Euro Vector) machine and an EA 1119 CHNS, CE machine, is within ±0.40% of the calculated values confirming a purity of >95%. artesunic acid (**2**) and DHA were obtained from ABCR (Karlsruhe, Germany).

4.1.3. 5-(3-Hydroxypropyl)-7-methoxy-2-(3,4-methylenedioxyphenyl) benzofuran (**3**)

R_f = 0.31 (PE/EtOAc 6:4, UV and sulfuric acid 10%); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.92 (m, 2 H), 2.75 (t, J = 7.5 Hz, 2 H), 3.69 (t, J = 6.4 Hz, 2 H), 4.01 (s, 3 H), 5.98 (s, 2 H), 6.61 (d, J = 1.4 Hz, 1 H), 6.77 (s, 1 H), 6.85 (d, J = 8.1 Hz, 1 H), 6.95 (d, J = 1.4 Hz, 1 H), 7.30 (d, J = 1.7 Hz, 1 H), 7.38 (dd, J = 8.1, 1.7 Hz, 1 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 32.4, 34.7, 56.2, 62.3, 100.3, 101.3, 105.5, 107.5, 108.6, 112.3, 119.2, 124.7, 131.0, 137.5, 142.5, 144.8, 148.0 (2x), 156.1 ppm; MS (ESI) m/z: 349 ([M + Na]$^+$); HRMS (ESI) m/z calcd. for [$C_{19}H_{18}NaO_5$]$^+$: 349.1046, found: 349.1047; Anal. calcd. for $C_{19}H_{18}O_5$: C, 69.93, H, 5.56, found: C, 69.72, H, 5.57, N, 0.04.

4.1.4. Ferrocene dicarboxylic acid (**14**)

R_f = 0.76 (MeOH, UV); ^{1}H NMR (400 MHz, CD_3OD): δ = 4.48 (t, J = 1.8 Hz, 4 H), 4.81 (t, J = 1.8 Hz, 4 H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ = 73.0, 74.4, 74.7, 174.5 ppm; MS (ESI) m/z: 297 ([M + Na]$^+$); HRMS (ESI) m/z calcd. for [$C_{12}H_{10}FeNaO_4$]$^+$: 296.9821, found: 296.9824; Anal. calcd. for $C_{12}H_{10}FeO_4$: C, 52.59, H, 3.68, found: C, 52.75, H, 3.72, N, 0.03.

4.1.5. Ferrocene dicarboxylic acid chloride (**15**)

To ferrocene dicarboxylic acid (**14**) (367 mg, 1.34 mmol, 1.0 eq) in anhydrous DCM (10 mL) oxalyl chloride (575 μL, 850 mg, 6.70 mmol, 5.0 eq) and a drop of anhydrous pyridine were added under N_2 and the reaction mixture was refluxed for 5 h. Follow-up, the solvent was removed under reduced pressure and the crude was extracted with boiling PE. The acid chloride **15** was obtained as red crystals (355 mg, 1.14 mmol, 85%): R_f = 0.84 (PE/EtOAc 1:1, UV); ^{1}H NMR (400 MHz, [D_6]acetone): δ = 4.88–5.03 (m, 4 H), 5.03–5.19 (m, 4 H) ppm; ^{13}C NMR (100 MHz, [D_6]acetone): δ = 75.3, 77.3, 78.3, 168.8 ppm.

4.1.6. 3-((3R,5aS,6R,9R,12R,12aR)-3,6,9-trimethyldecahydro-3H-3,12-epoxy[1,2]dioxepino[4,3-i]isochromen-10-yl)propan-1-ol (**16**)

R_f = 0.16 (PE/EtOAc 7:3, phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.85 (d, J = 7.5 Hz, 3 H), 0.94 (d, J = 6.0 Hz, 3 H), 0.95–0.99 (m, 1 H), 1.19–2.05 (m, 16 H), 2.26–2.35 (m, 1 H), 2.64 (sex, J = 6.8 Hz, 1 H), 3.61–3.75 (m, 2 H), 4.17–4.24 (m, 1 H), 5.31 (s, 1 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.9, 20.2, 24.7, 24.9, 26.0, 26.5, 30.5, 31.3, 34.4, 36.6, 37.5, 44.2, 52.3, 62.8, 75.4, 81.1, 89.2, 103.2 ppm; MS (ESI) m/z: 349 ([M + Na]$^+$); HRMS (ESI) m/z calcd. for [$C_{18}H_{30}NaO_5$]$^+$: 349.1986, found: 349.1999; Anal. calcd. for $C_{18}H_{30}O_5$: C, 66.23, H, 9.26, found: C, 66.20, H, 9.27, N, 0.01.

4.1.7. Ferrocene monocarboxylic acid (**17**)

R_f = 0.86 (MeOH, UV); ^{1}H NMR (400 MHz, CD_3OD): δ = 4.22 (s, 5 H), 4.46 (t, J = 1.9 Hz, 2 H), 4.77 (t, J = 1.9 Hz, 2 H) ppm; ^{13}C NMR (100 MHz, CD_3OD): δ = 71.0, 71.4, 72.3, 72.6, 176.1 ppm; MS (ESI) m/z: 230 ([M]$^+$); HRMS (ESI) m/z calcd. for [$C_{11}H_{10}FeO_2$]$^+$: 230.0025, found: 230.0030; Anal. calcd. for $C_{11}H_{10}FeO_2$: C, 57.43, H, 4.38, found: C, 57.83, H, 4.36.

4.1.8. 1,2,4-Trioxane–ferrocene–egonol hybrid **7**

In an evacuated flask ferrocene dicarboxylic acid chloride (**15**) (25.0 mg, 0.08 mmol, 1.0 eq), egonol (**3**) (26.2 mg, 0.08 mmol, 1.0 eq) and alcohol **16** (26.9 mg, 0.08 mmol, 1.0 eq) were dissolved in anhydrous DCM (3.5 mL) under N_2. The flask was wrapped with aluminum foil and DMAP (39.3 mg, 0.32 mmol, 4.0 eq) was added under N_2. The solution was stirred at rt o/n and during the reaction the color of the solution changed from red to brown. After removing the solvent under reduced pressure the obtained brown solid was purified by column chromatography (PE/EtOAc 1:1). Hybrid **7** was obtained as an orange solid (21.7 mg, 0.02 mmol, 30%): R_f = 0.30 (PE/EtOAc 1:1, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, [D_6]acetone): δ = 0.87 (d, J = 7.6 Hz, 3 H), 0.91 (d, J = 6.4 Hz, 3 H), 0.93–0.98 (m, 1 H), 1.12-1.20 (m, 1 H), 1.27–1.42 (m, 6 H), 1.55–1.64 (m, 2 H), 1.67–1.81 (m, 2 H), 1.84–1.95 (m, 1 H), 1.95–2.26 (m, 7 H), 2.48–2.57 (m, 1 H), 2.79–2.81 (m, 2 H), 4.02 (s, 3 H), 4.23–4.30 (m, 5 H), 4.48–4.52 (m, 4 H), 4.79–4.83 (m, 4 H), 5.31 (s, 1 H), 6.07 (s, 2 H), 6.87 (d, J = 1.3 Hz, 1 H), 6.96 (d, J = 8.2 Hz, 1 H), 7.09-7.12 (m, 2 H), 7.39 (d, J = 1.7 Hz, 1 H), 7.46 (dd, J = 8.1, 1.6 Hz, 1 H) ppm; ^{13}C NMR (100 MHz, [D_6]acetone): δ = 12.9, 20.4, 25.5, 25.6, 26.2, 27.0, 27.5, 31.5, 31.8, 33.2, 35.3, 37.4, 38.0, 45.1, 53.2, 56.5, 64.4, 64.9, 72.3 (3x), 73.6 (4x), 74.2, 74.4, 74.5, 81.8, 90.3, 101.7, 102.6, 103.3, 106.0, 108.8, 109.6, 113.4, 119.9, 125.8, 132.2, 138.7, 143.5, 146.1, 149.3, 149.4, 170.5 (2x) ppm; FT-IR (ATR): $\tilde{\nu}$ = 3104 (w), 2950 (w), 2862 (w), 2363 (m), 2009 (w), 1707 (s), 1598 (w), 1502 (w), 1452 (m), 1365 (m), 1273 (s), 1221 (m), 1140 (s), 1026 (m), 930 (m), 881 (w), 820 (m), 783 (w), 746 (w), 660 (w), 598 (w), 490 (m) cm^{-1}; MS (ESI): m/z = 913 ([M + Na]$^+$); HRMS (ESI) m/z calculated for [$C_{49}H_{54}FeNaO_{12}$]$^+$: 913.2858, found 913.2855; Anal. calcd. for $C_{49}H_{54}FeO_{12}$: C, 66.07, H, 6.11, found: C, 66.13, H, 6.24, N, 0.04.

4.1.9. Egonol–ferrocene–egonol hybrid **8**

Ferrocene dicarboxylic acid chloride (**15**) (28.6 mg, 0.09 mmol, 1.0 eq) and egonol (**3**) (60.0 mg, 0.18 mmol, 2.0 eq) were dissolved in anhydrous DCM (4.0 mL) under N_2. The flask was wrapped with aluminum foil and DMAP (44.7 mg, 0.37 mmol, 4.0 eq) was added. The solution was stirred at rt o/n and during the reaction the color of the solution changed from red to brown. After removing the solvent under reduced pressure the crude was purified by column chromatography (*n*-hexane/EtOAc 1:2). Hybrid **8** was received in 97% yield (78.0 mg, 0.09 mmol): R_f = 0.64 (PE/EtOAc 1:4, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.02–2.12 (m, 4 H), 2.75–2.82 (m, 4 H), 4.00 (s, 6 H), 4.25 (t, J = 6.6 Hz, 4 H), 4.41 (t, J = 1.9 Hz, 4 H), 4.83 (t, J = 1.9 Hz, 4 H), 5.98 (s, 4 H), 6.62 (d, J = 1.3 Hz, 2 H), 6.74 (s, 2 H), 6.84 (d, J = 8.1 Hz, 2 H), 6.95 (d, J = 1.2 Hz, 2 H), 7.28 (d, J = 1.6 Hz, 2 H), 7.36 (dd, J = 8.1, 1.7 Hz, 2 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 30.9, 32.6, 56.2, 63.9, 71.5, 72.8, 73.1, 100.3, 101.3, 105.5, 107.4, 108.6, 112.3, 119.2, 124.7, 131.1, 136.9, 142.5, 144.8, 148.0 (2x), 156.1, 170.4 ppm; FT-IR (ATR): $\tilde{\nu}$ = 3099 (w), 2949 (m), 2894 (m), 2840 (w), 2775 (w), 2362 (w), 2247 (w), 2033 (w), 1706 (s), 1620 (m), 1596 (m), 1501 (s), 1472 (s), 1447 (s), 1366 (m), 1334 (m), 1272 (s), 1223 (s), 1141 (s), 1108 (s), 1027 (s), 972 (w), 929 (m), 905 (m), 860 (m), 809 (s), 775 (m), 726 (s), 648 (m), 599 (m), 502 (m), 490 (m) cm^{-1}; MS (ESI): m/z = 913 ([M + Na]$^+$); HRMS (ESI) m/z calculated for [$C_{50}H_{42}FeNaO_{12}$]$^+$:

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913.1919, found 913.1912; Anal. calcd. for $C_{50}H_{42}FeO_{12}$: C, 67.42, H, 4.75, found: C, 67.07, H, 5.08.

4.1.10. 1,2,4-Trioxane–ferrocene hybrid **9**

After pre-drying ferrocene monocarboxylic acid (**17**) (62.0 mg, 0.27 mmol, 1.1 eq) in an evacuated Schlenk tube on the vacuum pump for 5 min, alcohol **16** (80 mg, 0.25 mmol, 1.0 eq) was added under N_2 and both compounds were dissolved in anhydrous THF (2.0 mL). The Schlenk flask was wrapped with aluminum foil and the reaction mixture was cooled to 0 °C. PPh_3 (64.0 mg, 0.25 mmol, 1.0 eq) and DIAD (48.0 μL, 49.6 mg, 0.25 mmol, 1.0 eq) were added and the reaction mixture was slowly warmed to rt and stirred o/n. Follow-up, the solvent was removed and a crude brown oil was obtained. Purification by column chromatography (PE/EtOAc 2:1) gave hybrid **9** as a red brown solid (101.0 mg, 0.19 mmol, 77%): $R_f = 0.85$ (PE/EtOAc 6:4, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.88$ (d, $J = 7.6$ Hz, 3 H), 0.94 (d, $J = 6.0$ Hz, 3 H), 0.95–0.98 (m, 1 H), 1.18–1.32 (m, 5 H), 1.41 (s, 3 H), 1.58–1.83 (m, 5 H), 1.83–1.95 (m, 1 H), 1.95–2.13 (m, 2 H), 2.31 (td, $J = 13.5$, 4.0 Hz, 1 H), 2.67 (sex, $J = 6.9$ Hz, 1 H), 4.18 (s, 5 H), 4.21–4.27 (m, 3 H), 4.36 (t, $J = 2.0$ Hz, 2 H), 4.78 (t, $J = 1.9$ Hz, 2 H), 5.31 (s, 1 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 12.9$, 20.1, 24.7, 24.9, 26.0, 26.1, 26.8, 30.3, 34.4, 36.5, 37.4, 44.2, 52.3, 64.1, 69.7, 70.1, 71.2, 74.6, 81.1, 89.2, 103.1, 171.8 ppm; FT-IR (ATR): $\tilde{\nu} = 3083$ (w), 2917 (m), 2857 (m), 2361 (m), 2350 (w), 2050 (w), 2000 (w), 1706 (s), 1461 (m), 1374 (m), 1276 (s), 1207 (w), 1185 (w), 1143 (s), 1102 (m), 1032 (s), 979 (m), 947 (m), 876 (m), 815 (m), 774 (m), 648 (w), 604 (w), 556 (w), 482 (s), 424 (m) cm^{-1}; MS (ESI): $m/z = 538$ ($[M]^+$); HRMS (ESI) m/z calculated for $[C_{29}H_{38}FeO_6]^+$: 538.2012, found 538.2013; Anal. calcd. for $C_{29}H_{38}FeO_6$: C, 64.69, H, 7.11, found: C, 64.57, H, 7.15, N, 0.04.

4.1.11. Egonol–ferrocene hybrid ***10***

Ferrocene monocarboxylic acid (**17**) (50.0 mg, 0.22 mmol, 1.0 eq) and egonol (**3**) (71.0 mg, 0.22 mmol, 1.0 eq) were dissolved in anhydrous THF (2.0 mL) in an evacuated 5 mL two-neck flask under N_2. The flask was wrapped with aluminum foil and the brown solution was cooled to 0 °C. After adding PPh_3 (57.0 mg, 0.22 mmol, 1.0 eq) and DIAD (43.0 μL, 44.0 mg, 0.22 mmol, 1.0 eq), the reaction mixture was slowly warmed to rt and stirred o/n. Follow-up, the solvent was removed under reduced pressure and the obtained brown oil was purified by column chromatography (PE/EtOAc 2:1). Hybrid **10** was gained as a dark orange solid (80 mg, 0.15 mmol, 68%): $R_f = 0.24$ (PE/EtOAc 2:1, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 2.02$–2.12 (m, 2 H), 2.80-2.84 (m, 2 H), 4.02 (s, 3 H), 4.21 (s, 5 H), 4.25 (t, $J = 6.5$ Hz, 2 H), 4.40 (s, 2 H), 4.82 (s, 2 H), 5.99 (s, 2 H), 6.63 (d, $J = 1.3$ Hz, 1 H), 6.78 (s, 1 H), 6.85 (d, $J = 8.2$ Hz, 1 H), 6.99 (d, $J = 1.3$ Hz, 1 H), 7.31 (d, $J = 1.6$ Hz, 1 H), 7.39 (dd, $J = 8.1$, 1.7 Hz, 1 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 31.0$, 32.6, 56.2, 63.5, 69.8, 70.2, 71.4, 100.4, 101.3, 105.6, 107.5, 108.7, 112.4, 119.3, 124.8, 131.2, 137.1, 144.9, 148.1 (2x) 156.3, 171.9 ppm; FT-IR (ATR): $\tilde{\nu} = 3121$ (w), 3090 (w), 3064 (w), 2962 (w), 2929 (m), 2856 (w), 2836 (w), 2361 (w), 2337 (w), 1704 (s), 1616 (m), 1593 (m), 1476 (s), 1368 (m), 1347 (m), 1271 (s), 1234 (m), 1218 (m), 1141 (s), 1039 (m), 1010 (m), 929 (m), 915 (m), 868 (m), 743 (w), 598 (w), 501 (s), 481 (s) cm^{-1}; MS (ESI): $m/z = 538$ ($[M]^+$), 1099 ($[2M + Na]^+$); HRMS (ESI) m/z calculated for $[C_{30}H_{26}FeO_6]^+$: 538.1073, found 538.1053; Anal. calcd. for $C_{30}H_{26}FeO_6$: C, 66.93, H, 4.87, found: C, 66.78, H, 4.98.

4.1.12. Artesunic acid–egonol hybrid ***11***

In an evacuated flask artesunic acid (**2**) (45.0 mg, 0.12 mmol, 1.0 eq) was dissolved in anhydrous DCM (2.90 mL) under N_2. egonol (**3**) (114 mg, 0.35 mmol, 3.0 eq) and DMAP (1.50 mg, 0.01 mmol, 10 mol%) were added under N_2. At 0 °C DCC (27.0 mg, 0.13 mmol, 1.1 eq) was added under N_2. The solution was allowed to warm up to rt and stirred for 1 h. The urea was removed by filtration. After removing the solvent the crude was purified by column chromatography (PE/Et_2O 3.5:6.5). Hybrid **11** was obtained as a white solid (76.0 mg, 0.11 mmol, 94%): $R_f = 0.37$ (PE/Et_2O 3.5:6.5, UV and sulfuric acid 10%); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.81$ (d, $J = 7.1$ Hz, 3 H), 0.92 (d, $J = 5.8$ Hz, 3 H), 0.93–1.00 (m, 1 H), 1.20–1.50 (m, 7 H), 1.54–1.77 (m, 3 H), 1.80–1.90 (m, 1 H), 1.93–2.04 (m, 3 H), 2.30–2.40 (m, 1 H), 2.49–2.76 (m, 7 H), 4.01 (s, 3 H), 4.12 (t, $J = 6.5$ Hz, 2 H), 5.39 (s, 1 H), 5.77 (d, $J = 9.9$ Hz, 1 H), 5.99 (s, 2 H), 6.59 (s, 1 H), 6.78 (s, 1 H), 6.85 (d, $J = 8.2$ Hz, 1 H), 6.94 (s, 1 H), 7.30 (d, $J = 1.7$ Hz, 1 H), 7.38 (dd, $J = 8.1$, 1.7 Hz, 1 H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 11.9$, 20.1, 21.9, 24.5, 25.9, 28.9, 29.2, 30.6, 31.7, 32.4, 34.0, 36.2, 37.2, 45.2, 51.5, 56.2, 64.1, 80.1, 91.5, 92.2, 100.4, 101.3, 104.5, 105.6, 107.5, 108.6, 112.4, 119.3, 124.8, 131.1, 137.0, 142.6, 144.9, 148.1 (2x), 156.2, 171.3, 172.2 ppm; FT-IR (ATR): $\tilde{\nu} = 2922$ (w), 2363 (w), 2156 (w), 2038 (w), 2011 (w), 1734 (m), 1599 (w), 1500 (m), 1473 (m), 1450 (w), 1361 (w), 1230 (m), 1211 (m), 1146 (m), 1101 (m), 1012 (s), 926 (m), 871 (m), 846 (w), 817 (m), 741 (w), 512 (w), 454 (w), 400 (w) cm^{-1}; MS (ESI) m/z: 715 ($[M + Na]^+$), 731 ($[M + K]^+$); HRMS (ESI) m/z calcd. for $[C_{38}H_{44}NaO_{12}]^+$: 715.2725, found: 715.2738; Anal. calcd. for $C_{38}H_{44}O_{12}$: C, 65.88, H, 6.40, found: C, 66.07, H, 6.54.

4.2. Cytotoxicity studies against leukemia cells

4.2.1. Cell culture

Cells were cultivated in RPMI 1640 medium supplemented with 10% (v/v) inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2 in humidified atmosphere (95% relative humidity). CEM/ADR5000 cells were treated with 5000 ng/mL doxorubicin every other week for three days to maintain overexpression of P-glycoprotein [33]. The multidrug resistance profile of CEM/ADR5000 has been reported [34].

4.2.2. Cell viability assay

CCRF–CEM or CEM/ADR5000 cells were plated in 96 well plates and maintained in RPMI 1640 medium. Cell viability was determined by the resazurin assay [35]. We described the performance of the assay in detail [36]. Each experiment was performed in 6 parallel measurements and repeated at least two times. Doxorubicin served as a positive control (Sigma, purity ≥98%).

4.3. Cytotoxicity studies against P. falciparum 3D7 strains

4.3.1. Cell culture

The *P. falciparum* 3D7 strain was kept in continuous culture in complete culture medium (RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 50 μg/mL gentamicin and 0.5% w/v AlbuMAX) at 5% hematocrit at 37 °C under reduced oxygen conditions (5% CO_2, 5% O_2, 90% N_2).

4.3.2. Growth inhibition assay

All tested substances were dissolved in DMSO to a stock concentration of 50 mM, further dilutions were made with complete culture medium. Final concentration of DMSO in the assay did not exceed 0.1%, a concentration that does not interfere with parasite growth. Growth inhibition of *P. falciparum* 3D7 was evaluated by the histidine rich protein 2 (HRP2) assay according to standard procedures [37]. In brief: 96 well plates were coated with a threefold dilution of each substance before ring stage parasites were added at a hematocrit of 1.5% and a parasitemia of 0.05% and cultivated for 3 days. After the cultivation period plates were stored at −20 °C until performance of the HRP2 enzyme-linked immunosorbent assay (ELISA). Each assay was performed in duplicate and at least two independent experiments were done for each

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substance. The 50% inhibitory concentration (IC_{50}) was determined by analyzing the nonlinear regression of log concentration–response curves using the drc-package v0.9.0 of R v2.6.1 [38].

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Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version at http://dx.doi.org/10.1016/j.ejmech.2014.01.043.

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