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Discovery of Novel Targets of Quinoline Drugs in the Human Purine Binding Proteome

PAUL R. GRAVES, JESSE J. KWIEK, PATRICK FADDEN, RUPA RAY, KLAAS HARDEMAN, ANDREW M. COLEY, MICHAEL FOLEY, and TIMOTHY A. J. HAYSTEAD

Department of Pharmacology and Cancer Biology, Center for Chemical Biology, Duke University, Durham, North Carolina (P.R.G., J.J.K., R.R., T.A.J.H.); Serenex Inc., Durham, North Carolina (P.F., K.H.); Department of Pharmacology and Program in Molecular Medicine, University of Virginia, Charlottesville, Virginia (J.J.K.); and Department of Biochemistry and the Cooperative Research Centre for Diagnostics, La Trobe University, Melbourne, Australia (A.M.C., M.F.)

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ABSTRACT

The quinolines have been used in the treatment of malaria, arthritis, and lupus for many years, yet the precise mechanism of their action remains unclear. In this study, we used a functional proteomics approach that exploited the structural similarities between the quinoline compounds and the purine ring of ATP to identify quinoline-binding proteins. Several quinoline drugs were screened by displacement affinity chromatography against the purine binding proteome captured with γ-phosphate-linked ATP-Sepharose. Screening of the human red blood cell purine binding proteome identified two human proteins, aldehyde dehydrogenase 1 (ALDH1) and quinone reductase 2 (QR2). In contrast, no proteins were detected upon screening of the *Plasmodium falciparum* purine binding proteome with the quinolines. In a complementary approach, we passed cell lysates from mice, red blood cells, or *P. falciparum* over hydroxychloroquine- or primaquine-Sepharose. Consistent with the displacement affinity chromatography screen, ALDH and QR2 were the only proteins recovered from mice and human red blood cell lysate and no proteins were recovered from *P. falciparum*. Furthermore, the activity of QR2 was potently inhibited by several of the quinolines in vitro. Our results show that ALDH1 and QR2 are selective targets of the quinolines and may provide new insights into the mechanism of action of these drugs.

The quinolines represent one of the most successful yet poorly understood classes of drugs. Notable examples of the quinoline compounds include the 4-aminoquinoline chloroquine, the 8-aminoquinoline primaquine, and the quinolinemethanols mefloquine and quinine. Until the emergence of drug-resistant parasites, these drugs were the most effective means to treat malaria, a disease that claims 1 to 3 million lives annually (Foley and Tilley, 1998). In addition to malaria, quinoline-containing and structurally related compounds have been used in the treatment of lupus erythematosus (Van Beek and Piette, 2001), arthritis (Fox, 1993), and HIV (Savarino et al., 2001) and have been shown to exhibit antiprion activity (Korth et al., 2001). Remarkably, there is no clear mechanism known for the therapeutic action of these drugs in any of these diseases. The most accepted theory for the action of the quinoline drugs in the treatment of malaria is interference with heme detoxification within the red blood cell (Foley and Tilley, 1997). However, the heme detoxification mechanism cannot explain the action of the quinoline compounds in the treatment of arthritis, lupus, or HIV.

The quinoline drugs share some common structural features with purine nucleotides, such as the heterocyclic quinoline ring that is analogous to the C1-C10 ring of purines (Fig. 1). We hypothesized that proteins that interact with purines might also be quinoline-interacting proteins. Proteins that are regulated by or use purines represent approximately 4% of the genome (Lander et al., 2001; Venter et al., 2001), and many of these proteins perform essential functions in the cell. Examples include enzymes involved in the synthesis of RNA and DNA, dehydrogenases, and protein and nonprotein kinases. In the present study, we used an affinity matrix, γ-phosphate-linked ATP-Sepharose, to isolate the entire purine binding proteome from an animal or cell lysate. We then screened the purine binding proteomes from mouse, human red blood cells (RBCs), and *Plasmodium falciparum* with quinoline drugs. We report the direct interaction between quinoline antimalarials and the human proteins alde-

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P.R.G., J.J.K., P.F., and R.R. contributed equally to this work. This work originated in the laboratory of T.A.J.H.

ABBREVIATIONS: HIV, human immunodeficiency virus; RBC, red blood cell; ALDH1, aldehyde dehydrogenase 1; QR2, quinone reductase 2; PQ, primaquine; HCQ, hydroxychloroquine; PAGE, polyacrylamide gel electrophoresis; QR1, quinone reductase 1; NMeH, *n*-methlydihydronicotinamide; GST, glutathione-*S*-transferase; CQ, chloroquine; MQ, mefloquine; QC, quinacrine; Q, quinine.

hyde dehydrogenase 1 (ALDH1) and quinone reductase 2 (QR2).

Materials and Methods

***P. falciparum* Cultures.** *P. falciparum* strain 3D7 was obtained from the Malaria Research and Reference Reagent Resource Center (MR4)/American Type Culture Collection (Manassas, VA) and grown according to the included specifications. Parasites were harvested by saponin lysis as described previously (Schlichtherle et al., 2000). *P. falciparum* growth was measured by [^{3}H]hypoxanthine uptake as described previously (Schlichtherle et al., 2000).

Reagents. All compounds were obtained from Sigma-Aldrich (St. Louis, MO) except for mefloquine-HCl, which was obtained from F. Hoffman-La Roche (Basel, Switzerland).

Preparation of ATP, primaquine (PQ), and Hydroxychloroquine (HCQ)-Sepharose. ATP-Sepharose was prepared as described previously (Haystead et al., 1993). PQ-Sepharose was prepared by coupling primaquine diphosphate to *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow obtained from Pharmacia (Peapack, NJ) in 100 mM HEPES, pH 8.3, for ~12 h at room temperature. HCQ-Sepharose was prepared by coupling hydroxychloroquine to epoxy-activated Sepharose 6B (Pharmacia) according to the manufacturer's instructions.

ATP, PQ, and HCQ-Sepharose Affinity Chromatography. *P. falciparum*-infected or noninfected RBCs were lysed by mixing with an equal volume of 2× buffer A and rocking for 30 min at 4°C (1× buffer A: 50 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 μg/ml leupeptin, 100 μg/ml pefabloc, and 1 μg/ml aprotinin). For mouse homogenates, a whole mouse (except for the tail, feet, skin, and intestines) was frozen in liquid N_2, crushed, and blended in buffer A. Mouse or RBC lysate was clarified by centrifugation for 1 h at 100,000*g* and applied to the ATP or quinoline drug-affinity columns equilibrated in buffer A. The columns were washed with ~100 column volumes of buffer A, followed by buffer A containing 1 M NaCl, and then reequilibrated in buffer A. For elutions, all compounds were dissolved in buffer A and adjusted to pH 7.5. Proteins were resolved by 12% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250 or silver nitrate. Alternatively, proteins were transferred to polyvinyl membrane (Kaysville, UT) for mixed peptide sequencing (Damer et al., 1998).

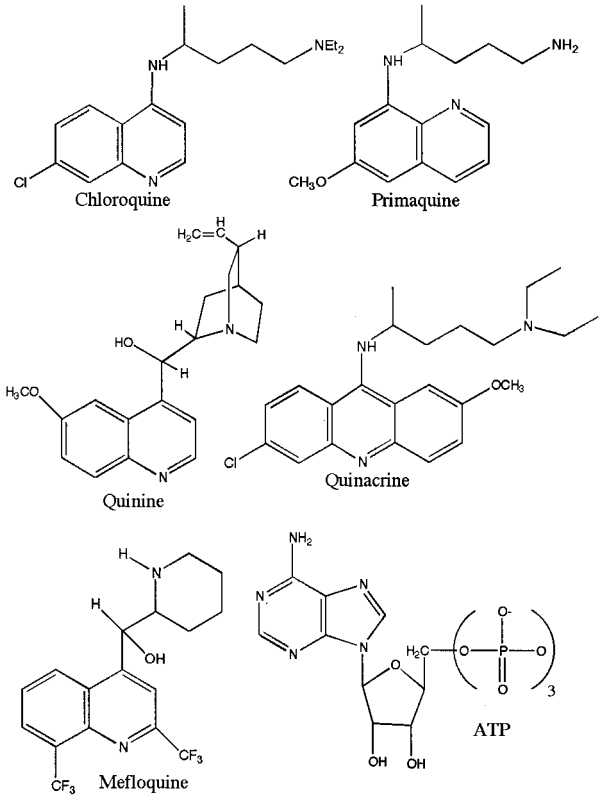


Fig. 1. Chemical structure of ATP and the antimalarial compounds studied.

Protein Sequencing. Edman-based mixed peptide sequencing was carried out as described previously (Damer et al., 1998). The mixed sequences were sorted and matched against the entire published protein (SWISS-PROT, NCBI, or mouse EST) or DNA databases with the FASTF or TFASTF algorithms, respectively (Damer et al., 1998; Mackey et al., 2002) For mass spectrometry, protein samples were in-gel digested with trypsin according to the method of Shevchenko et al. (1996). Extracted tryptic peptides were purified with Poros R2 (Applied Biosystems, Foster City, CA) according to a protocol on the Web site at http://protana.com. The extracted peptides were concentrated in a nano-electrospray capillary (Protana, Odense, Denmark) and placed in the source head of an API QSTAR Pulsar Hybrid mass spectrometer (Applied Biosystems). Mass spectra data were analyzed with Q-analyst software (Applied Biosystems) to derive de novo peptide sequences. Peptide sequences were searched against the nonredundant sequence database using FASTS (Mackey et al., 2002).

Purification of Native ALDH1, QR2, and Quinone Reductase 1. RBC extract was prepared as described above and applied to PQ-Sepharose equilibrated in buffer A. ALDH1 and QR2 were obtained by eluting the column with 5 mM β-NAD^+ and *n*-methyldihydronicotinamide (NMeH), respectively. QR1 was purified from rabbit liver as described previously (Lind et al., 1990). All enzymes were sequenced to confirm their identify and were >90% pure as judged by SDS-PAGE and silver staining.

Cloning of Human QR2. Human QR2 was PCR amplified from human liver cDNA (BD Clontech, Palo Alto, CA) with the following primers: 5′-GCTATGGCAGGTAAGAAAGTACTC-3′ and 5′-GCCACAGAGTTATTGCCCGAAGTG-3′ and cloned into the pGEX-4T-2 GST expression vector (Pharmacia). GST-tagged QR2 was purified and the GST tag removed according to the manufacturer's instructions.

ALDH1, QR2, and QR1 Activity Assays. ALDH1 activity was determined using an high-performance liquid chromatography-based assay because of the coabsorbance of chloroquine (CQ) and NADH at 340 nm. Reaction products were separated with a gradient of acetonitrile in 10 mM triethylamine acetic acid. CQ and NADH peaks were identified by their signature spectra using an online photodiode array detector. QR2 activity was assayed in triplicate with recombinant QR2 (at 96 ng/ml) by measuring the absorbance at 365 nm in a buffer containing 50 mM Tris-HCl, pH 8.5, 50 μM NMeH, 5 to 30 μM menadione, and 0.1% Triton X-100. NMeH was synthesized as described previously (Ortiz-Maldonado et al., 1999). QR2 K_i values were calculated using KinetAsyst II by fitting the experimental data to the equations of Cleland (1979). QR1 activity assays were performed in triplicate according to the method of Chen et al. (1999). QR1 IC_{50} values were calculated using Prism (GraphPad Software, San Diego, CA).

Results

Capture of the Mouse, RBC, and *P. falciparum* Purine Binding Proteomes on ATP-Sepharose. To better understand the mechanism of the quinolines, we attempted to identify all quinoline-interacting proteins in a cell or animal lysate. To achieve this, we used a functional proteomics approach as outlined in Fig. 2. In this strategy, three different, yet complementary approaches were conducted to identify and validate targets of the quinolines. In step 1, termed

displacement affinity chromatography, a specific subproteome from a cell is captured on an affinity matrix by virtue of its interaction with an immobilized ligand (Fig. 2, step 1). The subproteome is captured after application of saturating amounts of cell lysate and extensive washing of the resin. The compounds of interest (in this case, the quinolines) are then applied to the matrix in parallel and allowed to interact with the bound proteome. If a compound is capable of interacting with a bound protein and can displace it from the affinity matrix, the protein is recovered in the eluent and identified by mass spectrometry. Because the drug presumably has the potential to interact with all of the proteins bound to the matrix, information about drug specificity can be obtained by identification of the eluted proteins (Fig. 2, step 1). In the second step, we created affinity matrices by directly linking the quinoline drugs to Sepharose, and after application of cell lysates, identified all proteins that specifically eluted from these matrices in the presence of drug (Fig. 2, step 2). Finally, in the third step, protein targets identified in the first two steps were assayed for activity in the presence of the quinolines (Fig. 2, step 3).

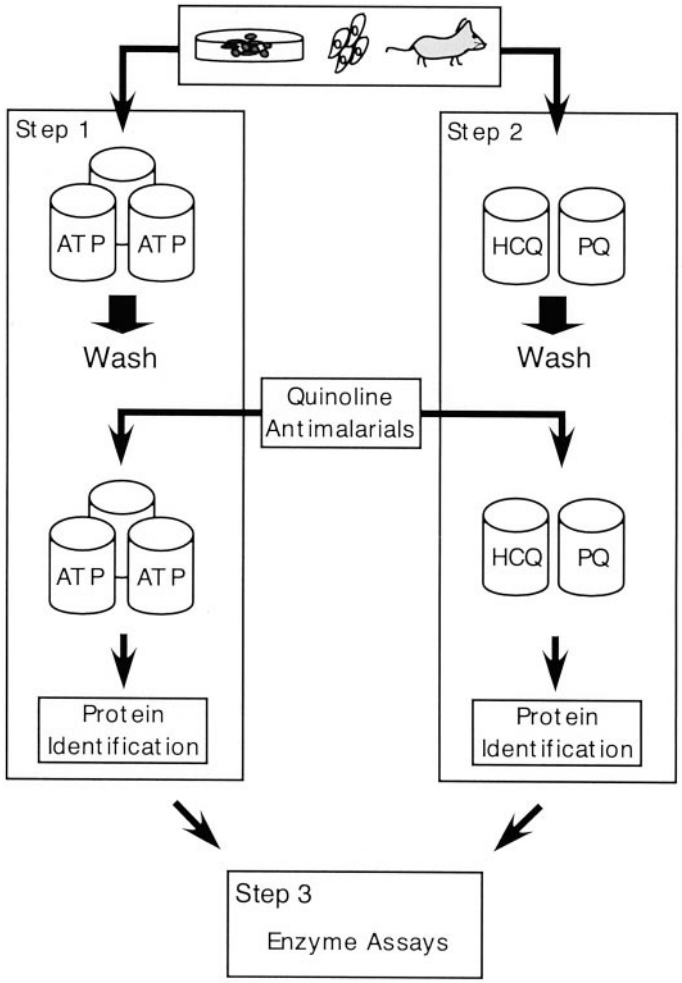


Fig. 2. Proteomics strategy for identification and validation of quinoline antimalarial drug targets. In step 1, a cell or animal lysate is passed over columns of ATP-Sepharose in parallel, washed to remove nonspecific proteins, and then proteins are eluted with quinoline antimalarials. In step 2, a cell or animal lysate is passed over quinoline antimalarial drug columns (HCQ-Sepharose and PQ-Sepharose), washed to remove nonspecific proteins, and then proteins eluted with quinoline antimalarials. All proteins are then sequenced and identified by mass spectrometry. In step 3, the isolated proteins from steps 1 and 2 are assayed for biological activity in the presence of quinoline antimalarials.

In this study, we used ATP linked to Sepharose via its γ-phosphate group to capture the purine binding proteome of cells for subsequent screening with the quinoline drugs (Haystead et al., 1993). To determine the specificity of γ-ATP-Sepharose, we saturated the affinity matrix with extract from a whole homogenized mouse. After extensive washing to remove nonspecific proteins, the resin was sequentially eluted with NADH, AMP, ADP, and ATP, and the eluted proteins were characterized by one-dimensional or two-dimensional SDS-PAGE (Fig. 3, A and B). Importantly, if ATP was linked to Sepharose through adenosine at N6 (N-6 linked resin) very few proteins were recovered from mouse extract (Fig. 3A).

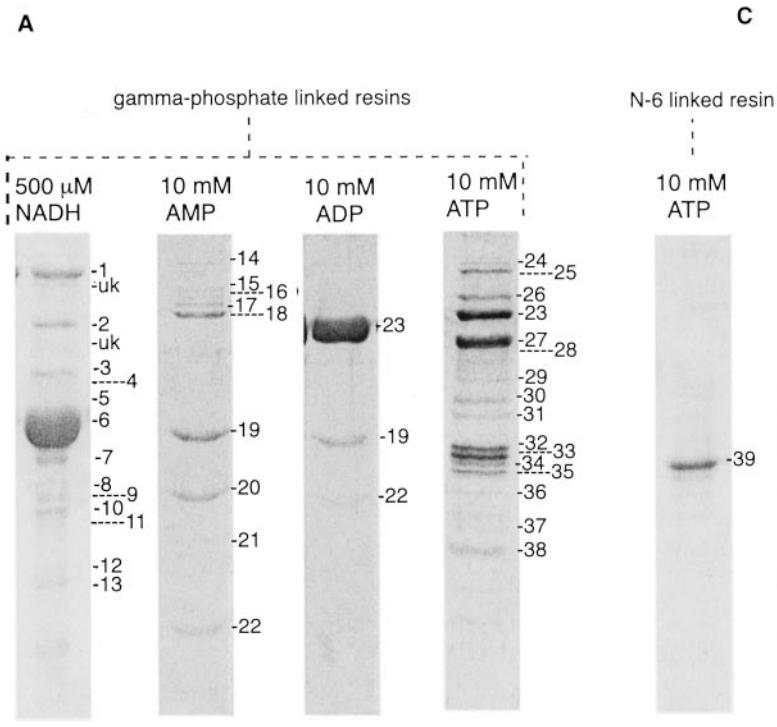
On average, ~400 distinct proteins ($n = 8$) were detected in the gels of which 72 were identified by mixed peptide sequencing (Damer et al., 1998) and mass spectrometry. Examination of the proteins that bound specifically to ATP-Sepharose (Fig. 3C) indicates that a diverse array of purine nucleotide using proteins was recovered. Moreover, the selectivity of ATP-Sepharose for purine binding proteins is demonstrated by the fact that all the proteins sequenced use purines or molecules closely resembling purines. Bound proteins identified include protein and nonprotein kinases, dehydrogenases, DNA ligases, mononucleotide ATPases, and nonconventional purine binding proteins (Fig. 3C).

To capture the purine binding proteome from human RBCs or *P. falciparum*, cell extracts from RBCs and *P. falciparum* parasites were passed in parallel over ATP-Sepharose. Sufficient cell mass (10^7–10^8 cells) was applied to each column to saturate all available ATP binding sites and to ensure detection and recovery of proteins expressed at 100 copies/cell (1 fmol). A selection of the bound proteins from each column was sequenced after elution with SDS (Fig. 4, A and B). Proteins were identified by FASTF or FASTS (Mackey et al., 2002) database searching algorithms with peptide sequences derived from mixed peptide sequencing or mass spectrometry, respectively. This search strategy was important because RBCs infected with *P. falciparum* contained a mixture of human and *P. falciparum* proteins. Using multiple peptide alignments, expectation (e) scores for top scoring *P. falciparum* proteins ranged from 10^{-6} to 10^{-33} compared with their respective human homologs that generally ranged from 10^{-2} to 10^{-14} (Fig. 4B). Because of the large diversity of proteins from human RBC and *P. falciparum* captured on ATP-Sepharose, this matrix is ideal for screening targets of the quinolines.

Identification of Quinoline Antimalarial Binding Proteins in the Human Red Blood Cell Purine Binding Proteome by Displacement Affinity Interaction. To identify quinoline binding proteins from human RBCs, ATP-Sepharose columns were charged with RBC extracts, washed, and eluted in parallel with 5 mM CQ, PQ, and mefloquine (MQ). All three drugs selectively eluted proteins of 55 and 26 kDa (Fig. 5A). The 55- and 26-kDa proteins were sequenced by mass spectrometry and identified as human ALDH1 (EC 1.2.1.3) and human QR2 (EC 1.6.99.2), respectively (Fig. 5C). Considering the number of other purine binding proteins captured by ATP-Sepharose from RBCs (Fig. 4A), these data indicate that the quinoline moieties of CQ, PQ, and MQ are highly selective toward ALDH1 and QR2.

To identify quinoline binding proteins from *P. falciparum*, parasites were isolated from *P. falciparum*-infected RBCs by

saponin lysis (Schlichtherle et al., 2000) and washed extensively to remove RBC proteins. The parasites were lysed, applied to ATP-Sepharose, washed, and eluted with 5 mM CQ. A single protein was detected in the eluate and was identified by mass spectrometry sequencing as human ALDH1 (Fig. 5B). The presence of human ALDH1 in the *P.*


A
gamma-phosphate linked resins
N-6 linked resin
500 µM NADH
10 mM AMP
10 mM ADP
10 mM ATP
10 mM ATP
B

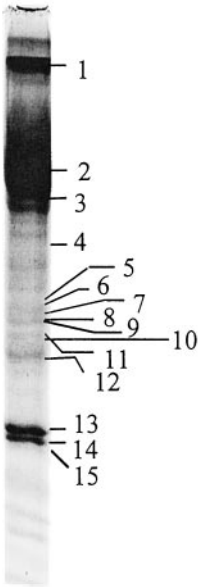
#	Protein ID	MW	(e)
1	2-Oxogluterate /dehydrogenase	95.1	9.3e-11
2	NADH dehydrogenase	65.0	1.9e-17
3	glutamate dehydrogenase	60.1	2.2e-16
4	aldehyde dehydrogenase	55.3	1.7e-33
5	6-phosphogluconate dehydrogenase	50.2	3.1e-17
6	GAPDH	45.3	2.2e-22
7	malate dehydrogenase	35.5	9.1e-6
8	sorbitol dehydrogenase	30.3	1.7e-14
9	lactate dehydrogenase	28.2	6.6e-33
10	isocitrate dehydrogenase	28.0	3.1e-44
11	3 hydroxyl CoA dehydrogenase	27.5	2.2e-6
12	alcohol dehydrogenase	25.2	1.7e-17
13	GAPDH fragment	20.2	5.5e-16
14	phosphoinositide kinase	150.2	5.6e-32
15	phosphorylase	86.3	4.2e-33
16	DUN1 protein kinase (related)	80.1	2.2e-11
17	AMP activated protein kinase	65.3	2.3e-8
18	formyltransferase	60.3	2.1e-17
19	purine synthetase(ADE2)	45.2	4.3e-37
20	ESTAA254816	35.6	9.1e-14
21	ESTAA571903	20.2	7.4e-11
22	purine synthetase (fragment)	19.3	9.3e-14
23	HSP90	83.5	1.7e-27
24	MLCK	65.4	2.1e-30
25	phosphorylase kinase	124.0	3.1e-31
26	p98 glucose induced kinase	92.0	2.9e-11
27	HSP70	55.5	2.6e-13
28	glutamate ammonium ligase	50.5	1.7e-33
29	HSPA5	72.1	2.8e-6
30	adenosine kinase	30.1	1.1e-33
31	MEK	44.4	3.1e-17
32	CSK	50.6	3.2e-12
33	MAPK	41.2	1.1e-14
34	PKA	40.5	1.3e-22
35	arginine deimidase	45.3	3.1e-17
36	pyridoxal kinase	35.1	6.1e-15
37	actin-like protein 2	44.7	4.7e-17
38	tubulin	40.5	3.1e-16
39	actin	45.2	2.2e-8
40	protein kinase kem	85.0	3.1e-19
41	cyclic G kinase	84.0	1.2e-20
42	T08777 PKC related	60.3	1.1e-6
43	p90 RSK	83.1	3.2e-11
44	stress-activated protein kinase-2	82.5	3.3e-12
45	PKC beta II	76.8	2.1e-15
46	PKC epsilon	73.1	1.1e-44
47	EST probable protein kinase T08748	70.2	2.1e-5
48	p70 RSK	54.7	3.3e-7
49	phosphofructokinase	85.4	5.5e-11
50	RNA helicase	72.6	1.6e-32
51	pp60 csrc	59.1	1.2e-11
52	CAMKII	54.3	5.5e-21
53	HSP60	61.0	3.1e-20
54	ferredoxin reductase	54.0	9.7e-11
55	glutathione synthetase	52.5	2.7e-16
56	argininosuccinate synthetase	46.4	3.9e-11
57	protein kinase pkxl	40.8	4.2e-15
58	fructose 1,6 bisphosphatase	43.4	3.4e-7
59	ZIP kinase	52.5	5.2e-16
60	tubulin alpha	50.1	1.7e-10
61	protein kinase PC-1	33.5	3.2e-10
62	GSKIII	46.7	4.2e-8
63	beta tubulin	50.3	9.2e-6
64	CKI	47.1	3.1e-11
65	CKII	45.1	3.3e-22
66	proliferating cell nuclear antigen	40.2	1.7e-19
67	Lim domain kinase	39.0	2.2e-11
68	Lupus nephritis protein LN1	38.6	3.2e-9
69	serine/threonine kinase 13	32.0	1.1e-6
70	HSP90 (fragment)	30.1	1.8e-15
71	Y12	27.7	1.1e-16
72	calmodulin	16.8	9.6e-11

Fig. 3. Capture and analysis of the mouse purine nucleotide binding proteome. A, proteins were eluted from γ-linked ATP-Sepharose (charged with whole mouse extract) with the indicated nucleotides, resolved by one-dimensional SDS-PAGE, visualized by silver staining, and sequenced by mixed peptide sequencing (Damer et al., 1998) or mass spectrometry. B, two-dimensional SDS-PAGE of the eluate from γ-ATP-Sepharose after elution with ATP. C, list of identified proteins that specifically bound γ-linked ATP-Sepharose. The proteins molecular weight (MW) and expectation score (e) are shown.

falciparum-enriched sample is most likely due to the inability to remove all human RBC proteins from the isolated parasites. No *P. falciparum* proteins eluted from ATP-Sepharose with 5 mM CQ, even though *P. falciparum* proteins bound ATP-Sepharose (Figs. 4B and 5B). A search of the annotated *P. falciparum* genome with multiple peptide sequences derived from human ALDH1 or QR2 did not identify a *P. falciparum* homolog of these proteins (W. R. Pearson, personal communication). These findings indicate the presence of two novel targets for the quinoline drugs encoded by the human genome.

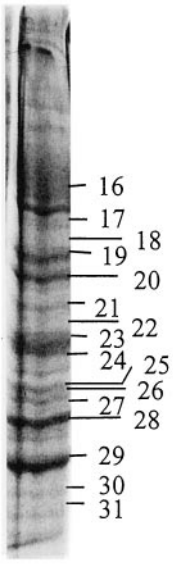
Primaquine and Chloroquine-Sepharose Selectively Bind ALDH1 and QR2 from Human RBCs. To investigate the selectivity of the quinolines further, PQ and HCQ affinity

A. Human RBCs



#	Human Protein ID	Mr	(e)
1	Spectrin	280.0	6.8e-8
2	Band-3 anion transporter	101.8	2.6e-36
3	Band-3 anion transporter (frag)	101.8	2.6e-36
4	Transitional ER atpase	89.0	1.7e-7
5	HSP90	84.6	1.5e-30
6	HSP70	70.9	6.5e-17
7	Aldehyde Dehydrogenase	55.0	1e-30
8	Glutathione synthase	52.4	3.9e-12
9	26S protease regulatory subunit	49.2	3.4e-11
10	PIP5 kinase Type II alpha	46.2	2.7e-7
11	ADE2	47.0	1.8e-25
12	ERK2	41.4	5.8e-12
13	Cinnamyl-alcohol dehydrogenase	39.0	9.7e-8
14	GAPDH	36.1	7.0e-27
15	Fructosamine-3-kinase	35.2	1.5e-16

B. *P. falciparum*-infected RBCs



#	Plasmodium falciparum Protein ID	Mr	(e)	Next Best Human (e)
16	101KD antigen	100	9.9e-10	1.7e-3
17	P98 glucose induced protein			
18	Rhoptry-associated protein1 (frag)	90.0	3.1e-22	
19	HSP90	90.0	9.3e-8	
20	HSP86	86.0	5.1e-6	0.13
21	HSP70	74.6	1.6e-33	2.0e-10
22	PEPCK fragment	69.2	3.6e-3	
23	Glutamate dehydrogenase			
24	Rhoptry-associated protein 2	46.7	2.2e-17	
25	RNA Helicase	45.3	1.1e-5	
26	Casein kinase I	37.8	4.8e-21	1.3e-14
27	GAPDH	36.6	1.4e-12	1.8e-4
28	ADE2			
29	LDH	34.0	2.6e-12	
30	dnak type molecular chaperone (frag)	33.6	3.2e-12	
31	Nucleosome assembly protein	31.8	2.8e-26	

Fig. 4. Capture and analysis of the human RBC and *P. falciparum* purine nucleotide binding proteome. Homogenates from noninfected RBCs (A) or *P. falciparum*-infected RBCs (B) were passed in parallel over columns containing γ-linked ATP-Sepharose, washed, and eluted with SDS. Proteins were resolved by SDS-PAGE and visualized by silver staining and identified by mixed peptide sequencing (Damer et al., 1998) or mass spectrometry. Expectation scores (e) are given for *P. falciparum* proteins and the next highest scoring human protein.

columns were generated. PQ and HCQ were immobilized to Sepharose via their primary amine and hydroxyl group, respectively (Fig. 1). This orientation of the immobilized PQ and CQ puts the quinoline moiety in a solvent-accessible position. PQ- and HCQ-Sepharose were charged with RBC extracts and eluted with 5 mM PQ or CQ, respectively (Fig. 6, A and B). Two major proteins eluted from PQ- and HCQ-Sepharose and were identified by microsequencing as human ALDH1 and QR2 (Fig. 6, A and B). To explore the specificity of PQ-Sepharose against a more complicated mixture of proteins, whole mouse extract was applied to PQ-Sepharose, washed, and then eluted with 5 mM PQ. Three proteins eluted with PQ and were identified by mass spectrometry as ALDH1, ALDH2, and QR2 (Fig. 6A). To test the strength of interaction between ALDH1, QR2, and PQ-Sepharose, the amount of Nonidet P-40 in the wash buffer was increased to 0.5%. Under these more stringent wash conditions, human QR2 was the only protein recovered from human RBCs after elution with CQ, PQ, QC, and quinine (Q) (Fig. 6A). This result suggests that ALDH1 binds PQ-Sepharose with a lower affinity than QR2. Significantly, when PQ- or HCQ-Sepharose was charged with *P. falciparum* lysate and eluted with PQ or CQ, respectively, no proteins were detected in the eluates (data not shown).

Inspection of the ALDH1 crystal structure suggests that the NAD^+ binding pocket is most likely responsible for the interaction of ALDH1 with ATP-Sepharose. This is supported by the elution of ALDH1 from ATP-Sepharose with NADH (Fig. 3A). The NAD^+ binding pocket is also probably the site where PQ binds ALDH1 because ALDH1 is selectively eluted from PQ-Sepharose with NAD^+ (Fig. 6C). For QR2, either the adenosine-binding pocket of the FAD^+ moiety or the substrate-binding pocket could explain its affinity for PQ-Sepharose. To determine which binding pocket was involved, PQ-Sepharose was charged with RBC extract and eluted with FAD^+ or the QR2 substrate analog NMeH (Ortiz-Maldonado et al., 1999) (Fig. 6C). No proteins were eluted with FAD^+, whereas NMeH eluted QR2, suggesting that the substrate binding pocket of QR2 is the site of interaction with the quinolines.

Quinolines Inhibit ALDH1 and QR2. To determine the effect of the quinolines on the activity of ALDH1, ALDH1 was assayed in vitro in the presence of CQ. Because of the coabsorbance of NADH and CQ at 340 nm, a high-performance liquid chromatography-based assay was developed to determine the effects of CQ on ALDH1 activity. At physiological concentrations of NAD^+, CQ was a relatively weak inhibitor of ALDH1, with an IC_{50} value in the high micromolar range (IC_{50} = 500 μM).

To test the ability of the quinolines to inhibit QR2 in vitro, QR2 activity was assayed in the presence of various concentrations of CQ, PQ, QC, MQ, and Q. As listed in Table 1, CQ, PQ, and QC were potent inhibitors of QR2 activity. In contrast, MQ and Q, both of which have large bulky substituents at the C-4 position (Fig. 1), are less potent inhibitors of the enzyme (Table 1). We also tested the effect of the quinolines on the activity of QR1, an enzyme that shares 49% amino acid identity with QR2. Interestingly, QR1 activity is not affected by CQ and QC and is weakly inhibited by MQ and PQ. These results indicate that the quinolines have specificity within the quinone reductase family of enzymes.

Effect of QR2 and ALDH1 Inhibitors on *P. falciparum* Growth. To determine the contribution of QR2 or ALDH1 inhibition to the antimalarial properties of the quinolines, known inhibitors of QR2 or ALDH1 were added to *P. falciparum* and its growth was measured. Known inhibitors of *P. falciparum* growth all had IC_{50} values in agreement with the literature (Fig. 7A). Two specific inhibitors of QR2, quercetin and chrysin, were lethal to the parasites at micromolar concentrations, with IC_{50} values of 81.8 ± 2.2 and 53.8 ± 6.3 μM, respectively (Fig. 7B). The growth of *P. falciparum* was also inhibited in vitro by a specific inhibitor of ALDH1, diethylaminobenzaldehyde (Fig. 7B), with an IC_{50} = 277 ± 15 μM. Although lethal to *P. falciparum*, the QR2 and ALDH1 inhibitors did not kill the parasites as effectively as the quinoline compounds. The explanation for this finding

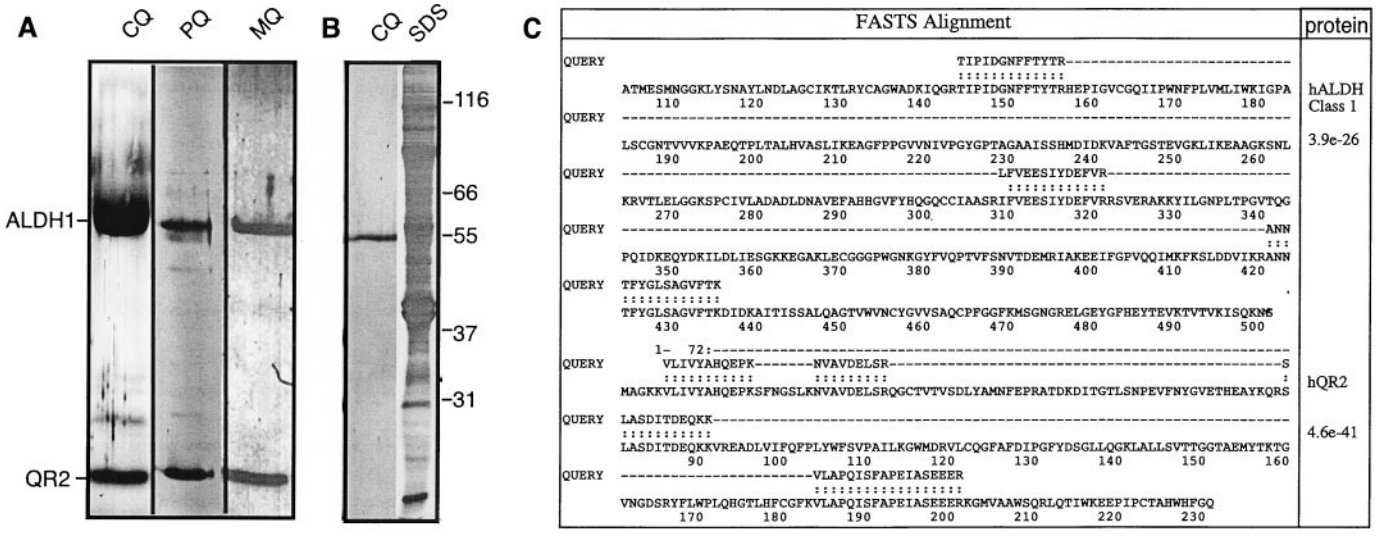


Fig. 5. Identification of quinoline antimalarial binding proteins in the human RBC or *P. falciparum* purine nucleotide binding proteome. A, elution of γ-ATP-Sepharose charged with noninfected human RBC extract with the indicated drugs. B, elution of γ-ATP-Sepharose charged with *P. falciparum*-infected RBC extract with CQ or SDS. Proteins were resolved by SDS-PAGE, visualized by silver staining, and sequenced by mass spectrometry. C, identification of human ALDH1 and QR2 by FASTS. Peptide sequences shown were obtained by mass spectrometry and used to search the NCBI/Blast NR database using the FASTS algorithm (Mackey et al., 2002). The expectation score (e) for each protein is shown.

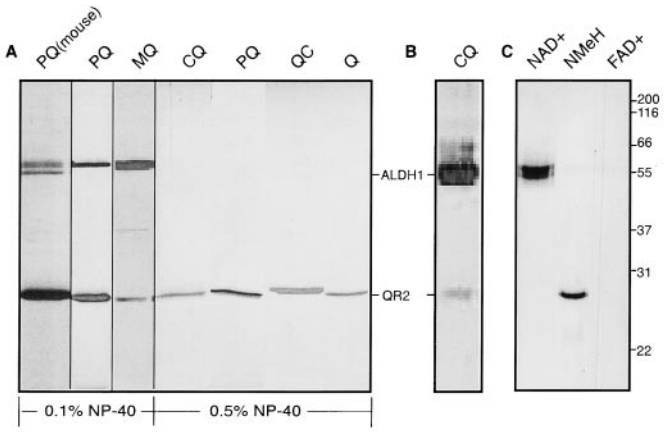


Fig. 6. ALDH1 and QR2 are recovered and selectively eluted from PQ and HCQ-Sepharose. A, PQ-Sepharose was charged with human RBC or mouse extract (indicated "mouse") and eluted with the indicated drugs. The amount of Nonidet P-40 included in the binding and wash buffer is indicated. B, HCQ-Sepharose was charged with human RBC extract and eluted with CQ. C, elution of RBC-extract charged PQ-Sepharose with NAD^+, NMeH, and FAD^+. The eluted proteins were resolved by SDS-PAGE, visualized by silver staining, and sequenced by mass spectrometry.

TABLE 1

Effect of antimalarial compounds on the activity of QR2 and QR1.

Data are presented as mean ± S.E.M.

Drug	QR2 (K_i)	QR1 (IC_{50})
	μM	
Chloroquine	0.61 ± 0.10	>1000
Primaquine	1.04 ± 0.38	124 ± 10
Quinacrine	0.51 ± 0.11	>1000
Mefloquine	17.0 ± 4.0	616 ± 60
Quinine	252 ± 50	9.6 ± 0.80
Dicumarol	—[a]	0.175 ± 0.01

[a] QR2 is insensitive to dicumarol (Zhao et al., 1997).

is likely to be related to the abilities of the drugs to penetrate the plasma membrane or their ability to become concentrated within *P. falciparum*-infected RBCs.

Discussion

Until the emergence of drug-resistant parasites, the quinolines were the cheapest and most effective antimalarial therapy. However, despite their widespread use, the precise mechanism of action of these drugs is the subject of great controversy. We used a functional proteomics approach to understand how these drugs function and to provide a comprehensive description of quinoline-interacting proteins. Identification of all quinoline binding proteins will allow for a better understanding of the mechanism of action of these drugs and the side effects associated with their use.

Using the proteomics approach described in this article, we demonstrated that several of the quinolines specifically target two human proteins, ALDH1 and QR2. The recovery of only these two proteins from ATP-Sepharose, even though hundreds of other purine nucleotide-using enzymes were present on the matrix, suggests that the quinolines are highly selective for these enzymes. In a complementary approach, we created affinity matrices by linking either PQ or HCQ to Sepharose and incubated these resins with whole cell lysates. In agreement with our initial results, both PQ- and HCQ-Sepharose selectively recovered ALDH1 and QR2. Furthermore, the specificity of PQ-Sepharose is demonstrated by its ability to bind only ALDH and QR2 from a whole mouse lysate.

In this work it was also shown that QR2 was potently inhibited by several of the quinolines in vitro (Table 1). The order of potency of the quinoline drugs against QR2 is CQ > PQ ≫ MQ, whereas an order of CQ > MQ ≫ PQ is observed with regard to parasite growth assays. The discrepancy between the ability of MQ and PQ to inhibit QR2 in vitro and

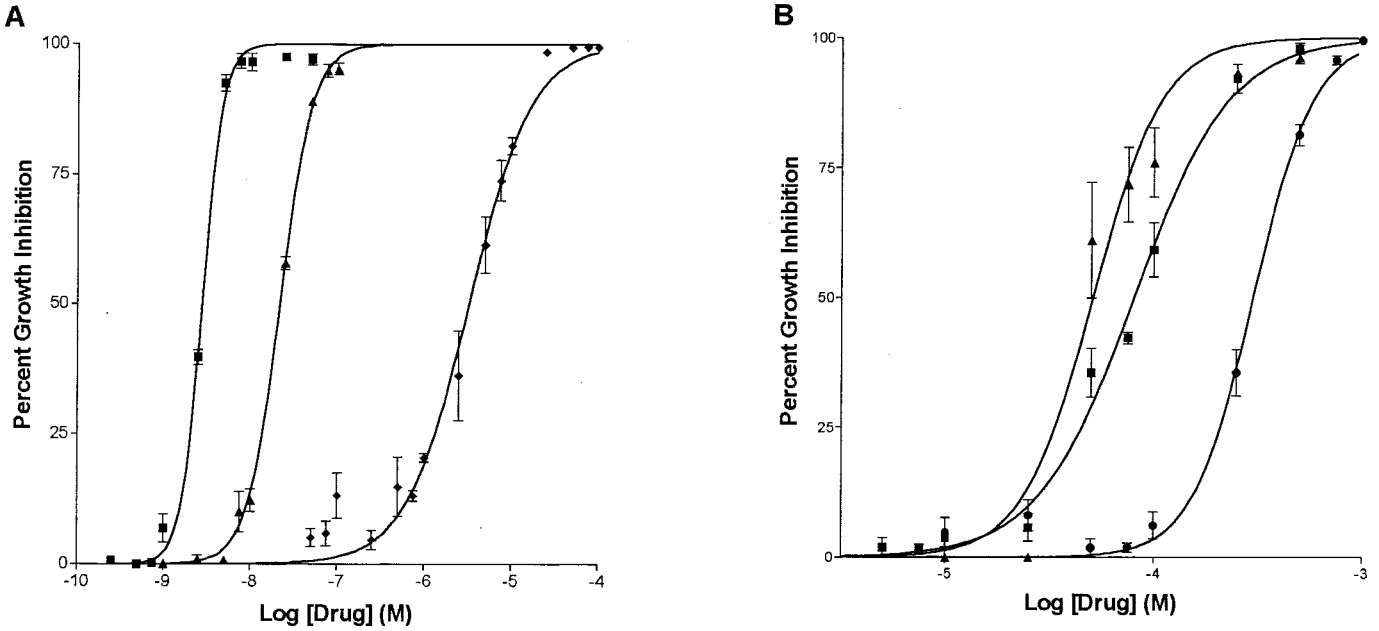


Fig. 7. QR2 and ALDH1 inhibitors have antimalarial activity in vitro. Growth of *P. falciparum* was measured in the presence of CQ (■), MQ (▲), and PQ (♦) (A) and quercetin (QU, ■), chrysin (CH, ▲), and diethylaminobenzaldehyde (●) (B). Data points are the mean ± S.E.M. Best fit lines were calculated with GraphPad Prism.

their ability to kill parasites in vivo may be related to inherent differences between an enzymatic and cell-based assay. For example, the bioavailability of the quinolines must be considered with cell-based assays, whereas the drugs are immediately accessible to QR2 in the enzyme assays. Because the K_i value of CQ toward QR2 is 0.61 μM and the concentration of CQ in uninfected red blood cells is estimated at 1 to 14 μM when taken at therapeutic doses (Adelusi et al., 1982; White, 1985), QR2 is a viable target of the quinolines in vivo.

The primary function of QR2 and its homolog QR1 is to catalyze the metabolic detoxification of quinones (e.g., menadione), a large class of potentially toxic compounds found in all respiring plant and animal cells (Chen et al., 2000; Dinkova-Kostova and Talalay, 2000; Long and Jaiswal, 2000). If not reduced to the hydroquinone form by QR1 or QR2, quinones can participate in redox cycling and generate reactive oxygen species (O'Brien, 1991). What effects then might the inhibition of QR2 have in relation to the diseases treated with the quinolines?

With regard to malaria, it is known that the parasite, *P. falciparum*, is very sensitive to oxidative stress (Green and Danubio, 1997). In fact, selective mutations within the human population suggest that we have exploited this weakness as a survival mechanism. The most striking example is the occurrence of mutations in glucose-6-phosphate dehydrogenase in malaria-endemic areas (Tishkoff et al., 2001). Mutations in glucose-6-phosphate dehydrogenase result in increased oxidative stress in RBCs (Lindquist, 1973; Chan et al., 1999) and are known to confer some protection against malaria (Martini and Ursini, 1996). Furthermore, in vitro cultures of *P. falciparum* are potently killed when a system capable of generating reactive oxygen species is introduced (Postma et al., 1996).

We hypothesize that the quinolines, by inhibiting quinone reductase activity in RBCs, may result in the generation of oxidative stress, thereby creating an inhospitable environment for the parasite. The parasite, through its digestion of hemoglobin as a food source, creates oxidative stress and this stress could become toxic if the host's antioxidant enzymes are compromised, as may be the case when QR2 is inhibited. In addition, menadione, which would presumably accumulate if QR2 was inhibited, has been reported to cause marked methemoglobin production and insertion of heme into the red cell membrane, resulting in hemolysis (Lopez-Shirley et al., 1994). Furthermore, the absence of QR1 in RBCs suggests that QR2 alone is responsible for the removal of reactive quinones in these cells.

The quinolines were also found to interact with human ALDH1. ALDH1 was efficiently recovered from whole blood using γ-ATP and PQ- and HCQ-Sepharose. However, in vitro assays of ALDH1 in the presence of CQ revealed that CQ was a relatively weak inhibitor of ALDH1 at physiological levels of NAD^+. This finding indicates that it is unlikely that ALDH1 is a target for the quinolines unless they accumulate to high levels within tissues. As a result, we hypothesize that ALDH1 is not involved in the antimalarial action of the drugs, but rather it may contribute to the side effects observed. For example, CQ actively accumulates to millimolar concentrations in tissues such as the skin and eye when administered at therapeutic levels (Lindquist, 1973; Rynes, 1997). One of the major functions of ALDH1 in the eye is to generate retinoic acid (visual pigment) from retinaldehyde, and the prolonged use of CQ or HCQ in the treatment of malaria, arthritis, or lupus can result in retinopathy and blindness as a result of the accumulation of retinaldehyde in the retina (Lindquist, 1973; Rynes, 1997; Van Beek and Piette, 2001). Our results also suggest a potential role for ALDH1 and QR2 in various other diseases for which the quinolines are currently indicated, namely, HIV, lupus, and rheumatoid arthritis.

In conclusion, we report the application of a novel proteomics strategy, termed proteome mining, for the identification of drug targets. Because of the unbiased nature of this methodology, all proteins that interact with the drug of choice can be identified, including proteins that may contribute to unwanted side effects. Reiteration of drug structure combined with proteome mining can result in the identification of compounds that display increased specificity toward given proteins. For example, identification of a drug that more specifically targets QR2 over ALDH1 may lead to the generation of new drugs for the treatment of arthritis and lupus that lack the retinopathy side effect.

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Address correspondence to: Dr. Tim Haystead, Department of Pharmacology and Cancer Biology, Center for Chemical Biology, Duke University Medical Center, Research Dr., C118 LSRC, Durham, NC 27710. E-mail: hayst001@mc.duke.edu