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New developments in synthetic peroxidic drugs as artemisinin mimics

Charles W. Jefford

Department of Organic Chemistry, University of Geneva, 1211 Geneva, Switzerland

The present review describes the current status of synthetic cyclic peroxides, trioxanes and trioxolanes that show significant promise as antimalarial drugs because of their artemisinin-like activity. The literature from 1996 onwards is critically surveyed to provide an update on how an age-old, persistent, debilitating and frequently deadly disease could be treated by new, affordable and effective medicines possessing the peroxide pharmacophore. The review is not exhaustive and does not cover recent progress on the lead structure artemisinin and its derivatives. Nevertheless, some mechanistic aspects gleaned from artemisinin that have relevance to synthetic peroxides are discussed.

The advent of antimalarial peroxides

Synthetic cyclic peroxides are commanding increasing attention as potential replacements for traditional antimalarial remedies such as chloroquine and mefloquine, which have lost their efficacy owing to the development of multidrug-resistant mutants of the *Plasmodium* parasite. They also offer a structurally simpler, synthetically accessible alternative to artemisinin (**1**) (Figure 1), the sesquiterpene trioxane found in the common shrub *Artemisia annua*, and its first generation derivatives, as exemplified by artemether (**2**), artesunic (**3**) and artelinic (**4**) acids. In other words, artemisinin, like any other natural substance endowed with promising pharmaceutical properties, should be regarded as a lead to be developed into products of improved performance.

About ten years ago, synthetic trioxanes and peroxides of the so-called second generation of artemisinin were reviewed [1]. Promising candidates were deemed to be the deoxa-artemisinins (**5**, **6**); the tricyclic trioxanes (**7**, **8**), in which three of the rings of the tetracyclic artemisinin skeleton are retained; the spirocyclic tetraoxane (**9**); the cyclopentene-1,2,4-trioxanes (**10**, **11**); and arteflene (**12**), which was developed by Hoffmann-LaRoche as a stable analogue of yingzhaosu A (**13**), but subsequently discontinued as a drug candidate after phase III trials.

More recent reviews have provided a fuller appraisal of candidate peroxides [2–7]. The tricyclic trioxanes (**14**, **15**) retain much of the framework of artemisinin (**1**). Despite its structural similarity, the dimethyl derivative **14** is about ten times less potent *in vitro* than artemisinin (**1**) [2,3]. By contrast, the spirocyclopentyl trioxane **15** is as active as artemisinin (**1**), demonstrating that the cyclopentane ring, like that in the bicyclic trioxanes **10** and **11**, is an essential part of the pharmacophore. The tricyclic trioxanes **16** and **17** can be regarded as partial versions of artemisinin (**1**), in that the lactone ring and the peripheral methyl substituents are missing (Figure 1). The *endo* epimer of the bridgehead methyl compound **16**, in which the methoxy group points inwards, has the same activity *in vitro* as artemisinin (**1**) [8]. When the methoxy substituent has the *exo* orientation, which is a minor molecular alteration, activity falls away ~5–10-fold. The *exo* and *endo* epimers of the bridgehead phenyl analogue **17** behave like those of **16**.

Many derivatives of the tricyclic trioxane core of **16** and **17** were tested (Figure 1). Unsurprisingly, most have activity close to that of artemisinin (**1**) [9]. The *exo* methoxy epimers of the water-soluble carboxylic acid **18** and the hydrophobic fluoro derivative **19** (Figure 1) are as active as artelinic acid (**4**) when tested subcutaneously against *Plasmodium berghei* in the mouse, requiring an effective dose of 8.3–14 mg/kg (per body weight) to decrease parasitemia by 90% (ED_{90}) [2,9].

Finally, the tricyclic trioxanes, although synthesized in great profusion since the first reported examples [8], have not been developed further because their preparation has proved impractical. They did, however, serve a most useful purpose by revealing how molecular structure correlates with radical formation and

Corresponding author: Jefford, C.W. (charles.jefford@chiorg.unige.ch)

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2007.04.009

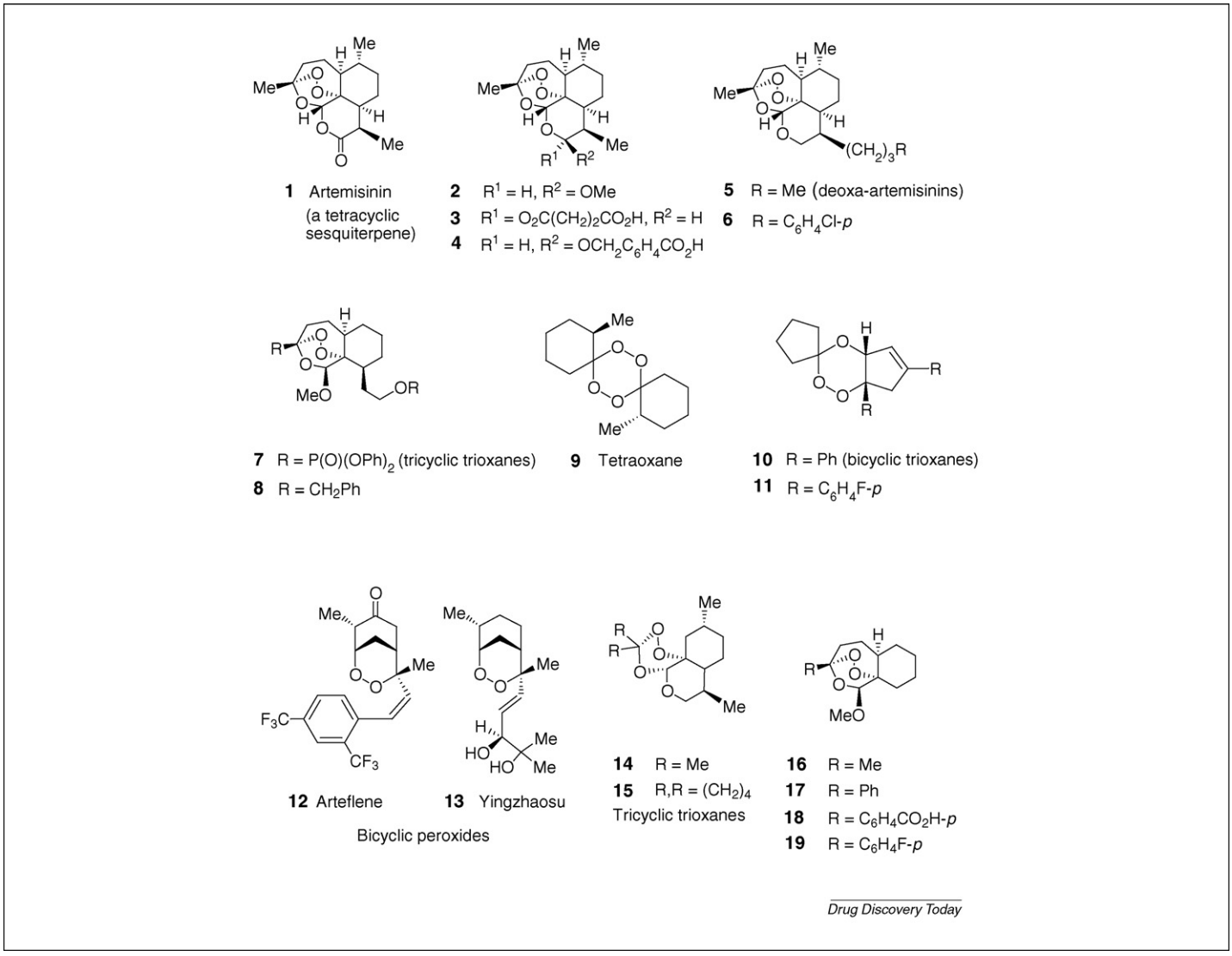

FIGURE 1

Early peroxidic antimalarial drug candidates. The correct configurations are shown for the enantiomerically pure compounds **1–4** and **12–15**. For clarity, only one enantiomer is drawn for the remaining racemic compounds.

antimalarial activity, thereby providing a rationale for the design of new antimalarial peroxides [10].

The most striking finding was the high artemisinin-like activity shown by the trioxanes **10** and **11**, and the tetroxane **9**, the carbocyclic skeletons of which bear no resemblance to that of artemisinin (**1**). This finding showed that it is not necessary to simulate the artemisinin framework, either wholly or partially, to secure superior antimalarial potency. The reason why these peroxides are as active as artemisinin is their ability to mimic its mechanism of action. It was realized that the key to activity – and thus the feature that is indispensable for efficacy – lies in the peroxide bond and its adjacent masked functionality. It is precisely this feature that defines the scope of the present review.

Mechanistic rationale for designing peroxidic drugs

The mode of parasiticidal action of all of these peroxides is attributed to transient carbon-centered radicals, which arise through interaction with heme in the parasitized erythrocyte. How do the radicals form? After the host is infected – in an *in vivo* experiment, for example – *Plasmodium* parasites invade the erythrocytes, where they ingest hemoglobin in the food vacuole to obtain amino acids for growth. Because it is soluble and toxic to the host, the excised prosthetic group (heme) is oxidized and polymerized to hemozoin, an insoluble substance. When the host is treated with an active peroxide, the detoxification of heme within the food vacuole is interrupted by a cascade of chemical events [11]. Taking the trioxane **10** as an example, the ferrous ion of heme ($Hem.Fe^{2+}$) attacks the peroxide function (the O–O bond), breaking it apart to create a ferric oxide bond and an oxy radical **20** (Figure 2). The oxy radical **20** then irreversibly rearranges to the thermodynamically stable ester function, simultaneously producing the pendent ethyl radical **21**, which reacts with the protein of a nearby parasite, thereby causing its death. On protonation, the radical product **22** liberates the disabled alkylated parasite **23**, hemin ($Hem.Fe^{3+}$) and, lastly, hemozoin.

Clearly, to be effectual a peroxide must be able to diffuse into the parasitized erythrocyte. But accessibility to the peroxide function by heme is also crucial for activity: it determines whether an

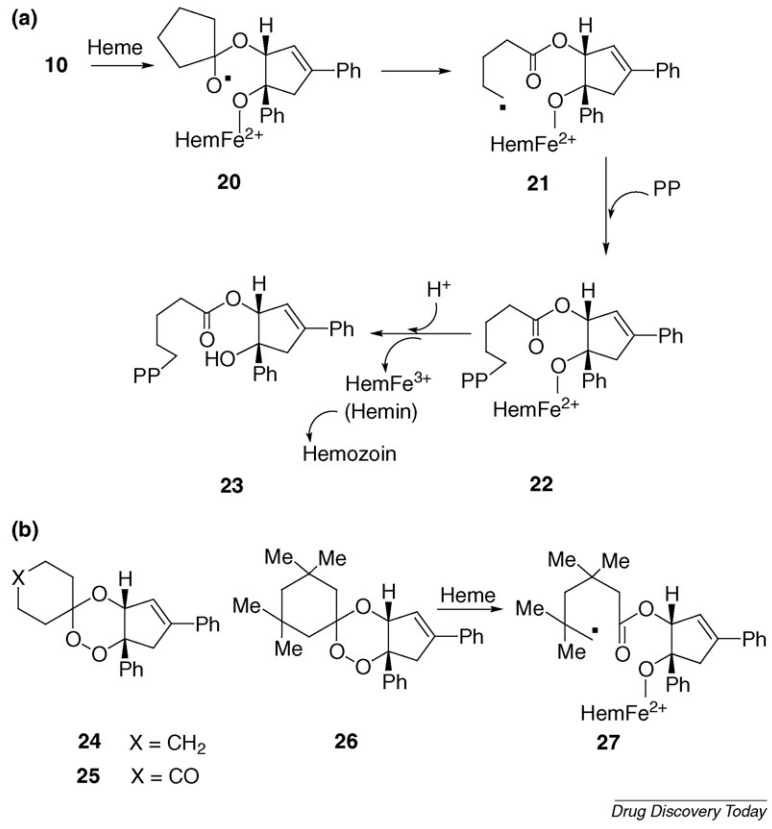

FIGURE 2

Formation of radicals from trioxanes. (a) Reaction of trioxane **10** with heme to give the oxy radical **20**, followed by the primary carbon-centered radical **21**, which alkylates parasite protein (PP) to **22** and **23**, releasing hemin and hemozoin. **(b)** Trioxanes **24** and **25** are active, whereas **26** and **27** are inactive because of their bulkiness.

appropriate carbon-centered radical can actually form. For example, spirocyclohexyl trioxanes **24** and **25** are highly active, having concentrations of 3.9 and 0.8 ng/ml that inhibit growth of the chloroquine-resistant Indochina W-2 clone by 50% (IC_{50}), similar to the value observed (1.2 ng/ml) for artemisinin (**1**) (Figure 2) [2,12]. When the peroxide ring becomes encumbered, however, activity is lost. The tetramethyl derivative **26** is essentially inactive, having an IC_{50} of 94 ng/ml against the same clone. It can be inferred either that heme cannot reach the peroxide bond in **26** because the methyl groups get in the way, or that, if heme does manage to get close enough to bind and react, the resulting neopentyl-type carbon radical **27** is prevented from alkylating the parasite because of its own bulkiness (Figure 2).

Despite the evidence for the intermediacy of radicals as the parasiticidal species (Figure 2), some alternative interpretations have been proposed, as discussed below. Nevertheless, the design of the new peroxidic antimalarial drugs has been based solely on the concept of the heme-mediated rupture of a peroxide bond to yield a lethal carbon radical.

Hybrid or chimeric compounds

Trioxaquines

Novelty has been introduced by reductively combining a known effective trioxane (**25**) with aminoquinoline **28** (Figure 3); the essential part of the traditional antimalarial remedy chloroquine [13]. The resulting hybrid or chimeric molecule **29**, dubbed a 'trioxaquine', is purported to show 'covalent bitherapy', with the aim of thwarting the rise in drug resistance. The quinoline component is thought to facilitate penetration of the parasitized erythrocytes by binding to free heme, enabling the attached trioxane ring to act as an alkylating agent [13]. The reasoning underlying such a supposition is questionable. Taking the behaviour of chloroquine and dihydroartemisin as a guide, either part of the hybrid, trioxane or quinoline, will bind equally strongly to heme [14–16]. The trioxane component does not need its quinoline companion to help it to get into the iron-rich food vacuole. Moreover, the trioxane **25** is sufficiently active by itself: its IC_{50} against the Indochina W-2 clone is 0.8 ng/ml, as compared with a value of 5 ng/ml for **29** against the chloroquine-resistant FcB1-Colombia strain [12,13].

The trioxaquine, because of its dual nature, poses a mechanistic conundrum. On the one hand, the quinoline part on binding with heme would prevent its degradation to hemozoin, thus potentiating its toxic effect on the parasite. On the other hand, the trioxane part reacts with heme differently, converting it to hemozoin while producing a carbon radical as the lethal agent. It therefore seems that, once bound to heme by its quinoline end, the hybrid would need a second molecule of heme to activate the pendent trioxane end. Evidently, both parts of the hybrid might well act independently. Consequently, a simple mixture of chloroquine and **25** could have the same therapeutic effect.

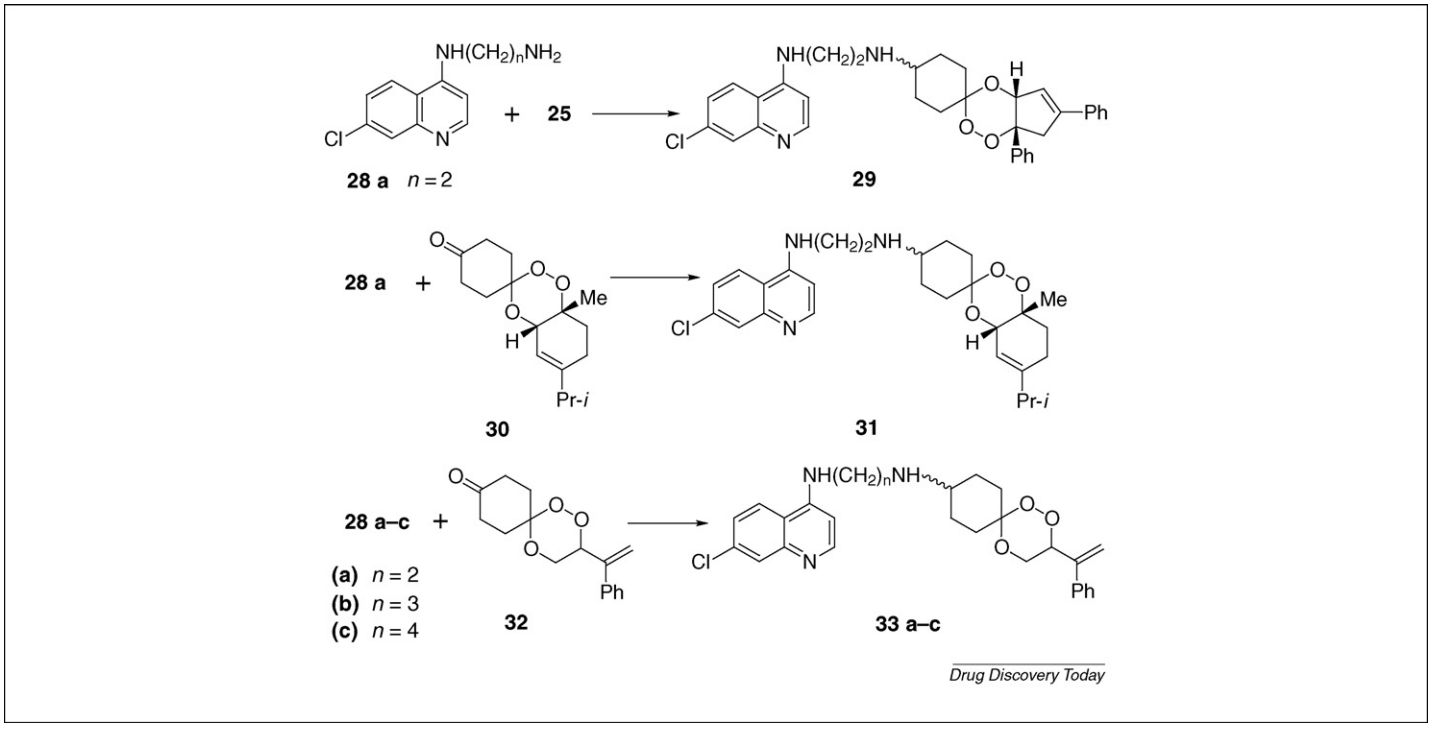

FIGURE 3

Preparation of trioxaquines (29, 31 and 33a–c) from trioxanes (25, 30 and 32) and 28.

The preceding findings have been extended [17]. The most significant results were obtained with a hybrid (**31**) derived from condensation of the trioxane **30** and **28a** (Figure 3). The ED_{50} values for the di-citrate of **31** against *Plasmodium vinckei* by the intraperitoneal and oral routes were found to be 5 and 18 mg/kg/day, respectively. At doses of 20 and 50 mg/kg/day, parasitemia was completely cleared over 60 days. As promising as these results appear, an appreciation of the benefits brought by the hybrids is lacking, because the activity data of the parent trioxane (e.g. **30**) from which they are derived were not reported.

Another variation on the hybrid theme is the reductive coupling of **28a–c** with the easily accessible trioxane **32** bearing a spirocyclic cyclohexanone entity (Figure 3) [18]. The resulting hybrids (**33a–c**) have been tested as their citrates against multidrug-resistant *Plasmodium yoelii* in the mouse by the oral and intramuscular routes. The hybrids showed no improvement in activity over the parent trioxane **32** when administered intramuscularly at a dose of 96 mg/kg/day. Suppression of parasitemia at day 4, however, was more effective for the hybrids (89–94%) than for **32** (7%) when given orally. Nevertheless, neither the hybrids nor **32** afforded protection of the treated mice after 28 days by either route because all the mice died. Furthermore, the hybrids did not supersede the performance of artemisinin (**1**), which at an intramuscular dose of 48 mg/kg/day suppressed parasitemia by 100% at day 4 and afforded total protection at day 28.

A related question concerns the therapeutic value of the aminoquinoline **28a**. Does it have antimalarial activity by itself? The answer is an equivocal yes. Suppression of parasitemia in mice given intramuscular **28a–c** was found to be 87–97% at day 4, but no treated mice survived the 28-day trial [18] (Figure 3).

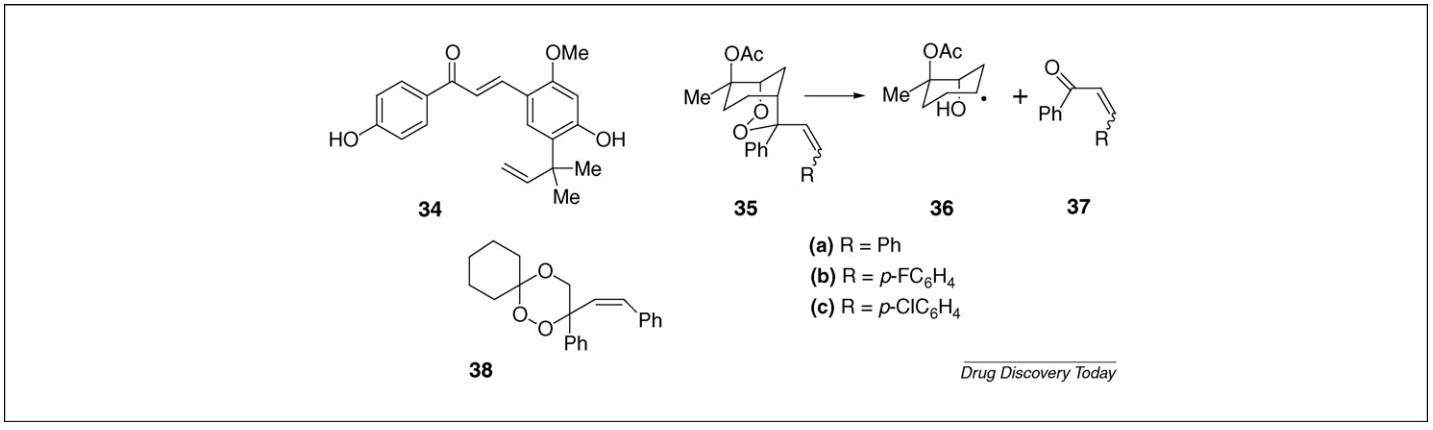

FIGURE 4

Licochalcone (34) and masked 'Trojan Horse' chalcones (35 and 38). Cleavage of 35 gives a carbon radical (36) and the chalcone (37).

Apart from the absence of any significant cooperative effect on activity, the hybrids **33a–c** are impaired by poor stability and poor solubility in oil and water, hence their evaluation as citrates. Moreover, like **29**, they are limited as drug candidates owing to their formation as inseparable diastereomeric mixtures.

Hybrid chalcones

Another version of the hybrid approach is the evocatively named 'Trojan Horse', in which a peroxide or trioxane is tethered to a latent chalcone [19]. Chalcones are well known for their wide-ranging biological properties. Licochalcone A **34** (Figure 4), isolated from Chinese licorice roots, has potent antimalarial activity against *P. yoelii* YM when administered intraperitoneally [20]. Other chalcones have a similar effect [21]. They act by inhibiting cysteine protease, which degrades hemoglobin to the amino acids needed for growth by the parasite.

Joining an active arteflene-like peroxide to chalcones as the masked ketones (**35a–c**) creates two prodrugs in one [22]. The peroxide entity targets heme in the food vacuole. Once the prodrug enters and attacks heme, it decomposes to the secondary carbon-centered radical **36**, releasing at the same time the chalcone **37a–c** (Figure 4). Apart from killing parasites by alkylation, the chalcone part of the hybrid assists by checking the growth of the survivors: at least, that is the alleged mechanism as deduced by test-tube reactions with ferrous salts. When tested *in vitro* against the K1 strain of *P. falciparum*, **35a–c** shows IC_{50} values of 23–34 nM, which are better than that of arteflene (**12**), but worse than that of artemisinin (**1**), as attested by their respective values of 47 and 15 nM.

A trioxane Trojan Horse (**38**), which should work against *Plasmodium* by the same *modus operandi*, has also been assembled [23]. It will be instructive to see how **38** compares in terms of activity with the individual trioxane and chalcone from which it is derived.

Spirocyclic trioxanes

Structurally reminiscent of the highly active *cis*-fused cyclopentene trioxanes (**10**,**11**) is a series of *trans*-fused cyclohexene trioxanes bearing spirocyclic alkyl groupings at the C3 position (**39–42a–c**) (Figure 5) [24]. Oral activity against multidrug-resistant *P. yoelii* at a dose of 96 mg/kg/day was reported for only **39c**, **40c**, **41c** and **42b**. At day 4 parasitemia was suppressed by 96–100%, a result similar to that obtained for artemether (**2**) (Figure 1), albeit at the lower dose of 48 mg/kg/day. It can be assumed that the activities of the other derivatives were too poor to be reported. It appears that the *trans* fusion of the cyclohexenyl ring to the 1,2,4-trioxane is detrimental to artemisinin-like potency. A pertinent example is **43**, which has IC_{50} and IC_{90} values of 311 and 643 ng/ml, respectively, against the drug-resistant W2 clone of *P. falciparum in vitro* [25]. The corresponding values for **10** under the same test conditions are 3.9 and 5.0 ng/ml, respectively [25].

Simplifying the trioxane structure, while keeping the spirocyclic element, results in high activity approaching that of artemisinin (**1**). When tested against *P. yoelii* by the intramuscular route in the mouse, the cyclopentane, cyclohexane and adamantane trioxanes as racemates (**44** to **46a–d**) were found to require a dose of 96 mg/kg/day to suppress parasitemia by 100% at day 4, as compared with **1**, which needed only a dose of 24 mg/kg/day [26]. In the crucial survival test over 28 days, only **45b**, **46a** and **46b** preserved the

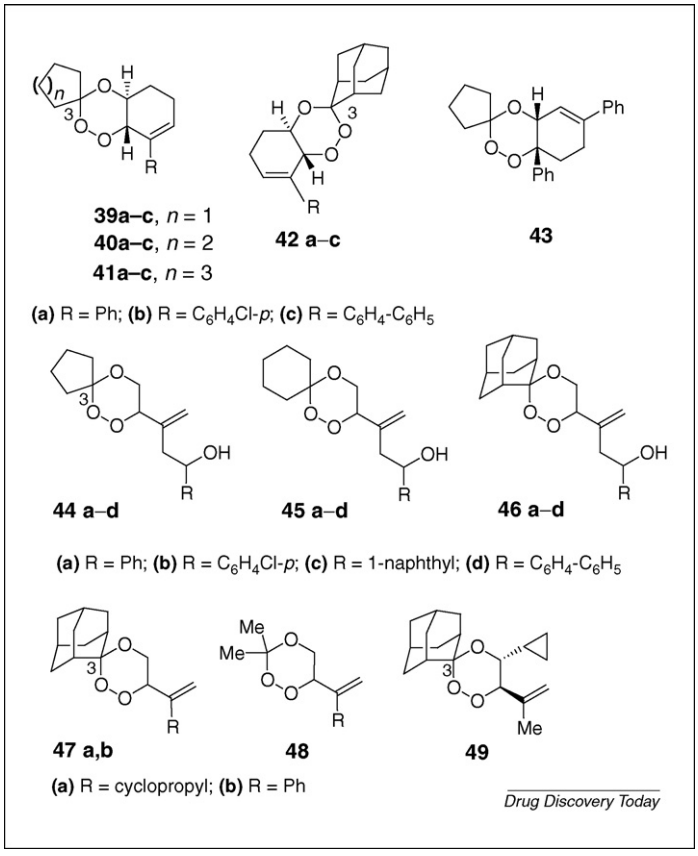

FIGURE 5

Examples of spirocyclic trioxanes.

lives of all of the treated mice. The mean survival times for the oral route at the same dose were 7–10 days for **44a–c**, 16 days for **44d**, 10–14 days for **45a–d**, 17 days for **46a**, **46c** and **46d**, and more than 28 days for **46b**. In summary, the adamantane entity strongly favors activity, which is reinforced by the more hydrophobic substituents on the side chain, such as biphenyl.

These findings have been confirmed in a subsequent study [27]. The cyclopropyl derivative **47a**, tested intramuscularly in the above manner, preserved the lives of 80% of the treated mice at 28 days, whereas the more hydrophobic, phenyl derivative **47b** protected no mice. Activity was destroyed on replacing the adamantane by a *gem*-dimethyl grouping, as exemplified by **48** (Figure 5).

The benefit accruing from adamantane as a spirocyclic appendage is also seen for trioxane **49**, which is as active as artemisinin (**1**) against *P. falciparum in vitro* (Figure 5) [28].

Monocyclic peroxides

The methyl esters of the peroxyplakoric acids A_3 and B_3 (**50** and **51**) (Figure 6), isolated from the marine sponge *Plakortis*, are endowed with antimalarial activity [29]. Taking **50** and **51** as leads, the substituents at the C3 and C6 positions have been modified with the aim of improving potency. Typical derivatives are the methyl, *t*-butyl and phenyl esters equipped with a C3 pentyl side chain (**52a–c**). The esters **52a** and **52b** have IC_{50} values of 0.12 μM against *P. falciparum in vitro*, whereas the phenyl ester **52c** is inactive. The *in vivo* activity of **52a** and **52b** against *P. berghei* is, however, insignificant.

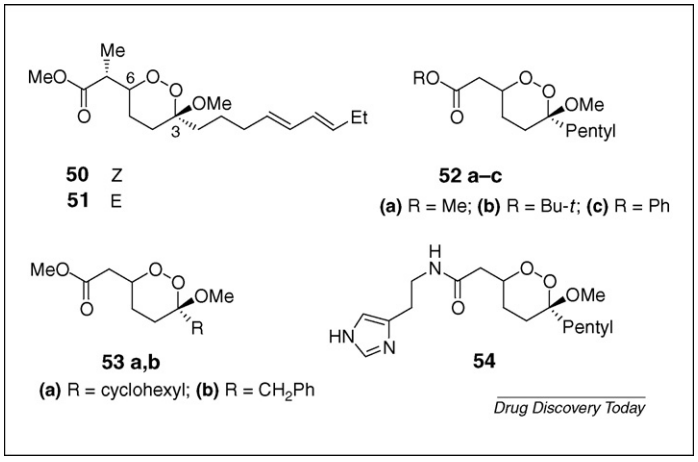

FIGURE 6

Peroxyplakoric esters (50 and 51) and their analogues (52–54). The correct configurations are shown for the enantiomerically pure compounds **50** and **51**.

The *in vitro* activity of **52a–c** is attributed to the formation of a primary pentyl radical, following attack by heme on the peroxide function. Analysis of the product mixture obtained by treatment of **52a** with ferrous sulfate in water has confirmed the excision of the pentyl substituent as the presumed radical [30]. The cyclohexyl and benzyl derivatives **53a** and **53b** show *in vitro* activities that are far poorer than that of **52a**. Artemisinin-type activity requires the intermediacy of an aggressive unstable carbon-centered radical, preferably a primary radical. More stable radicals, as exemplified by the cyclohexyl and benzyl radicals produced by **53a** and **53b**, are less toxic to the parasite.

The best result has been obtained with the imidazole derivative **54** (Figure 6), which shows an ED_{50} value of 18 mg/kg against *P. berghei*, as compared with a value of 5 mg/kg for artemisinin (**1**).

All of the above tests were carried out on isomeric mixtures and, significantly, the absolute configuration of the 3-methoxy-1,2-dioxane moiety had no bearing on activity [31].

Secondary ozonides or 1,2,4-trioxolanes

Ozonation of olefins to give primary ozonides that rearrange to secondary ozonides or 1,2,4-trioxolanes is a classic topic that is well known to organic chemists. It comes as a surprise to discover that 1,2,4-trioxolanes are stable compounds, some of which behave like 1,2,4-trioxanes by killing the *Plasmodium* parasite. Nonetheless, this discovery could easily have been missed. The prototype is the doubly spirocyclic derivative in which the crucial five-membered ring is sandwiched between adamantane and cyclohexane (Figure 7, **55**) [32]. It is highly active, just like artemether (**2**), against *P. falciparum in vitro* and *P. berghei in vivo*. Amazingly, the closely related bis-cyclohexane and bis-adamantane trioxolanes **56** and **57** are completely inactive.

Treating the cyclohexanone derivative **58** with ferrous acetate as a model for heme indicates that the peroxide bond breaks to give successively the oxy and the secondary carbon radicals (**59** and **60**) Evidently, the activity of **55** is due to a similar event occurring in the parasite, which is killed by the deadly carbon radical. It can be construed that **56** and **57** are prevented from binding to ferrous iron or heme for steric reasons, which accounts for their inactivity. On further comparing **55** to 1,2,4-trioxane analogues, it was

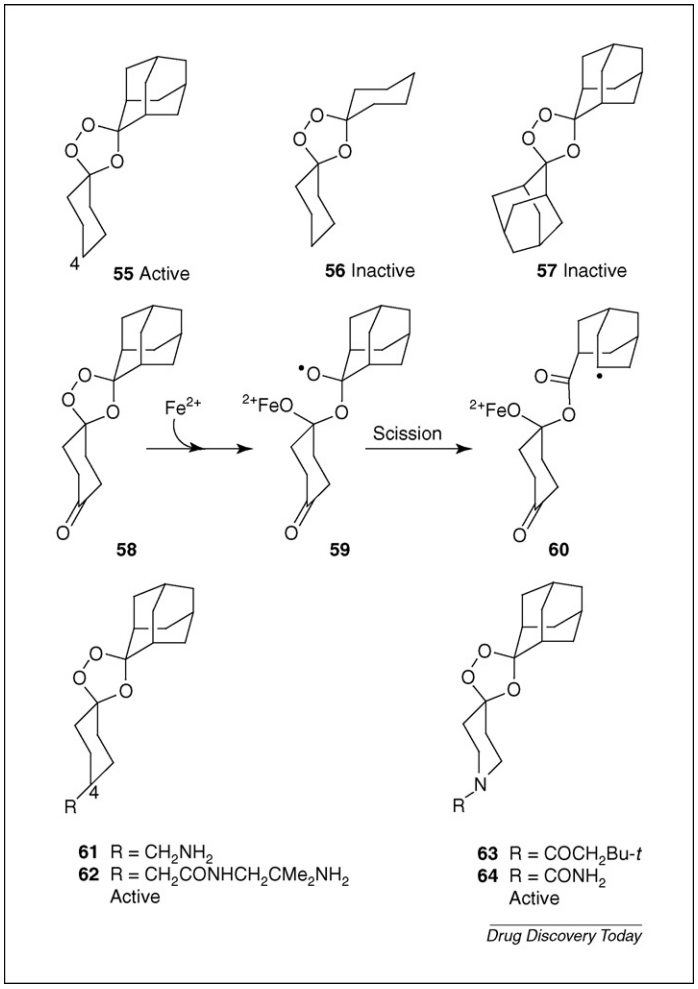

FIGURE 7

Active and inactive 1,2,4-trioxolanes. A secondary carbon-centered radical (**60**) is formed via the oxy radical (**59**) by the action of ferrous ion on **58**.

shown that access of ferrous ion to the latter is easier and less selective for the analogues than for **55** [33]. Evidently, the bis-spirocyclic trioxolane core is more sterically congested.

The problems that beset most peroxide and trioxane drug candidates are solubility, bioavailability and enantiomeric purity. Trioxolane **55** is no exception. It has poor solubility in water and low oral bioavailability. Fortunately, it has mirror plane symmetry. Consequently, mono-substitution at the C4 position of the cyclohexane ring in the mirror plane leads to *cis* and *trans* epimers, which are achiral (Figure 7). Such *meso* derivatives are favorable for development, because they are not subject to the drug registration strictures required for enantiomers. Many *meso* derivatives of **55** have been tested [34]. After much experimentation, the amine (**61**) and amide (**62**) of the 4-substituted derivatives turned out to be the best drug candidates. They proved to be superior to artemether (**2**) and artesunate (**3**) in every way. Apart from their *in vitro* and *in vivo* activity, they completely cured mice infected with *P. berghei* when given as an oral dose of 10 mg/day for three successive days. The half-lives of **61** and **62** on intravenous injection in the rat are 3–5 times longer than that of artesunate (**3**). Equally important is their low susceptibility to metabolic degradation by P450 monooxygenases. Finally, **62** has been chosen for drug

development by Ranbaxy Laboratories (http://www.ranbaxy.com) because of its better toxicology profile and lower accumulation in brain tissue [35].

In a structural modification of **55**, the cyclohexane ring has been replaced by piperidine to advantageous effect [36]. Some 27 *meso* derivatives have been prepared, most of which have IC_{50} values ranging from 0.2 to 7.0 ng/ml. Two of them, the amide (**63**) and the urea (**64**), have oral activities against *P. berghei* in the mouse that are superior to that of artesunate (**3**) and similar to that of artemisinin (**1**). Although these piperidines are easily accessible, they apparently suffer from metabolic instability.

In summary, it was found that lipophilic trioxolanes tend to have better oral activities than their more polar counterparts. In general, derivatives bearing neutral or basic substituents, but not acidic ones, have good antimalarial activity [37,38]. In addition, amino- or amide-substituted trioxolanes have the best combination of antimalarial and biopharmaceutical properties [39].

The mode of action

Heme as the receptor

How antimalarial agents act at the molecular level in a biological context has been difficult to ascertain. As might be expected, most work has dealt with the commercially available, long-established artemisinin and its congeners, whereas little has been done on the new synthetic peroxides. As mentioned above, all of the previously described trioxanes, peroxides and ozonides owe their pharmaceutical heritage to artemisinin; therefore, conclusions drawn from the artemisinins are relevant to the new peroxides.

Synthetic peroxides, in the same way as artemisinin, generate lethal carbon-centered radicals [11,19,40,41]. There are, however, subtle differences. Artemisinin (**1**) generates two, a primary radical (**65**) (Figure 8a) and a secondary radical (**66**), whereas bicyclic trioxanes and bis-spirocyclic trioxolanes produce only one each, a primary radical (**21**) (Figure 2) and a secondary radical (**60**) (Figure 7), respectively. Once formed, the radicals are obliged to react further by alkylating a nearby substrate. This substrate has been variously proposed to be a parasite protein, such as the translationally controlled tumor protein of the parasite; cysteine protease, as mentioned above; and even heme. In any event, when the carbon radical reacts with a substrate, the attached heme moiety becomes oxidized to hemin (Hem.Fe^{3+}) and thence to hemozoin – a result that has been demonstrated experimentally [42].

However, a recent report presents a different view [43]. Artemisinin was shown to react preferentially with hemoglobin over heme. The mono- and di-alkylated products so obtained bind to *Plasmodium falciparum* histidine-rich protein II, killing the parasite while inhibiting hemozoin formation. By way of a check and to confirm the scope of such a finding, similar experiments should be performed with synthetic peroxides, both active and inactive.

It has also been maintained that **66** can pursue an alternative decomposition route to give the olefin **67** by extruding a high-valent iron oxo species ($O{=}Fe^{2+}$), presumably bound to heme, which acts as the lethal agent [19]. Evidence for this reactive avenue has not been observed for synthetic peroxides.

Does chirality matter?

Artemisinin (**1**) is chiral and exists as a single enantiomer. The same is true of its derivatives **2**, **3** and **4**, which are often used as reference compounds. By contrast, most synthetic peroxides are

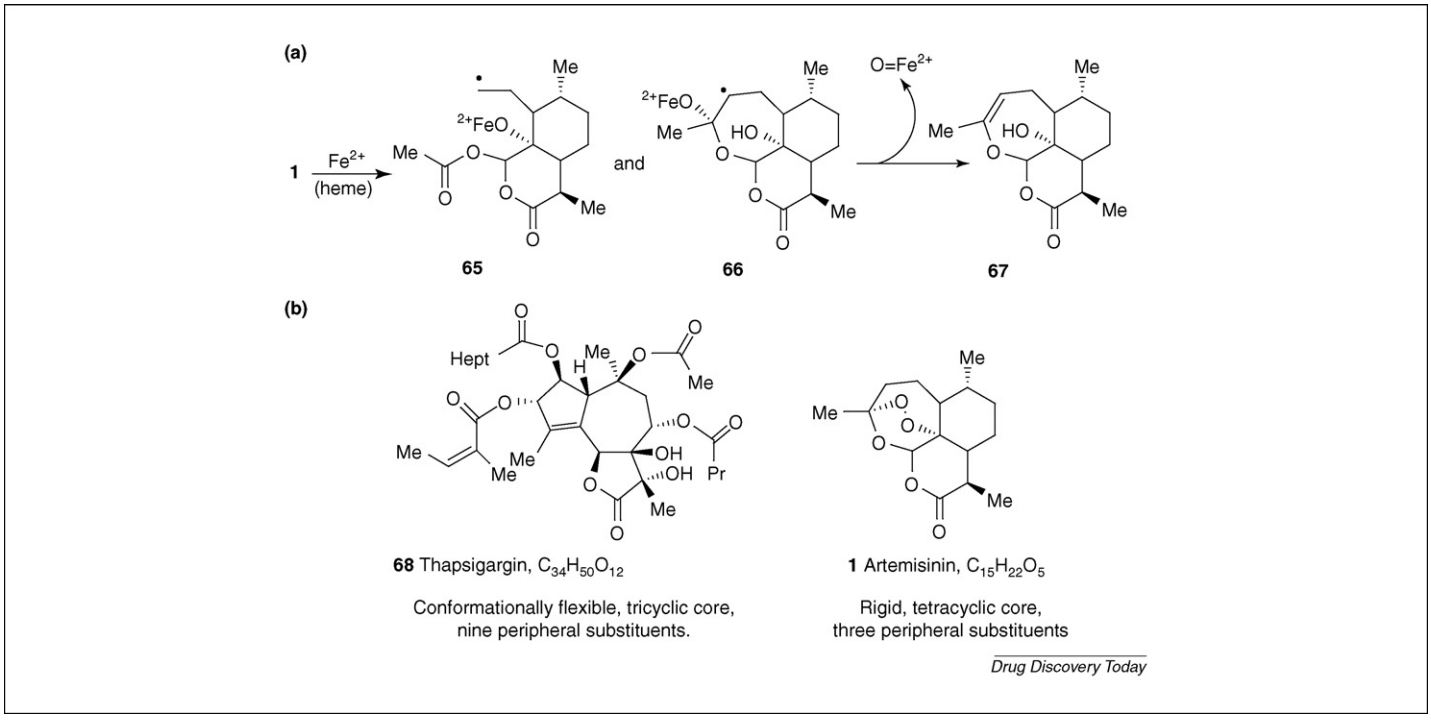

FIGURE 8

Artemisinin and thapsigargin. (a) Reaction of artemisinin (**1**) with heme (Fe^{2+}), giving primary and secondary carbon-centered radicals (**65** and **66**) and $O{=}Fe^{2+}$ by elimination from **66**. **(b)** Comparison of the structural and chemical features of the sesquiterpene lactones thapsigargin (**68**) and artemisinin (**1**). The correct configurations are shown for the enantiomerically pure compounds **65–68**.

prepared and tested as racemic mixtures in which the left-handed and right-handed forms are present in equal amounts. One might ask if the pure enantiomers, like most chiral pharmaceuticals, have different activities.

The answer is definitely no. An early crucial finding was the essentially identical activity *in vitro* against *P. falciparum* and *in vivo* against *P. berghei* of each of the pure enantiomers of **10** or **11**, and the racemic mixture itself [44]. This result is not surprising, because the receptor heme itself is achiral and thus unable to discriminate between the left- and right-handed forms of **10** or **11**, or for that matter any other active, racemic peroxide.

This finding has been recently corroborated [45]. Each enantiomer, as well as the racemate of the tricyclic trioxane **19**, which models part of the artemisinin skeleton, was tested *in vitro* against the chloroquine- and mefloquine-resistant Dd2 strain of *P. falciparum*. The IC_{50} values of the enantiomers and that of the racemate were found to be similar, falling in the 24.5–26.7 nM range, approximately twice that of artemisinin, revealing no correlation of activity with configuration. Whatever the nature of the receptor, heme or a protein, it is clear that chirality plays no role in the so-called 'recognition step', as it would in an enzymatic process. Traditional, chiral antimalarial drugs that target heme, such as chloroquine and benflumetol, also show no configurational correlation with activity [46,47].

PfATP6ase as a target

Quite a stir has been created by a report that artemisinin does not, after all, need heme to kill *Plasmodium* [48]. Instead, another target, PfATP6ase, was identified, lying not in the food vacuole, but in the endoplasmic reticulum of the parasite. This novel finding arose by a curious analogy. It was known that a plant constituent, thapsigargin (**68**) (Figure 8b), specifically inhibits at subnanomolar concentrations a mammalian Ca^{2+}-dependent ATPase, termed SERCA, that is located in the sarco/endoplasmic reticulum [49,50]. *Plasmodium falciparum* has just one enzyme that is orthologous to SERCA, PfATP6ase. It was thus reasoned that, because artemisinin (**1**) and thapsigargin (**68**) are both sesquiterpene lactones, they ought to behave similarly towards SERCA-type enzymes, because of supposed structural similarities. Paradoxically they do behave similarly, in spite of their markedly different chemical and molecular constitutions. Thapsigargin ($C_{34}H_{50}O_{12}$) has a relative molecular mass of 650.76, which is more than double that of artemisinin ($C_{15}H_{22}O_5$) at 282.34 (Figure 8b). Furthermore, artemisinin (**1**) is a rigid tetracyclic structure comprising a peroxide function and a six-membered lactone adorned with three methyl groups, whereas thapsigargin (**68**) is conformationally mobile and tricyclic, possesses a five-membered lactone, and is peripherally substituted by four aliphatic ester, three methyl, and two hydroxyl groups. The dissimilarity between **1** and **68** could not be greater.

Inhibition of PfATP6ase in *Xenopus* oocytes by **1** was found to be equipotent with that of **68**. Moreover, **68** antagonized the parasiticidal activity of **1**, showing that they both compete for the same receptor site on the enzyme. Addition of a chelator for ferrous ion attenuated the activity of **1**, thereby indicating that stimulation by radical formation is required for activity. Modeling studies confirm that both **1** and **68** fit into the same cleft in the enzyme [51], but that rupture of the peroxide bond by heme probably occurs after binding [52].

What are the implications of these findings for the many synthetic peroxides that do not even remotely resemble the structures of the above two disparate lactones? Several of them, as noted above, have activities equal to and sometimes greater than those of artemisinin and its semi-synthetic derivatives. It will be interesting to see whether these synthetic peroxides also inhibit PfATP6ase.

Another question concerns the lack of chiral discrimination by the target towards enantiomeric synthetic bicyclic and tricyclic trioxanes (*vide supra*) – behaviour that is not expected for an enzyme such as PfATP6ase. It was recently shown that mutation of a single amino acid can modulate susceptibility to artemisinin, an enantiomerically pure molecule [51]; a result suggesting that PfATP6ase as a target is sensitive to chiral interactions. It also suggests that PfATP6ase might be a specific target only for artemisinin. It would be informative to repeat the above experiments with a synthetic sample of the opposite enantiomer of artemisinin.

Clinical studies

Despite the vast amount of research carried out since the first trioxanes were tested in 1983, it is only now that a synthetic second generation peroxide has a chance of entering the market. The number of antimalarial candidates currently in clinical development is nine [53]. Astonishingly, four are formulations of artemisinin and its congeners: namely, combinations of artemether (**2**) and lumefantrine (Coartem™, Novartis), dihydroartemisinin and piperaquine (Artekin™, Holleykin Pharmaceuticals), artesunate (**3**) with dapsone and chloroproguanil (Lapdap™, Tropical Disease Research, World Health Organization, and partners), and artesunate (**3**) with pyronaridine (Zheng Laboratory).

Only one fully designed synthetic peroxide, the trioxolane **62** (OZ277/RBx11160) is currently in clinical trials. It was found to be well tolerated by healthy volunteers in a phase I trial. Under the aegis of the Medicines for Malaria Venture, the Swiss Tropical Institute and Ranbaxy Laboratories, trioxolane **62** is now undergoing a phase II dose ranging study on individuals affected with uncomplicated falciparum malaria (http://www.clinicaltrials.gov/ct/show/NCT00362050). The study is expected to be completed by May 2007.

Conclusion

The biggest hurdle to overcome in developing an effective drug against multidrug-resistant falciparum malaria is one of cost, not molecular design. The market for such drugs lies mainly in the malarious zones of the third and developing worlds where low purchasing power cannot afford the expense of prevention and cure. Consequently, a pharmaceutical company might not see its development costs recouped from sales in this market. Partnerships involving private industry, an international agency, a philanthropic foundation and academia, for example, provide an economical approach for sharing costs as a candidate proceeds through the stages of drug approval and registration. Trioxolane **62** is a successful illustration of such a cooperative endeavor.

Reformulation of traditional remedies is not going to provide an adequate response to the growing incidence of multidrug-resistant malaria. It now seems that peroxidic drugs, such as an analogue of arteflene (**12**) and the cyclopentene-1,2,4-trioxane **11**, are less liable to incur resistance by the parasite [54]. The reasons are

not known, but might be due to the non-specific agency of carbon radicals. Further advantages offered by synthetic peroxides are cheapness of preparation and versatility of structural modification, enabling them to be tailored to fit a drug profile characterized by potency superior to that of the natural product artemisinin, by enhanced bioavailability, and by minimal neurotoxicity. In the future, more of these peroxidic candidates will ultimately find a place in the medicine chest of antimalarial drugs.

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