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Research paper

New efficient artemisinin derived agents against human leukemia cells, human cytomegalovirus and *Plasmodium falciparum*: 2nd generation 1,2,4-trioxane-ferrocene hybrids

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ABSTRACT

In our ongoing search for highly active hybrid molecules exceeding their parent compounds in anticancer, antimalaria as well as antiviral activity and being an alternative to the standard drugs, we present the synthesis and biological investigations of 2nd generation 1,2,4-trioxane-ferrocene hybrids. *In vitro* tests against the CCRF-CEM leukemia cell line revealed di-1,2,4-trioxane-ferrocene hybrid **7** as the most active compound (IC_{50} of 0.01 μM). Regarding the activity against the multidrug resistant subline CEM/ADR5000, 1,2,4-trioxane-ferrocene hybrid **5** showed a remarkable activity (IC_{50} of 0.53 μM). Contrary to the antimalaria activity of hybrids **4**–**8** against *Plasmodium falciparum* 3D7 strain with slightly higher IC_{50} values (between 7.2 and 30.2 nM) than that of their parent compound DHA, hybrids **5**–**7** possessed very promising activity (IC_{50} values lower than 0.5 μM) against human cytomegalovirus (HCMV). The application of 1,2,4-trioxane-ferrocene hybrids against HCMV is unprecedented and demonstrated here for the first time.

1. Introduction

The natural 1,2,4-trioxane sesquiterpene artemisinin (**1**) (Fig. 1), isolated from the Chinese medicinal plant *Artemisia annua L.*, [1–3] has proven to be a versatile antimalarial [2,4–11], anticancer [6,8,12–24] and antiviral compound [25–30]. Its mode of action is not yet fully understood, but in the cases of malaria and cancer, the endoperoxide moiety plays a crucial role since it is activated and fragmented by intracellular Fe(II) leading to the formation of reactive oxygen species (ROS) and peroxyl free radicals. These species induce oxidative stress, DNA damage, alkylation of target proteins and apoptosis [7,11,19,31–37]. Regarding its activity against human cytomegalovirus (HCMV), artemisinin (**1**) inhibits its replication, most probably on the basis of interfering with virus-supporting cellular signaling pathways including NF-κB-specific signaling [27–29,38].

To avoid upcoming drug-resistance [39–42] against artemisinin (**1**), more effective drug candidates against malaria, cancer and HCMV can be obtained applying the concept of hybridization: the chemical combination of two or more natural product fragments leads to new structures possessing improved biological activities compared to the natural products itself [43–48]. Applying this concept, many hybrid molecules based on artemisinin (**1**) linked to a different subunit/natural product fragment are described in literature [6,8,11,49–57].

Although ferrocene-derived compounds such as ferroquine (**2**) (Fig. 1) showed improved antimalarial activity [58–61] and other derivatives such as ferrocifen possessed anticancer activity [62–68], hybrids containing a ferrocene and a 1,2,4-trioxane moiety like compound **3** were only reported twice until 2014 [69,70]. The combination of both entities in one molecule affords hybrids

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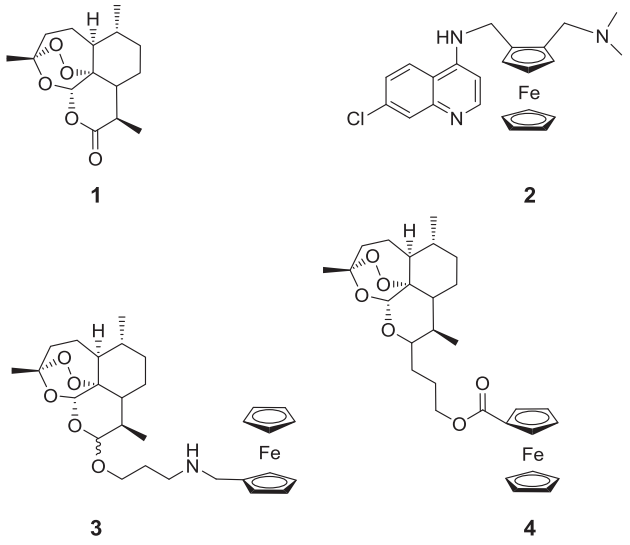


Fig. 1. Compounds with antimalaria and/or anticancer activity.

that could produce a greater amount of ROS as they have an interior iron source being able to break the endoperoxide bridge of the 1,2,4-trioxane moiety.

Recently, we reported [71] a study of different 1,2,4-trioxane-ferrocene hybrids and revealed that 1,2,4-trioxane-ferrocene hybrid **4** was the most active compound on wild-type as well as multidrug-resistant leukemia cells. Encouraged by the effective anticancer and antimalarial hybrids of this study [71], we designed four 2nd generation 1,2,4-trioxane-ferrocene hybrids (compounds **5**–**8**, Fig. 2) whose synthesis consist of only a few steps. Their biological activity against drug-sensitive CCRF-CEM leukemia cells, the multidrug-resistant sub-line CEM/ADR5000, *Plasmodium falciparum* and HCMV are reported here.

2. Results and discussion

2.1. Chemistry

All compounds of the present study (Fig. 2) could be regarded as derivatives of dihydroartemisinin (**10**) and were accessible from this commercially available starting material in only a few steps (Scheme 1).

In addition to dihydroartemisinin (**10**), its literature known derived alcohols **14** [50,72–77] and **11** [78] were chosen as building blocks for the 1,2,4-trioxane-ferrocene hybrids, since both alcohols proved to be versatile precursors for effective anticancer and antimalaria agents [50,78–85]. The approach of synthesizing a hybrid between 1,2,4-trioxanes and ferrocene derivatives has been applied a few times (e.g. hybrid **3**, Fig. 1) [69,70] before, but this motif still contains great potential to create powerful agents as our group recently demonstrated with hybrid **4** [71]. In relation to 1,2,4-trioxane dimers known from literature possessing a phenyl group as a part of bis-ether linker between the two 1,2,4-trioxane moieties [86], hybrids presented in this work are expected to benefit from biological interactions between the ferrocene moiety and the 1,2,4-trioxane unit possibly leading to strong efficacies against cancer cells, malaria parasites and viruses.

Two reaction types were used for the synthesis of hybrids **5**–**8** and their application is based on our recent experience with this type of compounds (Scheme 1) [71]. Hybrids **5** and **7** were synthesized by a Mitsunobu reaction between ferrocene monocarboxylic acid (**9**) and the corresponding alcohol (dihydroartemisinin (**10**) and di-1,2,4-trioxane-derived alcohol **11**) in the presence of PPh_3 and DIAD. Hybrid **7** was obtained in good yield (67%), whereas the yield for hybrid **5** was only 23%. This can probably be explained by the different reactivity of the alcohol functions in dihydroartemisinin (**10**) and di-1,2,4-trioxane-derived alcohol **11**. Additionally, we tried to prepare a hybrid containing a ferrocene linker and four 1,2,4-trioxane moieties starting from ferrocene dicarboxylic acid (**12**) and two molecules of alcohol **11** under Mitsunobu conditions (PPh_3/DIAD). Nevertheless, it was not possible to obtain the desired tetramer: only traces of the product could be observed in the ^{1}H NMR spectra. This might be due to the high steric demand of two alcohol **11** moieties.

As we found out before, the Mitsunobu reaction did not yield 1,2,4-trioxane-ferrocene-1,2,4-trioxane compounds and therefore, a different reaction type was needed to be applied for the synthesis of hybrids **6** and **8**: initially, ferrocene dicarboxylic acid dichloride (**13**) was synthesized from ferrocene dicarboxylic acid (**12**) using oxalyl chloride and pyridine. Afterwards, dichloride **13**, corresponding alcohol **10** or **14** and DMAP were stirred in dry DCM overnight (o/n) and hybrids **6** and **8** were obtained in 65% and 71% yield. Since all 1,2,4-trioxane-ferrocene hybrids exhibited less than 5% decomposition observable via ^{1}H NMR after 20 h at 60 °C, they have proven to be stable under similar conditions introduced by Posner [78].

1,2,4-Trioxane-ferrocene hybrids **5** and 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **6** were recrystallized from a mixture of DCM and *n*-hexane. A suitable crystal for the X-ray diffraction analysis was obtained for both compounds and their structure was unambigiously determined by X-ray crystallography (Fig. 3) [87]. To the best of our knowledge these are the first two X-ray structures of artemisinin derivate-ferrocene compounds so far. Interestingly, in the case of the 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **6** both endoperoxide units point in the same direction. It still has to be determined, whether this structural feature influences the activity or the mode of action of this hybrid.

The β-configuration of hybrid **5** visible in the X-ray structure is confirmed by the small coupling constant of 3.5 Hz for the proton at C-10 in the ^{1}H NMR spectrum which is only realizable if both adjacent protons show in the same directions. In contrast, hybrid **6** possesses a coupling constant of 9.8 Hz for the corresponding proton being typically for an α-configuration. This fact is in agreement with the X-ray structure. In case of hybrids **7** and **8** the β,β- and β-configuration is deduced from the configuration of their literature known and stereochemically already defined precursors, the alcohols **11** [78] and **14** [76].

2.2. Biological activity of the hybrids

2.2.1. Cytotoxicity towards sensitive CCRF-CEM and multidrug-resistant CEM/ADR500 leukemia cells

The cytotoxic potential of hybrids **5**–**8** against wild-type CCRF-CEM and multidrug-resistant P-glycoprotein overexpressing CEM/ADR5000 human leukemia cells (Table 1) was investigated in this study. As the 1,2,4-trioxane-ferrocene hybrid **4**, recently published by our group [71], already displayed promising activities against the CCRF-CEM cells with a 50% inhibition concentration (IC_{50}) of 0.25 ± (0.14) μM as well as against the CEM/ADR5000 cells (IC_{50} of 0.57 ± (0.22) μM). Thus, we assumed that our new hybrids would be even more potent.

Regarding their activity against CCRF-CEM leukemia cells (IC_{50} < 0.13 μM for all four compounds), all hybrids were more active than artemisinin (**1**) and dihydroartemisinin (**10**) with IC_{50} values of 36.90 ± (6.90) μM and 0.48 ± 0.27 μM, respectively (Table 1). Considering the fact that ferrocene monocarboxylic acid (**9**) was not cytotoxic in the dose range tested, these results show

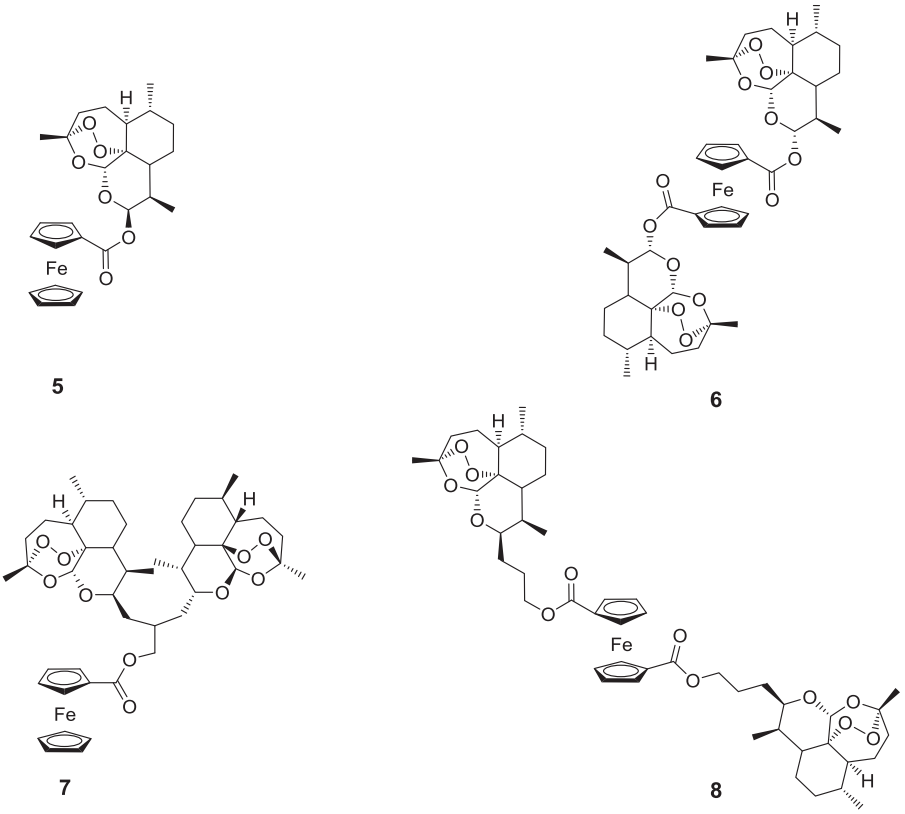


Fig. 2. New hybrids applied for biological tests against CCRF-CEM, CEM/ADR5000 cells, HCMV and *P. falciparum* 3D7 in this work: 1,2,4-trioxane-ferrocene hybrid **5**, 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **6**, di-1,2,4-trioxane-ferrocene hybrid **7** and 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **8**.

the successful application of the hybridization concept since the parent compounds are less active (or even not active) than the hybrids. The 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrids with two trioxane moieties (hybrid **6** and **8** with IC_{50} values of 0.07 ± (0.06) μM and 0.08 ± (0.03) μM, respectively) were approximately twice or even more effective against CCRF-CEM cells than corresponding hybrids with only one trioxane moiety (hybrid **5** (0.13 ± (0.04) μM) and **4** (0.25 ± (0.14) μM). Hybrid **7** was the most active compound against CCRF-CEM cells with an IC_{50} value of 0.01 ± (0.01) μM. The fact that multidrug-resistant CEM/ADR5000 cells displayed a 2585-fold resistance to the established anticancer drug doxorubicin (23.27 μM) compared to their drug sensitive parental CCRF-CEM cells (0.009 μM) (Table 1) demonstrates the great challenge of multidrug resistance in chemotherapy.

Artemisinin (**1**) as well as dihydroartemisinin (**10**) were similarly cytotoxic in CEM/ADR5000 (IC_{50} of 26.90 ± (4.40) μM and 0.68 ± (0.32) μM, respectively) and in CCRF-CEM cells (IC_{50} of 36.90 ± (6.90) μM and 0.48 ± (0.27) μM, respectively).

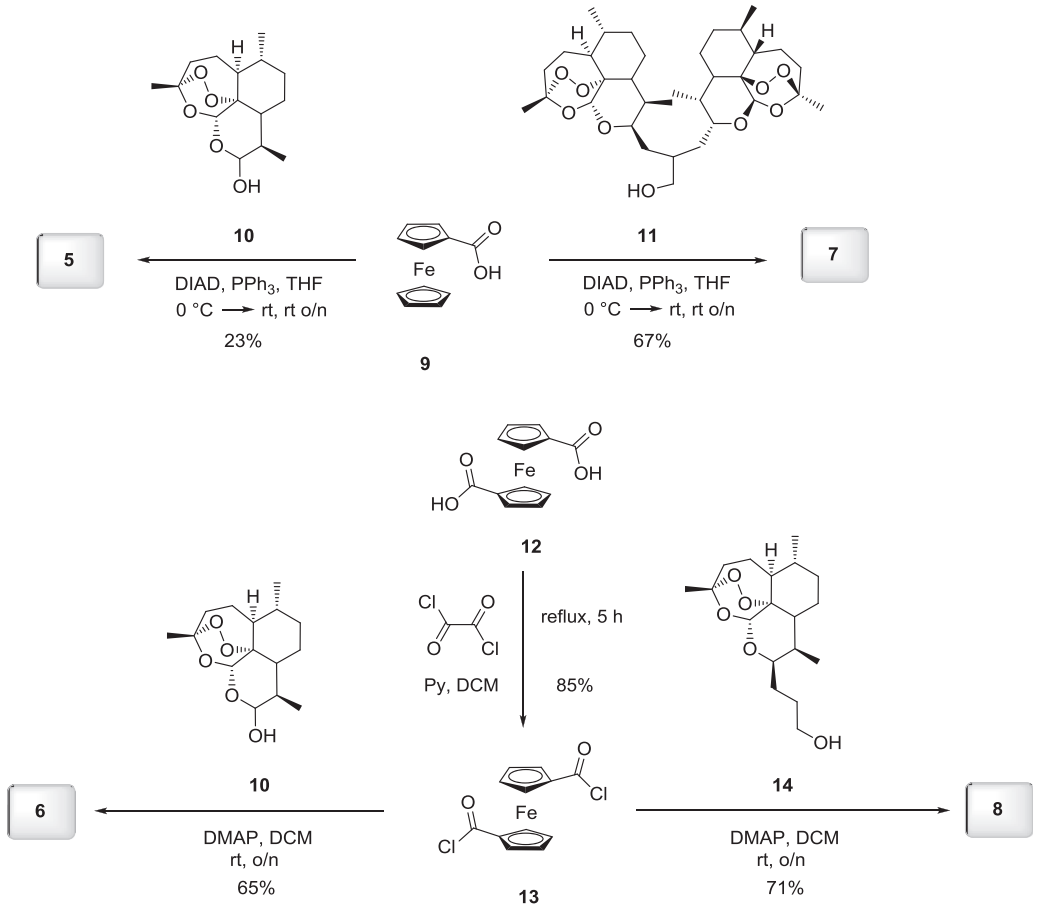
The 1,2,4-trioxane-ferrocene hybrids **5**–**8** were less active against the multidrug-resistant CEM/ADR5000 cells compared to CCRF-CEM cells, but all four hybrids were 3- to 50-fold more cytotoxic towards the multidrug-resistance cell line than artemisinin (**1**). Additionally, hybrid **5** with its IC_{50} value of 0.53 ± (0.33) μM was the most active hybrid against CEM/ADR5000 cells and was more active against this cell line than diyhdroartemisinin (**10**).

By comparing the cytotoxicities of the 1,2,4-trioxane-ferrocene hybrids **4** and **5** (IC_{50} of <1 μM) with a molecular weight around 500 g/mol and one trioxane moiety in the molecule to those of hybrids **6**–**8** (IC_{50} of >1.8 μM) with a molecular weight above 800 g/mol and two moieties, it seems that both or either the molecular weight of the trioxane-ferrocene hybrids and the amount of trioxane moieties plays a crucial role for the *in vitro* activity against multidrug-resistant CEM/ADR5000 cells.

In general, a cooperative and synergistic effect of the 1,2,4-trioxane and ferrocene moieties seems to play a significant role.

2.2.2. In vitro inhibitory activity towards HCMV in primary human fibroblasts

Antiviral activity of the new hybrid compounds was assessed by the use of an established GFP reporter-based replication assay of HCMV (recombinant strain AD169-GFP) in primary human foreskin fibroblasts (HFFs) [89,90]. We referred to the previously determined antiviral activity of artesunic acid [29,91,92] and ganciclovir, the latter being the standard drug of approved anti-HCMV therapy. These reference drugs exerted IC_{50} values of inhibition of HCMV replication in the low micromolar range (3.8 ± 0.4 μM and 2.6 ± 0.5 μM, respectively; Table 2) as measured in our quantitative experimental system. Importantly, three of the new 1,2,4-trioxane-ferrocene hybrids, namely **5**, **6** and **7**, proved to be an order of magnitude more efficient in anti-HCMV activity with IC_{50} values of 0.16 ± 0.06 μM, 0.46 ± 0.05 μM and 0.11 ± 0.01 μM, respectively (Table 2). This may indicate that the inhibition or binding of the so far unknown target might be highly intensified in case of these new artemisinin-ferrocene derivatives **5**, **6** and **7**, accompanied by putative improvements in target specificity, drug stability and pharmacological properties. In comparison, other 1,2,4-trioxane-derived compounds, such as compounds **1**, **8** and **10**, did not show a detectable antiviral activity. Thus, as illustrated by three examples, the chemical modification/hybridization of the artemisinin derivatives resulted in a strongly improved *in vitro* efficacy of anti-HCMV activity, exhibiting an unprecedented antiviral activity for artemisinin-ferrocene hybrids up to date (Table 2).



Scheme 1. Synthesis route for new hybrids **5**–**8**.

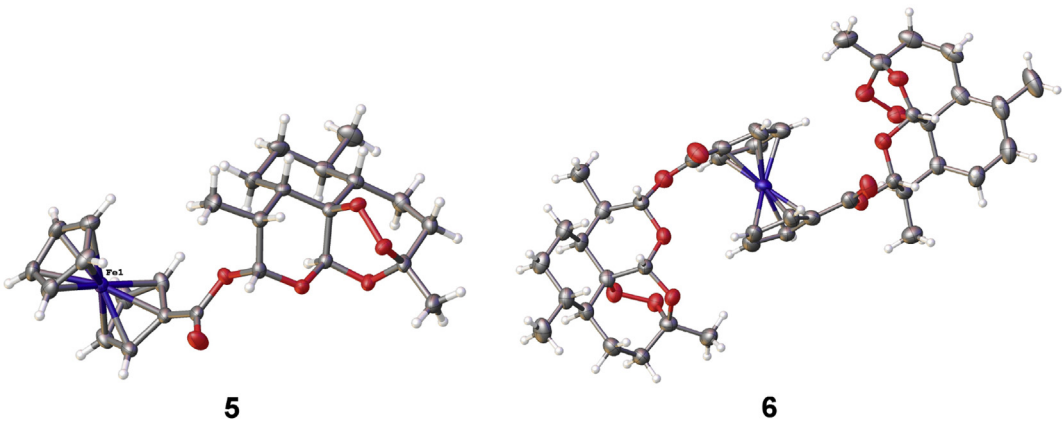


Fig. 3. X-ray crystal structure of 1,2,4-trioxane-ferrocene hybrid **5** and 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **6**.

2.2.3. Antiplasmodial activity

Furthermore, the antimalarial activity of new 1,2,4-trioxane-ferrocene hybrids **4**–**8** was evaluated and therefore, IC_{50} values on *P. falciparum* 3D7 growth *in vitro* were determined using chloroquine and DHA (**10**) as controls (Table 3).

Ferrocene monocarboxylic acid (**9**) did not exhibit any significant antimalarial activity (IC_{50} of 91 ± (9.7) μM). But if ferrocene derivatives are applied as linkers or subunits in 1,2,4-trioxane hybrids, like in case of compounds **4**–**8**, the resulting hybrids possess anti-plasmodial activity (IC_{50} of 7.2–30.2 nM). The activities are in a low nanomolar range comparable to that of the parent compound DHA (IC_{50} of 2.4 ± (0.4) nM). Interestingly, 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **6** (IC_{50} of 7.2 ± (1.2) nM) is more and 1,2,4-trioxane-ferrocene hybrids **4** and **5** similarly active (IC_{50} of 8.6± (1.9) and 13.4 ± (3.4) nM, respectively) as chloroquine (IC_{50} of 9.8 ± (2.8) nM) against *P. falciparum* 3D7 parasites.

Ferrocene as a fragment in the four new 1,2,4-trioxane-ferrocene hybrids **4**–**8** did not enhance the antimalaria activity of the 1,2,4-trioxane partners. This is in accordance to the findings by Delhaes et al. [69] and Bellot et al. [70], who also showed that their

Table 1
IC_{50} values for doxorubicin, artemisinin (**1**), dihydroartemisinin (**10**) and hybrids **5**–**8** in sensitive wild-type CCRF-CEM and multidrug resistant P-glycoprotein-overexpressing CEM/ADR5000 cells.

Compound	Molecular weight	CCRF-CEM IC_{50} (μM)	CEM/ADR5000 IC_{50} (μM)
Doxorubicin[a]	579.98	0.009	23.27
1	282.34	36.90 ± (6.90)	26.90 ± (4.40)
9[b]	230.04	–	–
10	284.35	0.48 ± (0.27)	0.68 ± (0.32)
4[c]	538.45	0.25 ± (0.14)	0.57 ± (0.22)
5	496.37	0.13 ± (0.04)	0.53 ± (0.33)
6	806.72	0.07 ± (0.06)	1.80 ± (0.46)
7	818.81	0.01 ± (0.01)	1.96 ± (0.66)
8	890.88	0.08 ± (0.03)	8.20 ± (4.43)

[a] IC_{50} values for both cell lines have been previously reported [88].
[b] Not active.
[c] IC_{50} values for both cell lines have been previously reported [71].

Table 2
IC_{50} values of anti-HCMV activity (AD169-GFP) displayed in virus-infected HFFs: ganciclovir, artemisinin (**1**), ferrocene monocarboxylic acid (**9**), dihydroartemsinin (**10**), and hybrids **4**–**8**.

Compound	Molecular weight	HCMV IC_{50} (μM)
Ganciclovir[a]	255.23	2.6 ± 0.5
Artesunic acid[a]	384.42	3.8 ± 0.4
1[b]	282.34	>10
9	230.04	>10
10[b]	284.35	>10
4	538.45	4.66 ± 2.07
5	496.37	0.16 ± 0.06
6	806.72	0.46 ± 0.05
7[c]	818.81	0.11 ± 0.01
8	890.88	>10

[a] IC_{50} values have been previously reported [91].
[b] IC_{50} value has been previously reported [92].
[c] Microscopically detectable cytotoxicity approx. 50% at 1 μM.

Table 3
IC_{50} values for chloroquine, ferrocene monocarboxylic acid (**9**), dihydroartemsinin (**10**) and hybrids **4**–**8** tested against *P. falciparum* 3D7 parasites.

Compound	Molecular weight	3D7 IC_{50} (nM)
Chloroquine	319.87	9.8 ± (2.8)
9	230.04	91 μM ± (9.7)
10	284.35	2.4 ± (0.4)
4	538.45	8.6 ± (1.9)
5	496.37	13.4 ± (3.4)
6	806.72	7.2 ± (1.2)
7	818.81	30.2 ± (4.2)
8	890.88	29.6 ± (4.2)

1,2,4-trioxane-ferrocene compounds were not able to surpass the activities of artemsinin (**1**) and dihydroartemisinin (**10**), respectively.

The IC_{50} values of the hybrid molecules tested were clearly above that of DHA (**10**) (2.4 nM) and ranged from 7.2 to 30.2 nM. These data imply that the iron center in the ferrocene part does not support the activation of the endoperoxide moiety that has been reported to play a crucial role in 1,2,4-trioxane antimalaria activity.

However, the physical proximity of the ferrocene part to the peroxide group/s seems to influence in most cases (except for hybrid **4**) the antiplasmodial activity of the hybrids. The compounds, in which the ferrocene moiety was in closer physical proximity to the peroxide group/s, showed a higher inhibitory potential: the most active being hybrid **6** (IC_{50} of 7.2 nM) followed by hybrid **5** (IC_{50} of 13.4 nM). In hybrid **7** (IC_{50} of 30.2 nM) and **8** (IC_{50} of 29.6 nM), the compounds with a lower inhibitory potential, the endoperoxide groups and the ferrocene moiety have a larger physical distance.

3. Conclusion

This study presents the successful preparation of new 1,2,4-trioxane-ferrocene hybrids **5**–**8** from ferrocene monocarboxylic acid (**9**), ferrocene dicarboxylic acid (**12**), DHA or DHA-derived precursors. Hybrids **5**–**8** were tested for their activity against two leukemia cells lines (the CCRF-CEM and the multidrug-resistant CEM/ADR5000 cell lines), against HCMV and against *P. falciparum 3D7* parasites. All four hybrids **5**–**8** were more active against CCRF-CEM cells ($IC_{50} \leq 0.13$ μM) than their parent compound DHA (IC_{50} of 0.48 μM). Regarding the multidrug-resistant CEM/ADR5000 cells, 1,2,4-trioxane-ferrocene hybrid **5** (IC_{50} of 0.53 μM) showed to be more potent than DHA (IC_{50} of 0.68 μM). Considering also compound **4** (IC_{50} of 0.25 μM), previously published by our group [71], a tendency is that hybrids with two 1,2,4-trioxane units (like compounds **6** and **8** with IC_{50} values of 0.07 and 0.08 μM, respectively) were at least 1.8-fold as active as the corresponding hybrids **4** and **5** with only one trioxane moiety.

On the contrary, the activity of hybrids **5** (IC_{50} of 0.53 μM) and **4** (IC_{50} of 0.57 μM) against the CEM/ADR5000 cells was at least 3-fold higher than the one of compounds **6** (IC_{50} of 1.80 μM) and hybrids **8** (8.20 μM). Besides 1,2,4-trioxane-ferrocene-1,2,4-trioxane hybrid **8** all new hybrids showed high activity against HCMV with IC_{50} values lower than 0.5 μM. Thereby, all three hybrids **5**–**7** surpassed their parent compound DHA with its IC_{50} of more than 10 μM and the standard drug ganciclovir (IC_{50} of 2.6 μM). As in the case of their anticancer and antiviral efficacy, 1,2,4-trioxane ferrocenes are promising antimalarial compounds, and while they did not exceed their parent compounds in their antimalarial potential, their activity was still in the nanomolar range (between 7.2 nM and 30.2 nM for all hybrids).

Summarizing, this study proves that trioxane-ferrocenes are an impressive example for the successful application of the concept of hybridization and still calls for further investigation to receive more structure-activity relationship information.

4. Experimental section

4.1. Chemistry

4.1.1. 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-hybrid **8**, were reprecipitated from DCM in *n*-hexane to yield a pure compound for elemental analysis and biological tests. Hybrid **8** was reprecipitated from

EtOAc in pentane. DCM was dried initially over $CaCl_2$ and then distilled from P_2O_5. THF was dried initially over KOH. Afterwards, THF was distilled from sodium/benzophenone. All 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 aluminium 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). All products were dried in high-vacuum (10^{-3} mbar). ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance or JEOL JNM GX 400 spectrometer operating at 300 MHz respectively 400 MHz. ESI Mass spectra were recorded on 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 Elementar vario MICRO cube machine, is within ±0.40% of the calculated values confirming a purity of >95%. DHA was obtained from ABCR (Karlsruhe, Germany).

Di-1,2,4-trioxane derived alcohol **11**: $R_f = 0.27$ (hexanes/EtOAc 6:4, phosphomolybdic acid); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 0.81–1.01$ (m, 8H), 0.93 (d, 6H, $J = 5.7$ Hz), 1.15–1.47 (m, 14H), 1.52–1.67 (m, 8H), 1.71–1.81 (m, 2H), 1.83–2.05 (m, 5H), 2.22–2.36 (m, 2H), 2.50–2.70 (m, 2H), 3.14 (dd, 1H, $J = 7.8$ Hz, $J = 6.0$ Hz), 3.57–3.66 (m, 1H), 3.72–3.81 (m, 1H), 4.31 (q, 1H, $J = 6.0$ Hz), 4.37–4.46 (m, 1H), 5.31 (s, 1H), 5.32 (s, 1H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 12.66, 12.92, 20.12, 20.17, 24.69, 24.72, 24.83 (2×), 25.91, 25.97, 30.65, 30.74 (2×), 31.21, 34.37, 36.51 (2×), 37.43, 37.46, 37.66, 44.00, 44.16, 52.06, 52.19, 65.12, 71.32, 73.90, 81.11 (2×), 89.20, 89.44, 103.00, 103.14 ppm; MS (ESI): $m/z = 607$ ($[M+H]^+$), 629 ($[M+Na]^+$); HRMS (ESI): calcd. for $[C_{34}H_{55}O_9]^+$: 607.3841; found: 607.3841; Anal. calcd. for $C_{34}H_{54}O_9$: C, 67.30; H, 8.97; found: C, 67.66; H, 9.07.

1,2,4-trioxane-derived alcohol **14**: $R_f = 0.16$ (PE/EtOAc 7:3, phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.85$ (d, $J = 7.5$ Hz, 3H), 0.94 (d, $J = 6.0$ Hz, 3H), 0.95–0.99 (m, 1H), 1.19–2.05 (m, 16H), 2.26–2.35 (m, 1H), 2.64 (sex, $J = 6.8$ Hz, 1H), 3.61–3.75 (m, 2H), 4.17–4.24 (m, 1H), 5.31 (s, 1H) 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): 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.

4.1.2. General procedure for the synthesis of hybrids **5** and **7**

A solution of alcohol **10** or **11** and ferrocene monocarboxylic acid (**9**) in dry THF was cooled to 0 °C. Then, PPh_3 and DIAD were added under N_2. The resulting reaction mixture was slowly warmed to rt and stirred o/n. Afterwards, the solvent was removed and the crude product was purified by column chromatography. Ester **5** or **7** was obtained as an orange solid. After reprecipitation from DCM in *n*-hexane, esters **5** and **7** were received as dark orange crystals, which were used for Elemental Analysis and biological tests.

4.1.3. Hybrid **5**

DHA (**10**) (100 mg, 0.35 mmol, 1.0 eq), ferrocene monocarboxylic acid (**9**) (89.0 mg, 0.39 mmol, 1.1 eq), THF (2.7 mL), PPh_3 (92.0 mg, 0.35 mmol, 1.0 eq), DIAD (69.0 μl, 71.1 mg, 0.35 mmol, 1.0 eq). Column conditions: PE/EtOAc 4:1 and PE/EtOAc 15:1 → PE/EtOAc 9:1. Yield: 41.0 mg, 0.08 mmol, 23%. $R_f = 0.14$ (PE/EtOAc 4:1, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.95$ (d, $J = 7.4$ Hz, 3H), 1.01 (d, $J = 6.2$ Hz, 3H), 1.01–1.11 (m, 1H), 1.22–1.53 (m, 6H), 1.56–1.64 (m, 1H), 1.76–2.06 (m, 5H); 2.37 (td, $J = 13.4$, 4.0 Hz, 1H), 2.82–2.91 (m, 1H), 4.20 (s, 5H), 4.42 (t, $J = 2.0$ Hz, 2H), 4.73–4.78 (m, 2H), 5.52 (s, 1H), 6.36 (d, $J = 3.5$ Hz, 1H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.6, 20.3, 24.4, 24.6, 25.9, 29.8, 34.6, 36.2, 37.8, 44.0, 52.5, 69.8, 70.1, 70.3, 70.8, 71.5, 71.6, 80.6, 88.7, 94.4, 104.4, 170.5 ppm; MS (ESI): $m/z = 519$ ($[M+Na]^+$); HRMS (ESI): calcd. for $[C_{26}H_{32}FeNaO_6]^+$ 519.1441, found 519.1452; FT-IR (ATR): $\tilde{\nu}$ = 3105 (w), 3087 (w), 2957 (m), 2939 (m), 2926 (m), 2843 (w), 2350 (w), 2183 (w), 2149 (w), 2042 (w), 1955 (w), 1707 (s), 1453 (m), 1377 (m), 1268 (s), 1202 (m), 1138 (m), 1092 (s), 1020 (m), 975 (s), 962 (m), 905 (s), 856 (s), 825 (s), 781 (m), 650 (w), 590 (w), 540 (m), 486 (s), 409 (w) cm^{-1}; Anal. calcd. for: $C_{26}H_{32}FeO_6$: C, 62.91; H, 6.50; Found: C, 62.62; H, 6.55.

4.1.4. Hybrid **7**

Di-1,2,4-trioxane-derived alcohol **11** (50.0 mg, 0.08 mmol, 1.0 eq), ferrocene monocarboxylic acid (**9**) (22.8 mg, 0.10 mmol, 1.2 eq), PPh_3 (20.9 mg, 0.08 mmol, 1.2 eq) and DIAD (19.4 μL, 19.9 mg, 0.10 mmol, 1.2 eq). Column conditions: PE/EtOAc 5:1. Yield: 45.0 mg, 0.06 mmol, 67%. $R_f = 0.63$ (PE/EtOAc 7:3), UV and phosphomolybdic acid; ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 0.80–1.00$ (m, 14H), 1.13–1.69 (m, 20H), 1.70–1.93 (m, 6H), 1.94–2.06 (m, 2H), 2.21–2.38 (m, 3H), 2.51–2.74 (m, 2H), 4.22 (s, 5H), 4.28–4.40 (m, 5H), 4.41–4.50 (m, 1H), 4.75–4.85 (m, 2H), 5.31 (s, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 12.7, 13.0, 20.0, 20.1, 24.6, 24.7, 24.8, 24.9, 26.0 (2×), 30.1, 30.5, 30.6, 31.1, 33.6, 34.4 (2×), 36.6, 36.7, 37.4, 41.2, 44.1, 44.4, 52.1, 52.3, 66.5, 69.9 (2×), 70.0 (2), 70.9, 73.0, 77.2, 81.1, 81.2, 89.0, 89.6, 102.9, 103.1, 171.7 ppm; MS (ESI): m/z = 818 $[M]^+$, 841 ($[M+Na]^+$), 857 ($[M+K]^+$); HRMS (ESI): calcd. for $[C_{45}H_{62}FeO_{10}]^+$: 818.3688; found: 818.3695; FT-IR (ATR): $\tilde{\nu}$ = 3102 (w), 2936 (m), 2873 (w), 2241 (w), 2157 (w), 1709 (m), 1455 (m), 1375 (m), 1274 (s), 1203 (w), 1137 (s), 1105 (s), 1043 (m), 1006 (s), 930 (m), 911 (m), 880 (m), 843 (m), 820 (m), 772 (w), 729 (m), 646 (w), 592 (w), 550 (w), 527 (w), 502 (m), 486 (m) cm^{-1}; Anal. calcd. for $C_{45}H_{62}FeO_{10} \cdot 0.45H_2O \cdot 0.082\ CHCl_3$: C, 64.71; H, 7.59; Found: C, 64.71; H, 7.52.

4.1.5. General procedure for the synthesis of hybrids **6** and **8**

In an evacuated flask ferrocene dicarboxylic acid chloride (**13**) and alcohol **10** or **14** were dissolved in anhydrous DCM under N_2. Follow-up, DMAP was added. The solution was stirred at rt o/n. After the removal of the solvent under reduced pressure, the dark brown solid was purified by column chromatography. To obtain a pure compound for Elemental Analysis and biological tests, dimers **6** and **8** were reprecipitated.

4.1.6. Hybrid **6**

DHA (**10**) (91.4 mg, 0.32 mmol, 2.0 eq), ferrocene dicarboxylic acid chloride (**9**) (50.0 mg, 0.16 mmol, 1.0 eq), DCM (7.0 mL), DMAP (78.6 mg, 0.64 mmol, 4.0 eq). Column conditions: PE/EtOAc 1:1. Yield: 65%, 84.9 mg, 0.11 mmol. Reprecipitation: DCM in *n*-hexane. $R_f = 0.91$ (PE/EtOAc 1:1, UV and phosphomolybdic acid); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.94$ (d, $J = 7.3$ Hz, 6H), 0.96 (d, $J = 6.2$ Hz, 6H), 1.02–1.08 (m, 2H), 1.21–1.38 (m, 8H), 1.40–1.50 (m, 4H), 1.62–1.90 (m, 10H), 1.97–2.07 (m, 2H), 2.36 (td, $J = 14.0$, 3.9 Hz, 2H), 2.61–2.73 (m, 2H), 4.50–4.65 (m, 4H), 4.93–5.01 (m, 4H), 5.47 (s, 2H), 5.83 (d, J = 9.8 Hz, 2H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.2, 20.2, 22.0, 24.6, 25.8, 31.4, 34.1, 36.2, 37.3, 45.3, 51.6, 71.4, 71.8, 72.1, 74.5, 74.6, 80.2, 91.2, 92.0, 104.3, 169.9 ppm; MS (ESI): m/z = 806 $[(M)^+]$, 829 ($[M+Na]^+$); HRMS (ESI): calcd. for $[C_{42}H_{54}FeNaO_{12}]^+$ 829.2858, found 829.2850; FT-IR (ATR): $\tilde{\nu}$ = 3115 (w), 2949 (m), 2921 (m), 2863 (m), 2362 (w), 2155 (w), 2025 (w), 1990 (w), 1720 (s), 1453 (m), 1399 (w), 1372 (m), 1268 (s), 1204 (m), 1135 (s), 1092 (s), 1029 (s), 1011(s), 944 (m), 924 (m), 918 (m), 875 (s), 868 (m), 827 (s), 790 (w), 762 (s), 692 (w), 606 (w), 577 (m), 502 (s), 438 (m) cm^{-1}; Anal. Calcd. for: $C_{42}H_{54}FeO_{12}$: C, 62.53; H, 6.75; Found: C, 62.53; H, 6.81.

4.1.7. Hybrid **8**

1,2,4-trioxane-derived alcohol **14** (52.5 mg, 0.16 mmol, 2.0 eq), ferrocene dicarboxylic acid chloride (**13**) (25.0 mg, 0.08 mmol, 1.0 eq), DCM (3.5 mL), DMAP (39.3 mg, 0.32 mmol, 4.0 eq), Column conditions: hexanes/EtOAc 1:4. Yield: 71% yield (51.2 mg, 0.06 mmol). Reprecipitation: EtOAc in pentane. $R_f = 0.85$ (PE/EtOAc 1:2, UV and phosphomolybdic acid); ^{1}H NMR (300 MHz, [D$_6$] acetone): δ = 0.92 (d, J = 7.6 Hz, 6H), 0.94 (d, J = 6.4 Hz, 6H), 0.95–1.06 (m, 2H), 1.13–1.49 (m, 16H), 1.54–1.98 (m, 16H), 2.15–2.31 (m, 2H), 2.48–2.61 (m, 2H), 4.21–4.33 (m, 6H), 4.48 (t, J = 2.0 Hz, 4H), 4.76–4.83 (m, 4H), 5.36 (s, 2H) ppm; ^{13}C NMR (75 MHz, [D$_6$]acetone): δ = 13.01, 20.4, 25.6 (2×), 26.3, 26.9, 27.5, 31.5, 35.3, 37.4, 38.0, 45.1, 53.2, 64.8, 72.2, 73.6, 74.2, 74.4, 81.7, 90.2, 103.2, 170.4 ppm; MS (ESI): m/z = 913 ([M+Na]$^+$); HRMS (ESI): calcd. for [$C_{48}H_{66}FeO_{12}$]$^+$ 890.3899, found 890.3906; FT-IR (ATR): $\tilde{\nu}$ = 3102 (w), 2593 (s), 2873 (m), 2732 (w), 2010 (w), 1705 (s), 1457 (s), 1371 (s), 1272 (s), 1227 (w), 1196 (w), 1139 (s), 1103 (s), 1055 (s), 1018 (s), 945 (m), 937 (m), 876 (m), 821 (m), 773 (m), 733 (s), 703 (m), 629 (w), 599 (w), 545 (m), 483 (s), 434 (w) cm^{-1}; Anal. calcd. for: $C_{48}H_{66}FeO_{12}$: C, 64.71; H, 7.47; Found: C, 64.89; H, 7.62.

4.2. Cytotoxicity studies against CCRF-CEM and CEM/ADR5000 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 [93]. The multidrug resistance profile of CEM/ADR5000 has been reported [94,95].

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 [96]. We described the performance of the assay in detail [97]. 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. HCMV GFP-based replication assay

HCMV GFP-based replication assays were carried out over a duration of seven days (multi-round infection) with primary human foreskin fibroblasts (HFFs) infected with a GFP-expressing recombinant human cytomegalovirus (HCMV AD169-GFP) as described before [89,92]. All data represent mean values of determinations in quadruplicate (HCMV infections performed in duplicate, GFP measurements of total cell lysates performed in duplicate). Processing and evaluation of data was performed by the use of Excel (means and standard deviations).

4.4. Cytotoxicity studies against Plasmodium falciparum 3D7 strains

4.4.1. P. falciparum culture

P. falciparum 3D7 parasites were cultured in type A-positive human erythrocytes at a hematocrit of 5% in RPMI 1640 supplemented with 25 mM HEPES, 0.1 mM hypoxanthine, 50 μg/ml gentamycin and 0.5% albumax I. Cultures were incubated at 37 °C under controlled atmospheric conditions of 5% O_2, 3% CO_2, and 92% N_2 at 95% relative humidity.

4.4.2. In vitro antimalarial activity assay

Cultures used in cell proliferation assays were synchronized by sorbitol treatment [98]. Concentrations to inhibit parasite growth by 50% (IC_{50}) were determined using the SYBR Green I malaria drug sensitivity assay [99]. 100 μl aliquots of a cell suspension containing ring stages at a parasitemia of 0.2% and a hematocrit of 2% were added to the wells of 96-well microtiter plates. Plates were incubated for 72 h in the presence of different drug concentrations. Subsequently, cells of each well were lysed with 100 μl lysis buffer (40 mM Tris, pH 7.5, 10 mM EDTA, 0.02% saponin, 0.08% Triton X-100) containing 8.3 μM SYBR green. Plates were incubated for 1 h in the dark at room temperature under constant mixing before fluorescence (excitation wavelength 485 nm; emission wavelength >520 nm) was determined using a microtiter plate fluorescence reader (Victor X4; Perkin Elmer). Drugs were serially diluted 1:3, with initial drug concentrations being 243 nM for chloroquine, artesunate and its derivates, 81 nM for dihydroartemisinin (**10**) and it derivates and 100 μM for ferrocene monocarboxylic acid (**9**). Each drug concentration was examined in triplicate and repeated at least three times. Uninfected erythrocytes (hematocrit 2%) and infected erythrocytes without drug served as controls and were investigated in parallel. Percent growth was calculated as described by Beez [100]. Data were analyzed using the SigmaPlot (version 12.0; Hill function, three parameters) and Sigma Stat programs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.053.

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