***In vitro* efficacy of 2,*N*-bisarylated 2-ethoxyacetamides against *Plasmodium falciparum*.**

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Abstract— Investigation of a series of 2,*N*-bisarylated 2-ethoxyacetamides resulted in the identification of four inhibitors **5**, **20**, **24**, **29** with single-digit micromolar *in vitro* efficacy against two drug-resistant *P. falciparum* strains. These compounds are analogs of structurally-related 1,3-bisaryl-2-propen-1-ones (chalcones), the latter showing efficacy *in vitro* but not in a malaria-infected mouse. The 2,*N*-bisarylated 2-ethoxyacetamides (e.g. **2**, **5**, **20**) were shown to possess significantly greater stability in the presence of metabolizing enzymes than the corresponding 1,3-bisaryl-2-propen-1-ones (e.g. **1**, **3**, **18**). ©2015 Elsevier Science Ltd. All rights reserved.

Each year, more than 500,000 people die from malaria.¹ The organism responsible for most of these deaths, *Plasmodium falciparum*, has developed resistance to most available drugs.^{2,3} Thus, there is an urgent need for novel and affordable new products.⁴ Chalcones (1,3-diphenyl-2-propen-1-ones) and their carbocyclic and heterocyclic analogs display a wide range of biological activities, including antiprotozoal, antibacterial, antifungal and antiproliferative activity.⁵⁻⁷ Numerous mechanisms of action for the chalcones have been proposed and supported with experimental data.⁷⁻⁹ Several groups have studied the relationship between the structure of substituted chalcones and their antimalarial activity.¹⁰⁻¹⁴ Chalcones possessing submicromolar efficacy against drug-resistant strains of *P. falciparum* *in vitro* have been identified. The emerging SAR does not correlate with the SAR of known antimalarial targets,^{10,12} implying that the mechanism by which the chalcones act is distinct from established antimalarial mechanisms. Several of these compounds have been tested in animal models of malaria (*P. yoelii*- or *P. berghei*-infected mice),^{10,12-14} and some possess significant *in vivo* efficacy.¹²⁻¹⁴ However, *in vitro* and *in vivo* efficacies often do not correlate.^{10,12} Although possibly due to the difference in

Plasmodium species employed in *in vitro* and *in vivo* assays, we suspect that rapid compound metabolism also contributes, because the chalcones tested by us were all rapidly biotransformed when exposed to a preparation of human liver microsomes *in vitro*.¹⁰ Despite the considerable interest in the biological activities of chalcones, few experimental studies of chalcone ADME, including metabolism, have been described.¹⁵

We therefore wished to identify a compound series structurally distinct from the chalcones, but that mediates antimalarial activity via the same mechanism. We aimed to achieve this through use of a 3D pharmacophore that we had developed using CATALYST software which models the structural and electronic features required by compounds to effect antimalarial activity via this novel chalcone-mechanism.¹⁶

Using this model we ascertained that the two aryl substituents appear to be involved in target binding, but not the linking 2-propen-1-one group. We therefore searched the Walter Reed Army Institute of Research's compound database for compounds possessing these substituents but containing an alternative linking group.^{16,17} The 2,*N*-bisphenyl-2-ethoxyacetamides emerged as one possibility. In contrast to the rapid

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degradation of 1,3-bis-(4-chlorophenyl)-2-propen-1-one **1** upon exposure to a preparation of human liver microsomes *in vitro*, the corresponding 1,3-bis-(4-chlorophenyl)-2-ethoxyacetamide **2** exhibited significant stability. The potency of this amide **2** against *Plasmodium in vitro* was 2-3-fold lower than that of the chalcone **1**, but we had already learned which substitution patterns enhance the antiplasmodial potency of the chalcones.^{10,16} We therefore proposed the synthesis of 2,*N*-bisaryl 2-ethoxyacetamides (“amides”) containing these potency-enhancing substitution patterns, with the aim of producing potent, metabolically-resistant analogs of the potent, but metabolically-labile, 2-propen-1-ones (“chalcones”).

The most potent chalcones **3**, **8**, **18** previously identified contain either 2,5- or 3,4-dichlorophenyl-substitution proximal to the carbonyl of the chalcone linker (the R₁ position) with either 3- or 4-quinoliny-substitution in the distal position (R₂).^{10,16} Our modeling suggested that only the aryl substituents (and not the atoms of the linker) are involved in target binding,¹⁶ but we nevertheless decided to produce both possible analogs of unsymmetrical amides, namely those possessing a dichlorophenyl-substituent at R₁ with a quinoliny-substituent at R₂ and those with a dichlorophenyl-substituent at R₂ and a quinoliny-substituent at R₁.

The preparation of the former series began with synthesis of the requisite dichloroglycolic acids, as described in the literature¹⁸ and shown in Scheme 1.¹⁹ Treatment of the compounds with diethyl sulfate, in the manner described with glycolic acid itself yielded the corresponding 2-ethoxyacetic acids.¹⁸ Reaction with a variety of amines using standard peptide coupling reagents yielded the novel dichlorophenyl-*N*-quinoliny-2-ethoxy-acetamides **5-7**, **10-12**, **20-22**, **25-27**, **30-32**.²⁰

Scheme 1. a. aq. NaHSO₃, ether; aq. NaCN. b. aq. HCl. c. Et₂SO₄, aq. NaOH. d. R₂NH₂, *N*-Hydroxybenzotriazole, *o*-Benzotriazolyl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, *i*-Pr₂NEt.

Preparation of the R₁/R₂-reversed compound series began by preparation of 4-quinolineglycolic acid as described in the literature²¹ and shown in Scheme 2.¹⁹ Ethylation of this compound using diethyl sulfate proved unsuccessful, but treatment with iodoethane in the presence of freshly prepared silver oxide yielded a bisethylated compound. This was directly reacted with various amines using a trimethyl aluminum catalyst to

yield novel *N*-dichlorophenyl-4-quinoliny-2-ethoxy-acetamides **9**, **19**, **29**.²² We also discovered that the analogous reactions could be performed with the corresponding 3-quinoline-containing compounds. Thus, novel *N*-dichlorophenyl-3-quinoliny-2-ethoxy-acetamides **4**, **14**, **24**, were also prepared.

Scheme 2. a. aq. NH₄Cl, ether; aq. NaCN, *i*-PrOH. b. aq. HCl. c. EtI, Ag₂O. d. R₂NH₂, Me₃Al, PhMe, CH₂Cl₂.

The structures of the compounds prepared, and the results from their biological testing, are shown in Table 1. Compound identity, and assurance of purity exceeding 95%, was established by ¹H-NMR and ¹³C-NMR. All the compounds were assayed *in vitro* against two strains of *P. falciparum*, and selected compounds for predicted metabolic stability by *in vitro* exposure to human and in some cases mouse liver microsome preparations.²³⁻²⁴

Most of the novel amides were active *in vitro* against *P. falciparum*, with several possessing single-digit micromolar (IC₅₀ < 10 μM) *in vitro* efficacy against the two drug-resistant strains tested, the chloroquine, quinine and pyrimethamine-resistant W2 strain and the mefloquine-resistant D6 strain. (One determination of each IC₅₀ was made; in other studies, when multiple determinations were made, standard deviations were generally small, such that a four-fold difference in activity is likely to be significant). As was previously seen with the chalcones, the efficacies of the amides in the W2 and D6 strains were similar; a preliminary indication that the compounds are devoid of significant cross-resistance (Resistance Indices ≈ 1).¹⁰

The most potent chalcones **3**, **8** identified previously combine either 3- or 4-quinoliny-substitution with 2,5-dichlorophenyl substitution. Activities of the 3-quinoline-containing amides **4**, **5** analogous to the 3-quinoliny-chalcone **3** were compared, together with those of the analogous 2- and 6-quinoline-containing derivatives **6**, **7**. Similarly, activities of the 4-, 5- and 8-quinoline-containing amides (**9-10**, **11**, **12** respectively) were compared to those of the analogous 4-quinoliny chalcone **8**. All of the amide analogs (**4-7**, **9-12**) were found to be less potent than the chalcones (**3,8**) against both strains of drug-resistant parasite. The most active amide **5** was found to possess potency ten-fold lower against the D6 strain and six-fold lower against W2 than that of its corresponding chalcone **3**.

Table 1. Analogs synthesized with *in vitro* efficacies against *P. falciparum* D6 (mefloquine-resistant) and W2 (chloroquine, quinine and pyrimethamine-resistant) strains, % biotransformed *in vitro* by human or human and mouse liver microsomes and calculated LogPs.

Compound Number	Compound type	R ¹	R ²	<i>P.f.</i> D6 IC ₅₀ , μM ^a	<i>P.f.</i> W2 IC ₅₀ , μM ^a	Resistance Index ^b	% Biotransformed after ½ hr (HLM) or Biotransformation half-life (HLM, MLM) ^c	LogP ^d
chloroquine				0.24	0.013	19	-	-
1	Chalcone	4-chlorophenyl	4-chlorophenyl	2.6	5.3	2.0	85%	5.30±0.39
2	Amide	4-chlorophenyl	4-chlorophenyl	7.3	11	1.5	0%	5.14±0.55
^e 3	Chalcone	2,5-dichlorophenyl-	3-quinoliny-	0.62	1.7	2.7	100%	4.78±0.40
4	Amide	3-quinoliny-	2,5-dichlorophenyl-	14	14	1.1	-	-
5	Amide	2,5-dichlorophenyl-	3-quinoliny-	6.7	9.6	1.4	t _{1/2} = 27 min, 9 min	5.18±0.56
6	Amide	2,5-dichlorophenyl-	2-quinoliny-	19	>27	-	-	-
7	Amide	2,5-dichlorophenyl-	6-quinoliny-	>17	>17	-	-	-
^e 8	Chalcone	2,5-dichlorophenyl-	4-quinoliny-	0.19	0.23	1.2	100%	4.78±0.40
9	Amide	4-quinoliny-	2,5-dichlorophenyl-	14	15	1.0	-	-
10	Amide	2,5-dichlorophenyl-	4-quinoliny-	12	14	1.2	-	-
11	Amide	2,5-dichlorophenyl-	5-quinoliny-	11	15	1.4	-	-
12	Amide	2,5-dichlorophenyl-	8-quinoliny-	14	>27	-	-	-
^e 13	Chalcone	3,4-dichlorophenyl-	3-quinoliny-	3.1	12	4.0	-	-
14	Amide	3-quinoliny-	3,4-dichlorophenyl-	18	13	0.71	-	-
15	Amide	3,4-dichlorophenyl-	3-quinoliny-	13	5.9	1.2	-	-
16	Amide	3,4-dichlorophenyl-	2-quinoliny-	19	15	0.76	-	-
17	Amide	3,4-dichlorophenyl-	6-quinoliny-	7.7	9.1	0.46	-	-
^e 18	Chalcone	3,4-dichlorophenyl-	4-quinoliny-	1.7	0.84	0.48	100%	5.31±0.39
19	Amide	4-quinoliny-	3,4-dichlorophenyl-	15	12	0.80	-	-
20	Amide	3,4-dichlorophenyl-	4-quinoliny-	3.2	1.7	0.53	t _{1/2} = 27 min, 30 min	5.18±0.56
21	Amide	3,4-dichlorophenyl-	5-quinoliny-	>27	>27	-	-	-
22	Amide	3,4-dichlorophenyl-	8-quinoliny-	20	16	0.84	-	-
^e 23	Chalcone	2,4-dichlorophenyl-	3-quinoliny-	7.9	6.3	0.80	-	-
24	Amide	3-quinoliny-	2,4-dichlorophenyl-	4.5	2.0	0.43	t _{1/2} > 60 min, > 60 min	4.60±0.56
25	Amide	2,4-dichlorophenyl-	3-quinoliny-	9.8	27	2.7	-	-
26c	Amide	2,4-dichlorophenyl-	2-quinoliny-	15	14	0.88	-	-
27	Amide	2,4-dichlorophenyl-	6-quinoliny-	15	14	0.92	-	-
^e 28	Chalcone	2,4-dichlorophenyl-	4-quinoliny-	6.2	18	3.0	-	-
29	Amide	4-quinoliny-	2,4-dichlorophenyl-	6.9	1.8	0.26	t _{1/2} > 60 min, > 60 min	4.60±0.56
30	Amide	2,4-dichlorophenyl-	4-quinoliny-	14	15	1.0	-	-
31	Amide	2,4-dichlorophenyl-	5-quinoliny-	>27	>27	-	-	-
32	Amide	2,4-dichlorophenyl-	8-quinoliny-	>27	>27	-	-	-

^a Inhibition of [³H] hypoxanthine uptake by *P. falciparum*; values from one experiment.²³ ^b IC₅₀ against W2 / IC₅₀ against D6. ^c HLM, Human liver microsomes, MLM Mouse liver microsomes, values from one experiment.²⁴ ^d LogP calculated using ACD ChemSketch 12.0. ^e *In vitro* efficacy against *P. falciparum* W2 and D6 reported previously.¹⁰

Additionally, it was found that reversing the location of R₁/R₂ has a minimal impact on activity; evidenced by the comparable activity of amide **4** versus **5**, and of amide **9** versus **10**, against both strains.

Other potent chalcones **13**, **18**, identified previously, combine either 3- or 4-quinoliny-substitution with 3,4-dichlorophenyl substitution. Once again, the activities of these chalcones were compared with those of their analogous amides **14-17** and **19-22**, respectively. The

range of potencies of these 3,4-dichlorophenyl-containing amides was greater than for their 2,5-dichlorophenyl-containing analogs **4-7**, **9-12**; with most of the amides being moderately active against both parasite strains. Notably, amide **20**, which possesses significant activity, is just two-fold less potent than its corresponding chalcone **13** against both strains. In contrast to the 2,5-dichlorophenyl-containing series, where the most potent amide **5** was *N*-substituted with a 3-quinoliny-group; in the 3,4-dichlorophenyl series the

most potent amide against both strains **20** was *N*-substituted with a 4-quinolinyl-group.

Finally the activities of the amides containing 2,4-dichloro-substitution **24-27**, **29-32** were compared with those of the analogous 3- and 4-quinolinyl-containing chalcones **23**, **28**. In these series the two most potent amides are more potent against both strains than their corresponding chalcones (**23** compared to **24**, **28** compared to **29**). In contrast to the previous series, these more active amides are *N*-substituted with the 2,4-dichlorophenyl-group and possess either 3- or 4-quinolinyl-substitution at the 2-position.

Having achieved the goal of producing amides with comparable *in vitro* antiparasmodial efficacy to the chalcones we have described previously,¹⁰ the important question became how they compared in terms of predicted metabolic stability. Compound stability in the presence of human versus mouse liver microsomes was similar (**5**, **20**, **24**, **29**), so only the former was measured for most compounds. The same compounds, four of our most potent amides, were found to undergo biotransformation upon exposure to a human liver microsome preparation much more slowly than do the structurally-related chalcones **3**, **8**, **18**. In contrast to all the chalcones that we have tested (including those described herein) that are 100% biotransformed within a 30 min of exposure to a human liver microsome preparation, the half-life for the most stable of the amide analogs exceeds 60 min (**24**, **29**). Moreover, stability in the presence of microsomes appears to correlate with amide structure: being greater in the compounds where the quinoline group is proximal to the α -ethoxy (**24**, **29**), compared to those where it is proximal to the amide functional group (**5**, **20**). In contrast, calculated LogP values suggest that there is not an obvious relationship between compound stability in the presence of microsomes versus compound lipophilicity.

In conclusion, five 2,*N*-bisphenyl-2-ethoxyacetamides **5**, **17**, **20**, **24**, **29** with single-digit micromolar efficacy against two different drug-resistant strains of *P. falciparum* *in vitro* have been identified. These compounds possess considerably greater predicted metabolic stability compared to structurally-related 1,3-bisaryl-2-propen-1-ones (chalcones). This is important because chalcones with significant antiparasmodial efficacy *in vitro* have failed to demonstrate efficacy in a malaria-infected mouse *in vivo*, which we suspect is due in part to metabolic lability. In future studies, we plan to investigate the amides described herein in malaria-infected mice, with the expectation that their improved resistance to metabolism may allow their *in vitro* antiparasmodial efficacy to be realized *in vivo*.

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23. *In vitro* efficacy was determined by a modified version of Desjardins' method, in which parasites were pre-exposed to test compound prior to measurement of their [³H]-hypoxanthine uptake, as reported previously.¹⁰
24. To predict metabolic stability a 25 μ M solution of test compound was prepared in a mixture containing pooled human liver microsomes (0.5 mg/mL total protein from BD Gentest) and 0.1 M sodium phosphate buffer (pH 7.4) with an NADPH-regenerating system (1.25 mM β NADP⁺, 3.3 mM glucose-6-phosphate, and 3.3 mM MgCl₂). Glucose-6-phosphate dehydrogenase (1 unit/mL final concentration) was added to initiate reaction. The amount of parent compound remaining at various time points was determined using LC-MS analysis; half-life was calculated assuming first-order decay, as described in Shearer, T. W.; Kozar, M. P.; O'Neil, M. T.; Smith, P L, Schiehser, G. A.; Jacobus, D. P.; Diaz, D. S.; Yang, Y.-S.; Milhous, W. K.; Skillman, D. R. *J. Med. Chem.* 2005, 48, 2805.

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Four inhibitors with single-digit micromolar *in vitro* efficacy against two drug-resistant *P. falciparum* strains were identified, including compound **20**. They possess greater stability in the presence of metabolizing enzymes than do the corresponding 1,3-bisaryl-2-propen-1-ones, of which they are analogs.