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HYDROXY-ANTHRAQUINONES AS ANTIMALARIAL AGENTS

R. W. Winter,[a] Kenneth A. Cornell,[b] Linda L. Johnson,[a] Loren M. Isabelle,[c] David J. Hinrichs,[a] and Michael K. Riscoe*[a,b]

[a]Medical Research Service, 151-0, Department of Veterans Affairs Medical Center, 3710 SW U.S. Veterans Hospital Road, Portland, Oregon 97201, [b]Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Parkway, Portland, Oregon 97201 and [c]Oregon Graduate Institute of Science and Technology, 20000 NW Walker Rd., Portland, Oregon 97291

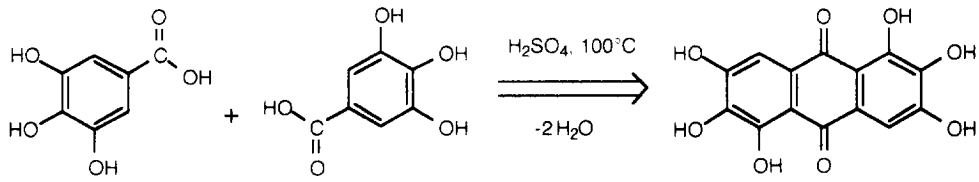
Abstract: A series of hydroxy- and polyhydroxy-anthraquinones were screened for inhibitory activity against the malarial parasite, *Plasmodium falciparum*. Rufigallol demonstrated the most potent effects with a 50% inhibitory concentration (IC_{50}) value of ~10.5 ng/ml (~35 nM). Deleterious effects were exerted by rufigallol toward bone marrow progenitor cells at concentrations ≥ 10 μM (3 μg/ml) where ~30% suppression of colony growth was noted. Taking into consideration its potency and relative lack of toxicity, we believe that rufigallol should be advanced for *in vivo* studies. At the very least, rufigallol represents a simple, inexpensive lead drug for the development of more potent analogs.

The World Health Organization estimates that nearly 1 million deaths are caused by malaria each year in Africa alone; most of these are children under the age of five. In addition, over 300 million people worldwide are believed to be chronically infected, and each year, nearly one third of these will suffer acute manifestions of the disease.[1] While the world awaits the development of a vaccine to control malaria, millions of lives are dependent upon intervention with chemotherapeutic agents. However, available drugs for the treatment of malaria are becoming increasingly ineffective due to the rapid emergence of drug-resistant strains of *P. falciparum*, causative agent for the most severe form of the disease. As a result, there is an urgent need for the development of new antimalarial agents.[2]

A recent addition to the arsenal of drugs for malaria treatment is the hydroxy-naphthoquinone, 2-[*trans*-4-(4-chlorophenyl-cyclohexyl)-3-hydroxy-1,4-naphthoquinone (BW 566C80).[3] Because of the success of this compound, we hypothesized that hydroxylated anthraquinones, acting as analogs of the naphthoquinones, could exert potent antimalarial effects. In this paper we present structure-activity relationships for a series of hydroxy-anthraquinones.

Many of the compounds tested were obtained from commercial vendors (9,10-anthraquinone, anthraflavic acid, anthrarufin, and purpurin-as indicated in Table 1); others were synthesized by modifications of published methods.[4] Rufigallol, one of the first compounds to be synthesized in the history of organic chemistry (by Robiquet in 1836) was obtained by dehydration of gallic acid as described by Grimshaw and Haworth in 1956[5] (Scheme 1). Octahydroxyanthraquinone was prepared from rufigallol by the Bohn-Schmidt reaction.[6] 2,3,6,7-Tetrahydroxy-anthraquinone was synthesized by condensing veratrole and paraldehyde to form diveratrylethane. A second condensation reaction between the diveratrylethane and acetaldehyde produced a 9,10-dimethyl-anthracene derivative which was then oxidized by $Na_2Cr_2O_7$.[7] Hexa-acetoxyrufigallol was obtained as an intermediate in the rufigallol synthesis. The methyl ethers of rufigallol and other selected anthraquinones were synthesized from the free hydroxy compounds.[8]

Scheme 1. Synthesis of rufigallol.



Gallic acid Rufigallol

Hexamethyl-rufigallol was further converted to the dibromide derivative with trifluoroacetyl-hypobromite. Briefly, red mercuric oxide (3.00 gm, 13.8 mmol) was stirred at room temperature with 100 ml of trifluoroacetic acid, followed by addition of trifluoroacetic anhydride (3.00gm, 14.3mmol). After 5 minutes, hexamethyl-rufigallol (3.00 gm, 7.7 mmol) was dissolved into the solution. The powerful electrophilic brominating agent, trifluoroacetyl-hypobromite,[9] was formed *in situ* by addition of bromine (5.4 gm, 33.8 mmol) which immediately caused the formation of a precipitate. After stirring for several minutes, the solution was poured into a flask containing 500ml water and extracted with dichloromethane (3 x 50 ml). The combined organic phases were dried with sodium sulfate, and the solvent removed under vacuum, leaving 5.1 gm of a sticky, orange-red residue. This material was subjected to column chromatography on silica gel (benzene:dioxane = 15:1) from which an intense yellow-colored band was collected. The yellow fraction was dried *in vacuo* to yield a mixture of yellow crystals and an orange gum. Further purification was accomplished by heating the mixture in a solution of petroleum ether and ethyl acetate (150 ml each). This process dissolved the orange component(s) leaving behind a crop of yellow crystals. After recrystallization from ethyl acetate, 0.75 gm of stout bright yellow crystals were obtained. Additional product was obtained by the same extraction process from the mother liquor (total yield: 1.35 gm, 31%).[10] The product, dibromo-hexamethoxy-rufigallol (0.5 gm, 0.92 mmol), was hydrolyzed to the free hydroxy compound with 48% hydrobromic acid (20 ml) in a Carius tube flushed with N_2 at 100-110°C for 89 hours. After this period, a flocculent, bright cinnabar solid was collected by filtration and washed with cold water. Part of the sample was subjected to chromatography on cellulose with glacial acetic acid and collected as a bright yellow band. (overall yield= 29.6 mg, extrapolated 13%).[10]

The anthraquinone derivatives were tested for their ability to inhibit the growth of *P. falciparum* and standard IC_{50} values were obtained by following the incorporation of radiolabeled ethanolamine.[11] As shown in Table 1, most of the compounds proved to be relatively poor antimalarial agents, requiring concentrations of greater than 1,000 nM to achieve demonstrable effects. The tetrahydroxy- and octahydroxy-derivatives of rufigallol were moderately effective, exhibiting IC_{50} values of 300 nM and 800 nM, respectively. Rufigallol proved to be the most potent of the anthraquinones tested producing an antimalarial effect equivalent to the *in vitro* activity of chloroquine in parallel experiments. The concentration of rufigallol required to inhibit the growth of *P. falciparum* by 50% was estimated to be ~35 nM (10.5 ng/ml, n=10). Results from a representative experiment are displayed in Figure 2. The drug was equally effective against the multi-drug resistant form of *P. falciparum* strain W2 (data not shown). Protected forms of rufigallol (e.g., the hexamethylether and hexaacetoxy-derivatives) were less potent than the parent compound (IC_{50} values of >5000 nM and 350 nM, respectively). In addition, insertion of two bromine atoms onto the rufigallol structure yielded a compound devoid of antimalarial activity.

Table 1. Inhibitory effect of various anthraquinones on *P. falciparum* (strain D6) in vitro.

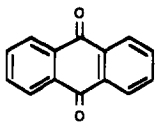
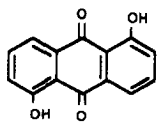
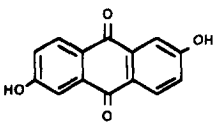
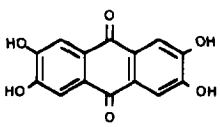
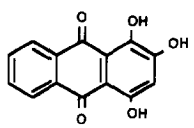
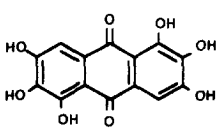
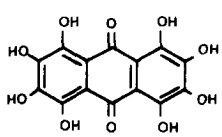
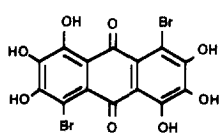
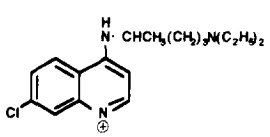
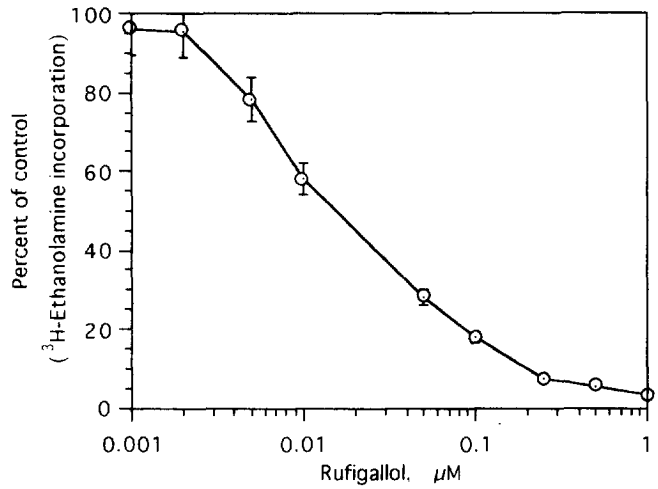
Chemical Structure	Name(s)	IC_{50}, nM	Notes
	9,10-anthraquinone	5000	Source: Janssen Chimica (Belgium) Purity: ~97%
	1,5-dihydroxy-9,10-anthraquinone, anthrarufin	>1000	Source: Aldrich Chemical Company Milwaukee, Wisconsin (USA) Purity: 85%
	2,6-dihydroxy-9,10-anthraquinone, anthraflavic acid	>1000	Source: Aldrich Chemical Company Purity: 97%
	2,3,6,7-tetrahydroxy-9,10-anthraquinone	300	Synthesized according to Boldt[7]. Tetra-methyl ether form, $IC_{50} \geq$ 5000nM.
	1,2,4-trihydroxy-9,10-anthraquinone, purpurin	>1000	Source: Aldrich Chemical Company Purity: ~90%
	1,2,3,5,6,7,-hexahydroxy-9,10-anthraquinone, rufigallol	35	Synthesized according to Haworth and Grimshaw[5]. Hexa-methyl ether form, $IC_{50} \geq$ 5000nM.
	octahydroxy-9,10-anthra-quinone	800	Synthesized according to Georgievics[6]. Octamethyl ether[8], $IC_{50} \geq$ 5000nM
	1,5-dibromo-hexahydroxy-9,10-anthraquinone	5000	Synthesized as described in the text. Hexamethyl ether form, $IC_{50} \geq$ 5000nM
	Chloroquine, a standard antimalarial agent	20	

Figure 2. Antimalarial activity of rufigallol demonstrated against the D6 clone of *Plasmodium falciparum*.



Toxicity tests were carried out to determine the effects of rufigallol on the growth and differentiation of normal human bone marrow stem cells (colony forming unit-granulocyte/macrophage-CFU-GM) *in vitro*. Deleterious effects were exerted by the drug at concentrations ≥ 10 μM (~3 μg/ml) where ~35% suppression of colony growth was noted (Table 2).[12] In parallel experiments conducted under the same test conditions, chloroquine produced a modest degree of suppression at a concentration of 10 μM.

Table 2. Toxicity of rufigallol toward human bone marrow stem cells (CFU-GM) *in vitro*.

Culture Conditions	**Average colony count, CFU-GM**	
[Drug], μM	Rufigallol	Chloroquine
Control (no additions)	56 ± 11	56 ± 11
0.1	61 ± 11	56 ± 4
1.0	57 ± 8	54 ± 5
5.0	47 ± 6	54 ± 5
10.0	36 ± 9	48 ± 3
100.0	5 ± 2	5 ± 3

In considering the mode of action of rufigallol in particular, and hydroxyanthraquinones in general, it is possible that they act in fashion similar to the hydroxynaphthoquinones, i.e., futile redox cycling.[13] Thus, rufigallol could act as a catalytic oxidizing agent capable of undergoing cyclic one-electron oxidation-reduction reactions. Such futile "redox-cycling" would lead to the catalytic reduction of oxygen to superoxide (O_2^-) at the expense of reducing equivalents such as NAD(P)H.[14] Hydroxy-naphthoquinones are also known to perturb pyrimidine biosynthesis in *P. falciparum* through inhibition of the enzyme dihydrooratate dehydrogenase and the linked respiratory system.[3,15] Another plausible explanation for the action of rufigallol involves intercalation of

the drug into DNA, as described for the anthraquinone adriamycin, a powerful anticancer agent.[16]

Iron chelation is also a possible mechanism of action for the antimalarial activity of rufigallol. The antimalarial effect of desferroxamine has been shown to involve chelation of parasite-associated iron which is necessary for cellular metabolism and DNA synthesis.[17,18] More recently, it has been shown that desferroxamine extracts ferric iron from the hemozoin deposit; a crystalline structure constructed by the *Plasmodium* parasite to protect itself from the toxic effects of ferric iron and hemin produced by digestion of hemoglobin.[19] By analogy to desferroxamine, it should be noted that since its structural determination in 1877 by Klobukowsky,[20] the most notable attribute of rufigallol has been the ability to form colored chelates with certain metals-including iron.[21,22]

Taken together, we believe that the anthraquinone, rufigallol, should be considered for *in vivo* testing against malaria. Synthesis of rufigallol is inexpensive, relatively simple, and the product is stable to extremes of heat and acid, suggesting that the drug would be suitable for oral administration. At the very least, rufigallol represents a promising lead compound for synthesis of a novel class of antimalarial agents. The mechanism of action of rufigallol is not known, but various attributes of the compound lead us to speculate that multiple actions may combine to produce the surprising antimalarial activity shown here. Our results highlight the effect of the degree of hydroxylation and positioning of the hydroxyl groups around the anthraquinone backbone on antimalarial activity. Further investigations to define the mode(s) of action of rufigallol and additional structure-activity screening may identify analogs suitable for use in combating the devastating consequences of malaria in humans.

Acknowledgements

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(8) Hydroxyanthraquinones were converted to their corresponding methoxy ethers by refluxing in the presence of potassium carbonate in acetone with an excess of dimethylsulfate. For example, 1,2,3,4,5,6,7,8-octamethoxyanthraquinone was synthesized as follows: K_2CO_3 (15.00 gm, 109 mmol), 15 ml of dimethyl sulfate (159 mmol) and 1.00 gm of octahydroxyanthraquinone (3 mmol) were dissolved in 60 ml acetone and refluxed for 2 h. The desired product was purified by column chromatography (silica gel, benzene:dioxane 15:1) and recrystallized from ethanol (yield = 16 % of theory, yellow crystals, mp = 165.5-167.5 °C). It should be noted that rufigallol-hexamethylether, which had been previously prepared by heating the potassium salt of rufigallol in dimethylsulfate (Fischer, O.; Gross, H. *J. Prakt. Chemie* **1911**, *84*, 369), exhibited a slightly higher melting point than the product obtained by the methods described above (246.5-248.6 °C vs. 240 °C).
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(10) 1,5-Dibromo-hexamethoxy-9,10-anthraquinone: Anal. calcd.: C, 44.12; H, 3.34; found C, 43.71, H, 3.13%. The material chromatographed as a single peak by GC-MS with a retention time of 31 min. Conditions: Hewlett Packard, Model 5970, Column: DB-5, 25 m (J & W Scientific), Linear temperature

gradient profile: 50-280 °C increasing at the rate of 10 °C per minute. Consistent with a characteristic of dibrominated compounds, dibromo-hexamethyl-rufigallol exhibited 3 mass parent ions centered on 546 amu. Characterization of dibromorufigallol was based primarily on ^{1}H-magnetic resonance spectroscopy which indicated that all methyl groups had been removed from the parent compound. This compound was refractory to confirmatory mass spectral analysis, including fast atom bombardment.

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