Bioorganic & Medicinal Chemistry Letters 20 (2010) 563–566

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The comparative antimalarial properties of weak base and neutral synthetic ozonides

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ARTICLE INFO

Article history:
Received 28 October 2009
Accepted 18 November 2009
Available online 22 November 2009

Keywords:
1,2,4-Trioxolanes
Secondary ozonides
Antimalarial
Peroxides
Artemisinin

ABSTRACT

Thirty-three *N*-acyl 1,2,4-dispiro trioxolanes (secondary ozonides) were synthesized. For these ozonides, weak base functional groups were not required for high antimalarial potency against *Plasmodium falciparum* in vitro, but were necessary for high antimalarial efficacy in *Plasmodium berghei*-infected mice. A wide range of Log $P/D_{pH\ 7.4}$ values were tolerated, although more lipophilic ozonides tended to be less metabolically stable.

First generation antimalarial peroxides (Fig. 1) such as the semisynthetic artemisinins artemether, artesunate[1] and artelinic acid,[2] and the synthetic peroxides arteflene[3] and fenozan B07[4] are all neutral or acidic compounds. More recently, the discovery of artemisone,[5] trioxaquine PA1103/SAR116242[6] and OZ277[7] (Fig. 2) illustrate that both semisynthetic artemisinins[8] and synthetic peroxides[9,10] with weak base functional groups have high antimalarial efficacy, although it remains to be seen whether any of these will ultimately be registered as a new antimalarial drug.

Amino ozonide OZ209 (**1**)[11] (Scheme 1) has substantially better antimalarial efficacy than OZ277, but it inhibits several CYP450 isoforms and has a less than ideal therapeutic index.[7] In a quest to identify a derivative of **1** with high oral activity, good biopharmaceutical properties, and low toxicity, we recognized that the primary amine functional group of **1** was ideally suited to prepare a wide range of acylated derivatives (Scheme 1) including neutral/acidic amides **2** (Table 1), weak base amides **3** (Table 2), oxamides **4** (Table 3), aryl amides **5** (Table 4), and ureas **6** (Table 5). In this Letter, we describe the synthesis[12] and antimalarial properties of these five types of ozonides. Metabolism and pharmacokinetic data

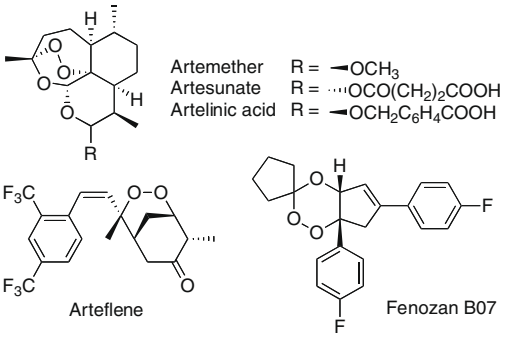

Figure 1. First generation antimalarial peroxides.

are presented for selected compounds. For these ozonides, the data reveal that acidic functional groups decrease antimalarial activity and that weak base functional groups are not required for high antimalarial potency against *Plasmodium falciparum* in vitro, but are necessary for high antimalarial efficacy in *Plasmodium berghei*-infected mice.

Ozonide **1** and ozonide hydantoin **2e** were obtained as previously described.[13] Ozonide amides **2a** (45%), **5b** (60%), and **5c**

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doi:10.1016/j.bmcl.2009.11.088

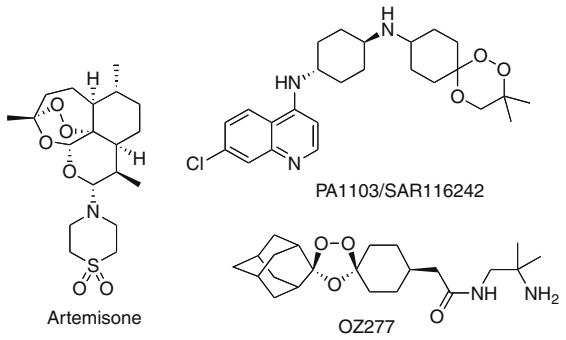
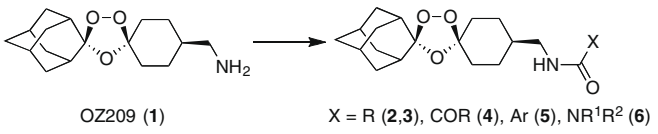
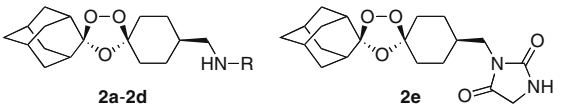


Figure 2. Weak base antimalarial peroxides.



Scheme 1. Synthesis of ozonides **2**–**6** from **1**.

Table 1
Lipophilicity, metabolic stability, and activity of neutral and acidic carboxamide and imide ozonides against *P. falciparum* in vitro and *P. berghei* in vivo (1 × 10 mg/kg po)



Compd	R	Log *P*/ $D_{pH\ 7.4}$[a]	IC_{50}[b] (ng/ml) K1/NF54	Activity[c] (%)	ER[d]
2a	$COCH_3$	4.7	0.63/0.84	98	ND
2b	$COCH_2NHCOH$	4.1	0.90/1.5	98	ND
2c	$COCH_2CH_2COOH$	1.9*D*	71/97	<40	ND
2d	$COCH_2SO_2CH_3$	3.8	1.1/2.2	99.2	ND
2e	NH	4.3	1.0/2.7	92	ND
1[e]	H	2.6*D*	0.39/0.42	>99.9	0.24
AS[f]	—	3.5*D*	1.3/1.6	67	0.43[g]

[a] Calculated as previously described[7,21] Log $D_{pH\ 7.4}$ denotes the octanol/buffer partition coefficient at pH 7.4 which is relevant for the ionizable analogues.
[b] Mean from $n = 2$–3. Individual measurements differed by less than 50%.
[c] Groups of five *P. berghei*-infected NMRI mice were treated orally one day post-infection with trioxolanes dissolved or suspended in SSV. Antimalarial activity was measured by percent reduction in parasitemia on day three post-infection. Individual measurements differed by less than 10%.
[d] Predicted hepatic extraction ratios (ER) using human microsomes.[16]
[e] Data from Tang et al.[11]
[f] Data from Dong et al.[20]
[g] Value for dihydroartemisinin (DHA), the primary metabolite of artesunate (AS).

(100%) were obtained by reaction of **1** with the requisite acid chlorides and 1 equiv of Et_3N in CH_2Cl_2 at 0 °C. Ozonide amide **2c** (41%) was obtained by reaction of **1** with succinic anhydride and 1 equiv of Et_3N in CH_2Cl_2 at 0 °C. Ozonide amides **2b** (71%), **3b** (81%), **3c** (62%), **3d** (79%), **3i** (75%), **5a** (23%), **5d** (83%), **5e** (15%), and **5f** (81%), and oxamide **4a** (88%) were obtained by reaction of **1** with the corresponding carboxylic acids and 1.5 M equiv of EDCI and HOBt in DMF at 25 °C. Using these same conditions, reaction of **1** with (methylthio)acetic acid and the protected amino acids *N*-phthaloylglycine and Fmoc-β-alanine followed by *m*-CPBA oxidation and deprotection with hydrazine and 10% piperidine, respectively, afforded ozonide amides **2d** (45% overall) **3a** (59% overall) and **3e** (41% overall). Ozonide amides **3f** (65%), **3g** (87%), **3h** (98%), and **3j** (72%) were obtained by treatment of the bromoacetamide of **1** with the requisite primary or secondary amines in acetonitrile at 0–25 °C. The latter intermediate was obtained in quantitative yield by treatment of **1** with bromoacetyl chloride in 8% aq $NaOH/CH_2Cl_2$ at −10 °C. Ozonide oxamides **4c** (93%), **4d** (32%), **4e** (37%), and **4f** (47%) were obtained by treatment of the methyloxamate of **1** with the requisite primary or secondary amines at 25 °C. The latter intermediate was obtained in 96% yield by treatment of **1** with methyloxalyl chloride and 1 equiv of Et_3N in CH_2Cl_2 at 0–25 °C. Hydrolysis of the methyloxamate of **1** with 15% aq KOH followed by acidification with 1 N HCl afforded carboxy oxamide ozonide **4b** (72%). Ozonide urea **6a** (74%) was obtained by treatment of **1** with KOCN in a HOAc/pyridine buffer. Ozonide ureas **6c** (74%), **6d** (71%), and **6f** (74%) were obtained by reaction of **1** with the requisite carbamoyl chlorides/Et_3N at 0–25 °C. Ozonide urea **6b** was obtained by reaction of **1** with ethyl isocyanate in the presence of Et_3N at 0–25 °C. Ozonide urea **6e** (71%) was obtained by reaction of piperazine with the 4-nitrophenylcarbamate of **1**. The latter intermediate was obtained in 59% yield by treatment of **1** with 1 equiv each of 4-nitrophenyl chloroformate and pyridine in refluxing CH_2Cl_2 according the method of Liu et al.[14]

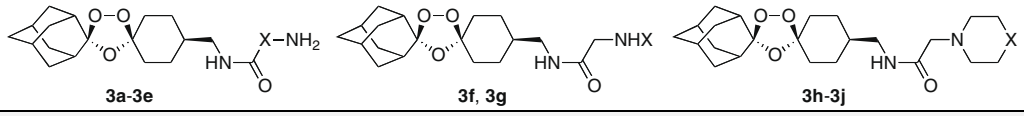
In vitro and in vivo antimalarial activities[7] were measured using the chloroquine-resistant K1 and chloroquine-sensitive NF54 strains of *P. falciparum*, and *P. berghei*-infected mice, respectively. In vivo data were determined using single 10 mg/kg oral doses of the trioxolanes administered on day 1 post-infection in a non-solubilizing, standard suspension vehicle (SSV) formulation comprising 0.5% w/v carboxymethyl cellulose, 0.5% v/v benzyl alcohol, 0.4% v/v Tween 80, and 0.9% w/v sodium chloride in water.

Several overarching SAR trends are immediately apparent from the data in Tables 1–5. First, with the exception of the weakly potent acidic ozonides **2c** and **4b** (IC_{50s} >50 ng/mL), each of the neutral and weak base ozonides had IC_{50} values in the relatively narrow range of 0.2–5 ng/mL; these values are within an order of magnitude to those for artesunate and **1**. This narrow IC_{50} range indicates that the *N*-acyl substructure plays only a minor role in the in vitro potency of these ozonides. Second, only ozonides with weak base functional groups had high antimalarial efficacy ($\geqslant$99.9%) in *P. berghei*-infected mice. For example, the neutral ozonides **2a**, **2b**, **2d**, **2e** (Table 1), **4a** (Table 3), and **6a**–**6d** (Table 5) with in vivo activities ranging from 92% to 99.5% were more than 5–80-fold less effective than **1** which had an activity of >99.9%.[15] Third, there was no correlation between calculated Log *P* (for the neutral compounds) or Log $D_{pH\ 7.4}$ (for the ionizable compounds) values and in vitro IC_{50} values or in vivo activities for these ozonides. Fourth, where measured, the predicted hepatic extraction ratios (ER) based on degradation rates measured in human liver microsomes[16] did correlate (0.66, $p = 0.0098$) with the calculated Log $P/D_{pH\ 7.4}$ values. Fifth, the most metabolically stable (ER $\leqslant$0.29) ozonides were primary (**1**, **3a**, **5f**) or secondary (**6e**) amines, whereas neutral (**6c**), pyridine (**5b**, **5c**), and tertiary (**6f**) amine ozonides were rapidly metabolized (ER $\geqslant$0.7).

We selected seven of the most promising ozonides with activity $\geqslant$99.9% and ER <0.5 to assess whether they could cure *P. berghei*-infected mice using multiple doses. In this experiment, the ozonides were administered at 3 × 3 mg/kg oral doses on days +1, +2, and +3 post-infection (Table 6). With this multiple dose administration, **4e** cured 1/5 of the infected mice, and **4f** and **6e** cured 2/5 of the infected mice. In contrast, ozonide control **1** and the antimalarial drugs artesunate (AS), chloroquine (CQ), and mefloquine (MF) did not cure any of the mice.[7] Although ozonides **3a**, **3b**, **3e**, and **4c** were also not curative at this dose, they did increase survival time compared to the untreated control and were as or more effective than **1**, AS and CQ in this respect. Of these, **3e** and **4c** were as or more effective than MF at increasing survival time.

Selected trioxolanes were administered intravenously (IV) and orally (PO) to rats[17] and pharmacokinetic data for **1**, **3a**, **4f**, **6e**, and dihydroartemisinin (DHA) are shown in Table 7. The data

Table 2
Lipophilicity, metabolic stability, and activity of amino amide ozonides against *P. falciparum* in vitro and *P. berghei* in vivo (1 × 10 mg/kg po)

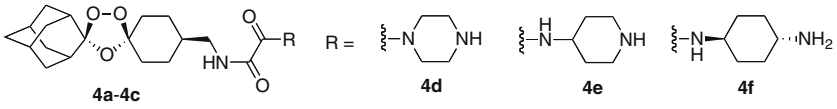


3a-3e 3f, 3g 3h-3j

Compd	X	Log $D_{pH\ 7.4}$	IC_{50} (ng/ml) K1/NF54	Activity (%)	ER
3a[a]	CH_2	3.4	0.36/0.29	99.9	<0.29
3b	$C(CH_3)_2$	3.7	0.50/0.36	99.9	0.32
3c	$C(CH_2)_4$	3.8	0.33/0.36	99.9	0.76
3d	$C(CH_2)_5$	4.8	0.24/0.44	99.9	0.82
3e[b]	CH_2CH_2	2.6	1.4/0.91	99.9	0.38
3f[b]	$CH(CH_2)_2$	3.4	0.39/1.2	99.6	ND
3g[b]	$CH_2CH(CH_2)_2$	4.1	0.36/1.2	99.5	ND
3h[c]	O	5.0	0.88/1.8	99	ND
3i[b]	SO_2	4.5	4.6/5.5	86	ND
3j[d]	NH	3.7	0.59/1.4	99.7	ND

[a] Mesylate salt.
[b] Tosylate salt.
[c] Hydrogen maleate salt.
[d] Ditosylate salt.

Table 3
Lipophilicity, metabolic stability, and activity of oxamide ozonides against *P. falciparum* in vitro and *P. berghei* in vivo (1 × 10 mg/kg po)

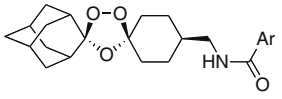


4a-4c 4d 4e 4f

Compd	R	Log *P*/$D_{pH\ 7.4}$	IC_{50} (ng/ml) K1/NF54	Activity (%)	ER
4a	NH_2	4.2	0.44/0.71	99.5	0.62
4b	OH	0.81*D*	58/59	<40	ND
4c[a]	$NHCH_2C(CH_3)_2NH_2$	3.6*D*	0.40/0.54	99.9	0.43
4d[a]	—	3.5*D*	0.69/0.54	99.9	ND
4e[a]	—	2.2*D*	0.56/0.79	>99.9	0.45
4f[b]	—	2.2*D*	0.70/1.3	99.9	0.48

[a] Mesylate salt.
[b] Tosylate salt.

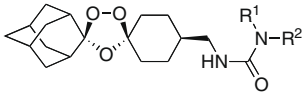
Table 4
Lipophilicity, metabolic stability, and activity of heteroaryl amide ozonides against *P. falciparum* in vitro and *P. berghei* in vivo (1 × 10 mg/kg po)



Compd	Ar	Log $D_{pH\ 7.4}$	IC_{50} (ng/ml) K1/NF54	Activity (%)	ER
5a	2-Pyridyl	5.6	1.6/2.3	98	ND
5b	3-Pyridyl	5.8	0.97/1.0	99.6	0.83
5c	4-Pyridyl	5.4	2.2/1.1	99.5	0.71
5d	2-Pyridizinyl	5.2	1.4/1.1	99.6	ND
5e[a]	4-Imidazolyl	5.3	0.84/1.1	99.5	ND
5f	5-(3-Amino-1,2,4-triazolyl)	4.2	1.7/2.0	99.8	<0.29

[a] Mesylate salt.

Table 5
Lipophilicity, metabolic stability, and activity of urea ozonides against *P. falciparum* in vitro and *P. berghei* in vivo (1 × 10 mg/kg po)



Compd	R^1, R^2	Log *P*/$D_{pH\ 7.4}$	IC_{50} (ng/ml) K1/NF54	Activity (%)	ER
6a	H, H	4.6	0.56/1.6	98	ND
6b	H, CH_2CH_3	5.3	0.52/0.72	98	ND
6c	CH_3, CH_3	4.8	1.1/1.5	99.4	0.84
6d	$CH_2CH_2OCH_2CH_2$	4.1	1.1/1.3	98	ND
6e[a]	$CH_2CH_2NHCH_2CH_2$	3.3*D*	0.43/0.68	>99.9	0.27
6f	$CH_2CH_2NCH_3CH_2CH_2$	4.5*D*	0.20/0.34	99.9	0.80

[a] Tosylate salt.

indicated that all of these compounds had oral bioavailabilities exceeding 30% at the doses administered, and half-lives varied from about 30 min for **3a** (similar to that for DHA) to 60–90 min for **1**, **4f** and **6e**. For **4f** and **6e**, the longer half lives are likely due to lower clearance, whereas for **1**, the high volume of distribution is probably responsible for the longer half life. For **1** and **3a**, the blood clearance values were significantly higher than hepatic blood flow in a rat that suggests that other non-hepatic mechanisms contribute to the clearance of these compounds. Inhibition assays[18] with CYP3A4, 2C9, and 2D6 revealed that **4f** had no effect on enzyme activities at concentrations up to 50 μM. Similarly, **1** had no effect on CYP2D6 isoform, but it did inhibit CYP3A4, CYP2C9, and CYP2C19 isoforms with IC_{50s} of 9.7, 17. and 0.11 μM.

Preliminary 5-day toxicological experiments in male rats given daily oral doses of **4f** indicated that high plasma levels were achieved—3700 and 5700 ng/mL on day 5 at 100 and 300 mg/kg/day. Even at the high dose, this ozonide, like artesunate, was minimally toxic with the liver, lymphatic organs, and possibly the kidneys, as target organs. No signs of neurotoxicity were seen. Findings tended to be reversible at the end of a 1-week recovery period. The overall toxicity of **4f** was significantly lower than that of **1**.[7]

Table 6
In vivo activity in *P. berghei*-infected mice following three consecutive daily oral doses

Compd	3 × 3 mg/kg po[a]		
	Activity (%)	Survival (days)	Cure[b] (%)
Control	0	6–7	0
3a	>99.9	16.2	0
3b	>99.9	12.4	0
3e	>99.9	20.8	0
4c	>99.9	22.8	0
4e	>99.9	22.4	20
4f	>99.9	25.2	40
6e	>99.9	27.0	40
1[c]	>99.9	12.8	0
AS[c]	70	9.2	0
CQ[c]	99.7	9.4	0
MF[c]	(%)	(%)	0

[a] Groups of five *P. berghei*-infected NMRI mice were treated orally on days +1, +2, and + 3 post-infection with trioxolanes dissolved or suspended in SSV. Antimalarial activity was measured by percent reduction in parasitemia on day 4 post-infection.
[b] No detectable parasites at 30 days post-infection.
[c] Data from Vennerstrom et al.[7]

Table 7
Pharmacokinetic parameters[a] after intravenous and oral administration to rats

Compd	Intravenous administration			Oral administration
	Half-life (min)	Vol of distribution (L/kg)	Blood clearance (mL/min/kg)	Bioavailability (%)
3a	32	8.8	126	76
4f	81	7.9	36	64
6e	62	7.4	35	48
1	90	46	129	31
DHA[b,c]	26	3.0	72	Not dosed PO

[a] Values represent the average of 2–3 determinations.[17]
[b] Dihydroartemisinin (DHA), the primary metabolite of artesunate (AS).
[c] Data from Dong et al.[21]

In summary, data for these ozonides provide guidance for the ongoing design[19] of synthetic peroxide antimalarials. First, acidic functional groups decrease antimalarial potency, consistent with our previous observations.[20] Second, weak base functional groups are not required for high antimalarial potency against *P. falciparum* in vitro, but are essential for high antimalarial efficacy in *P. berghei*-infected mice. Third, the *N*-acyl substructure played only a minor role in the in vitro potency of these ozonides. Fourth, a wide range of Log $P/D_{pH\ 7.4}$ values were tolerated, although more lipophilic ozonides tended to be less stable metabolically. Finally, we identified three new ozonides (**4e**, **4f**, **6e**) with better antimalarial efficacy and ADME profiles than **1**. Ongoing studies will determine the potential of these and other weak base trioxolanes as antimalarial drug development candidates.

Acknowledgment

This investigation received financial support from Medicines for Malaria Venture (MMV).

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15. Activity is defined as percent reduction on day 3 post-infection compared to an untreated control group. For example, a compound with an activity of 99.9% is 10-fold more active than one with an activity of 99.0% and 100-fold more active than one with an activity of 90%.
16. As fully described in Ref. 7, compounds were incubated with human liver microsomes and appropriate co-factors (BD Gentest, Discovery Labware Inc., Woburn, MA) at a substrate concentration of 1–10 μM and a microsomal protein concentration of 0.4 mg/mL. Loss of parent compound was monitored by LC/MS. Extraction ratios were calculated using appropriate scaling factors as previously described by Obach, R. S. *Drug Metab. Dispos.* **1999**, *27*, 1350.
17. In vivo pharmacokinetic studies were conducted in conscious male Sprague Dawley rats. Compounds were dosed by intravenous infusion over 5–10 min through a cannula previously implanted in the jugular vein at doses of 2.5 mg/kg (**4f** and **6e**), 7.5 mg/kg (**1**) or 10 mg/kg (**3a**). The IV formulations consisted of either normal saline (**1**), 10% v/v DMSO in 15 mM pH 3 citrate buffer (**6e**), 10% v/v ethanol in 15 mM pH 4 citrate buffer (**3a**) or 20% v/v propylene glycol, 10% v/v ethanol in 15 mM pH 3 citrate (**4f**). Oral doses (10 mg/kg for **4f** and **6e**, 40 mg/kg **1** and **3a**) were administered by gavage as aqueous suspensions prepared in 0.5% w/v hydroxypropylmethyl cellulose, 0.4% Tween 80, and 0.5% benzyl alcohol. Blood samples were collected via a cannula previously implanted in the carotid artery, and plasma was immediately obtained by centrifugation after which samples were stored at −20 °C. For analysis, samples were thawed, plasma proteins precipitated with acetonitrile, and aliquots of the supernatant analysed by LC/MS. Quantitation was conducted by comparison to the response obtained for calibration standards also prepared in plasma using the same methods. Pharmacokinetic parameters were calculated using non-compartmental methods.
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