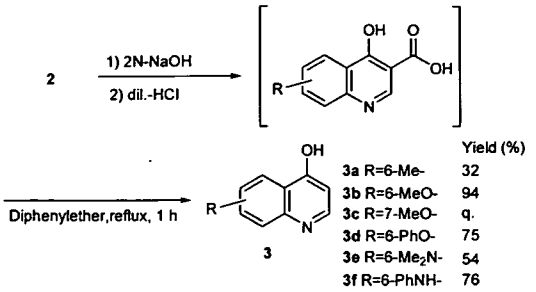

Chart 2

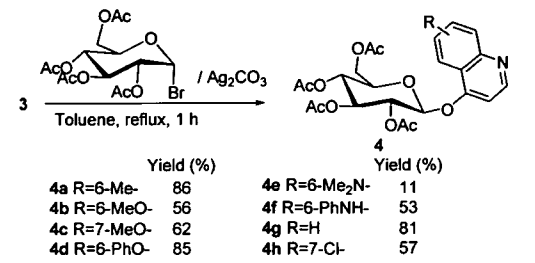

Chart 3

products. A mixture of **3**, 1-bromo-tetraacetyl-α-D-glucose, and 2-fold equiv. silver carbonate in toluene previously dehydrated by passage through molecular sieve 4A was heated under reflux for 1 h under a stream of argon gas. The reaction mixture was subjected to silica gel column chromatography with ethyl acetate as the eluent and afforded quinoline-β-glucoside tetraacetates **4**. Although unreacted **3** was recovered, the formation of α-anomers and glucoside-orthoesters was not confirmed in all cases, as shown in Chart 3. The low yield of **4e** was considered due to insolubility of **3e** in boiling toluene.

Deprotection of acetylated saccharides is generally performed with a strong base, such as sodium methoxide, sodium hydroxide, or potassium hydroxide. Deacetylation of **4** with sodium methoxide was carried out and the reaction mixture was neutralized with sulfuric acid. The workup had some problems: (1) large amounts of mineral salts were produced, and so it was not easy to isolate glucosides **5**, and (2) the reaction showed poor reproducibility. When an acidic ion-exchange resin was employed in the neutralization step, quinolinium salts were obtained. Therefore, deacetylation of **4** with a mineral base, such as sodium methoxide, was not useful. With the use of ammonia gas for deacetylation of **4**, acetamide is produced, which makes it difficult to separate to **5**. Therefore, deprotection of **4** with a basic ion-exchange resin was carried out. A mixture of **4** and a 2- or 3-fold excess of Amberlyst® A-26 (OH) by weight in methanol was stirred at RT. After 1—2 h, the disappearance of acetates **4** was determined by TLC. Use of an ion-exchange resin allowed separation without difficulty using only filtration. The precipitates thus produced were dissolved in methanol and the filtrates were combined. Evaporation of methanol afforded **5** in high yield, as shown in Chart 4.

With regard to anti-malarial activity, **5g** showed an EC_{50} value of 0.15 μg/ml in an *in vitro Plasmodium falciparum* culture system, which was almost the same value obtained with quinine used in commercial anti-malarial preparations.

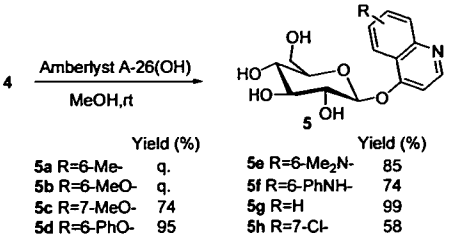

Chart 4

Anti-malarial assays of other **4** and **5** are currently underway and the results will be reported in a subsequent paper.

Experimental

The NMR spectra were obtained using a JEOL JNM-ECA 500 spectrometer with tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded with a PE Biosystems QSTAR spectrometer. IR spectra were performed with a JASCO FT/IR-420 spectrometer.

Preparation of 2 Typical Procedure: A mixture of **1** (10.7 g, 100 mmol) and ethyl ethoxymethylenediethylmalonate (23.3 g, 110 mmol) in toluene (100 ml) was heated under reflux for 1 h and then evaporated *in vacuo*. Diphenylether (150 ml) was added to the residue and the mixture was heated under reflux for 1 h or heated at 130 °C, as shown in Chart 1. After the reaction mixture was cooled to room temperature, *n*-hexane was added and the precipitates thus produced were filtered. The filtrate was washed with ethyl acetate, which yielded **2a** (10.5 g, 45%). Compounds **2** were not subjected to further purification.

Preparation of 3 Typical Procedure: A mixture of **2a** (10.5 g) and 2 N-sodium hydroxide (100 ml) was heated under reflux for 1 h. The reaction mixture was cooled to room temperature and 2 N-hydrochloride was added until precipitates were produced. The precipitates were filtered, washed with water, and dried. The obtained quinoline carbonic acid was not purified further. Diphenylether (50 ml) was added to the colorless powder, and the mixture was heated under reflux for 1 h. The reaction mixture was cooled to room temperature and *n*-hexane was added. Compound **3a** (2.3 g, 32%) was obtained by filtration and washing with ethyl acetate.

Preparation of 4 Typical Procedure: A suspension of **3a** (548 mg, 3.44 mmol), 1-bromo-tetraacetyl-α-D-glucose (1.56 g, 3.79 mmol) and silver carbonate (1.24 g, 3.79 mmol) in toluene previously dehydrated by passage through molecular sieve 4A (25 ml) was heated under reflux for 1 h in a stream of argon gas and cooled to room temperature. The insoluble products were filtered off and the filtrate was evaporated *in vacuo*. Ethyl acetate was added to the residue. Compound **4a** (1.48 g, 86%) was obtained by silica gel column chromatography of the solution with ethyl acetate as the eluent.

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-6-methylquinoline (**4a**) as Colorless Needles (Diisopropylether) (86%): mp 175—177 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.05 (3H, s), 2.06 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 2.57 (3H, s), 4.07 (1H, ddd, J=9.8, 5.3, 2.4 Hz), 4.22 (1H, dd, J=12.4, 2.4 Hz), 4.33 (1H, dd, J=12.4, 5.3 Hz), 5.25 (1H, dd, J=9.8, 9.4 Hz), 5.41 (1H, dd, J=9.4, 9.2 Hz), 5.44 (1H, d, J=7.6 Hz), 5.52 (1H, dd, J=9.2, 7.6 Hz), 7.00 (1H, d, J=5.2 Hz), 7.64 (1H, dd, J=8.6, 2.0 Hz), 7.88 (1H, d, J=2.0 Hz), 8.13 (1H, d, J=8.6 Hz), 8.75 (1H, d, J=5.2 Hz). IR (KBr) cm^{-1}: 1749. ESI-MS *m/z*: 512.1551 (Calcd for $C_{24}H_{27}NNaO_{10}$ (M+Na)$^+$: 512.1533).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-6-methoxyquinoline (**4b**) as Colorless Needles (Diisopropylether) (56%): mp 171—173 °C. ^{1}H-NMR (CD_3OD) δ: 2.02 (3H, s), 2.04 (3H, s), 2.05 (3H, s), 3.95 (s, 3H), 4.21 (1H, dd, J=12.2, 2.3 Hz), 4.25 (1H, ddd, J=10.0, 4.9, 2.3 Hz), 4,35 (1H, dd, J=12.2, 4.9 Hz), 5.19 (1H, dd, J=10.0, 9.4 Hz), 5.41 (1H, dd, J=9.5, 7.7 Hz), 5.49 (1H, dd, J=9.5, 9.4 Hz), 5.70 (1H, d, J=7.7 Hz), 7.04 (1H, d, J=5.5 Hz), 7.23 (1H, dd, J=9.2, 2.5 Hz), 7.32 (1H, d, J=2.5 Hz), 7.98 (1H, d, J=9.2 Hz), 8.64 (1H, d, J=5.5 Hz). IR (KBr) cm^{-1}: 1742. ESI-MS *m/z*: 528.1484 (Calcd for $C_{24}H_{27}NNaO_{11}$ (M+Na)$^+$: 528.1482).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-7-methoxyquinoline (**4c**) as Colorless Needles (Diisopropylether) (62%): mp 170—172 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.06 (3H, s), 2.07 (3H, s), 2.08 (6H, s), 3.96 (s, 3H), 4.08 (1H, ddd, J=10.1, 5.3, 2.3 Hz), 4,23 (1H, dd, J=12.5, 2.3 Hz), 4.36 (1H, dd, J=12.5, 5.3 Hz), 5.24 (1H, dd, J=10.1, 9.5 Hz), 5.43 (1H, d, J=7.6 Hz), 5.43 (1H, dd, J=9.5, 9.5 Hz), 5.54 (1H, dd, J=9.5, 7.6 Hz), 7.03 (1H, d, J=5.3 Hz), 7.39 (1H, d, J=2.8 Hz), 7.47 (1H, dd, J=9.1, 2.8 Hz), 8.22 (1H, d, J=9.1 Hz), 8.68 (1H, d, J=5.3 Hz). IR (KBr) cm^{-1}: 1742. ESI-MS *m/z*: 528.1485 (Calcd for

$C_{24}H_{27}NNaO_{11}$ $(M+Na)^+$: 528.1482).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-6-phenoxyquinoline (**4d**) as Colorless Needles (Diisopropylether) (85%): mp 136—138 °C. ^{1}H-NMR (CD_3OD) δ: 1.90 (3H, s), 2.00 (3H, s), 2.03 (3H, s), 2.04 (3H, s), 4.22 (1H, dd, *J*=12.4, 2.3 Hz), 4.22 (1H, ddd, *J*=10.0, 4.9, 2.3 Hz), 4.32 (1H, dd, *J*=12.4, 4.9 Hz), 5.16 (1H, dd, *J*=10.0, 9.4 Hz), 5.28 (1H, dd, *J*=9.6, 7.8 Hz), 5.43 (1H, dd, *J*=9.6, 9.4 Hz), 5.65 (1H, d, *J*=7.8 Hz), 7.08 (2H, ddd, *J*=6.7, 2.0, 1.0 Hz), 7.14 (1H, d, *J*=5.3 Hz), 7.22 (1H, tt, *J*=7.4, 1.0 Hz), 7.43 (2H, ddd, *J*=7.4, 6.7, 2.0 Hz), 7.45 (1H, d, *J*=2.6 Hz), 7.53 (1H, dd, *J*=9.1, 2.6 Hz), 7.98 (1H, d, *J*=9.1 Hz), 8.64 (1H, d, *J*=5.3 Hz). IR (KBr) cm^{-1}: 1749. ESI-MS *m/z*: 590.1663 (Calcd for $C_{29}H_{29}NNaO_{11}$ $(M+Na)^+$: 590.1638).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-6-*N*,*N*-dimethylaminoquinoline (**4e**) as Ochre Needles (Diisopropylether) (11%): mp 167—169 °C. ^{1}H-NMR (CD_3OD) δ: 2.03 (3H, s), 2.04 (3H, s), 2.05 (3H, s), 2.06 (3H, s), 3.09 (6H, s), 4.25 (1H, dd, *J*=12.6, 2.3 Hz), 4.25 (1H, ddd, *J*=9.7, 5.2, 2.3 Hz), 4.36 (1H, dd, *J*=12.6, 5.2 Hz), 5.20 (1H, dd, *J*=9.7, 9.2 Hz), 5.43 (1H, dd, *J*=9.7, 7.4 Hz), 5.51 (1H, dd, *J*=9.7, 9.2 Hz), 5.61 (1H, d, *J*=7.4 Hz), 7.06 (1H, d, *J*=5.2 Hz), 7.04 (1H, d, *J*=2.9 Hz), 7.45 (1H, dd, *J*=9.7, 2.9 Hz), 7.81 (1H, d, *J*=9.7 Hz), 8.39 (1H, d, *J*=5.2 Hz). IR (KBr) cm^{-1}: 1743. ESI-MS *m/z*: 541.1812 (Calcd for $C_{25}H_{30}N_2NaO_{10}$ $(M+Na)^+$: 541.1798).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-6-*N*-phenylaminoquinoline (**4f**) as Pale Brown Needles (Diisopropylether) (53%): mp 167—169 °C. ^{1}H-NMR (CD_3OD) δ: 1.93 (3H, s), 2.01 (3H, s), 2.03 (3H, s), 2.05 (3H, s), 4.22 (1H, dd, *J*=12.4, 2.3 Hz), 4.22 (1H, ddd, *J*=9.8, 4.9, 2.3 Hz), 4.32 (1H, dd, *J*=12.4, 4.9 Hz), 5.16 (1H, dd, *J*=9.8, 9.6 Hz), 5.29 (1H, dd, *J*=9.5, 7.7 Hz), 5.44 (1H, dd, *J*=9.6, 9.5 Hz), 5.65 (1H, d, *J*=7.7 Hz), 6.98 (1H, tt, *J*=7.4, 1.1 Hz), 7.05 (1H, d, *J*=5.3 Hz), 7.24 (2H, ddd, *J*=7.7, 2.0, 1.1 Hz), 7.43 (2H, ddd, *J*=7.7, 7.4, 2.0 Hz), 7.48 (1H, dd, *J*=9.1, 2.5 Hz), 7.64 (1H, d, *J*=2.5 Hz), 7.81 (1H, d, *J*=9.1 Hz), 8.44 (1H, d, *J*=5.3 Hz). IR (KBr) cm^{-1}: 1748. ESI-MS *m/z*: 567.1995 (Calcd for $C_{29}H_{31}N_2O_{10}$ $(M+H)^+$: 567.1979).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-quinoline (**4g**) as Colorless Needles (Diisopropylether) (81%): mp 175—176 °C. ^{1}H-NMR (CD_3OD) δ: 2.02 (3H, s), 2.04 (3H, s), 2.05 (3H, s), 2.06 (3H, s), 4.22 (1H, dd, *J*=12.3, 2.3 Hz), 4.27 (1H, ddd, *J*=10.0, 4.9, 2.3 Hz), 4.36 (1H, dd, *J*=12.3, 4.9 Hz), 5.21 (1H, dd, *J*=10.0, 9.2 Hz), 5.44 (1H, dd, *J*=9.6, 7.7 Hz), 5.51 (1H, d, *J*=9.6, 9.2 Hz), 5.73 (1H, d, *J*=7.7 Hz), 7.17 (1H, d, *J*=5.3 Hz), 7.60 (1H, ddd, *J*=8.4, 7.0, 1.2 Hz), 7.79 (1H, ddd, *J*=8.5, 7.0, 1.4 Hz), 7.98 (1H, dd, *J*=8.5, 1.2 Hz), 8.10 (1H, dd, *J*=8.4, 1.4 Hz), 8.73 (1H, d, *J*=5.3 Hz). IR (KBr) cm^{-1}: 1752. ESI-MS *m/z*: 476.1559 ($C_{23}H_{26}NO_{10}$ $(M+H)^+$: 476.1551).

4-(β-*O*-Tetraacetyl-D-glucosyloxy)-7-chloroquinoline (**4h**) as Colorless Prisms and Colorless Needles (Diisopropylether) (58%): mp 136—138 °C (prisms) and 144—145 °C (needles). ^{1}H-NMR (CD_3OD) (prisms and needles) δ: 2.02 (3H, s), 2.04 (3H, s), 2.05 (3H, s), 2.06 (3H, s), 4.22 (1H, dd, *J*=12.3, 2.3 Hz), 4.26 (1H, ddd, *J*=10.0, 4.9, 2.3 Hz), 4.35 (1H, dd, *J*=12.3, 4.9 Hz), 5.21 (1H, dd, *J*=10.0, 9.2 Hz), 5.43 (1H, dd, *J*=9.6, 7.7 Hz), 5.50 (1H, dd, *J*=9.6, 9.2 Hz), 5.73 (1H, d, *J*=7.7 Hz), 7.18 (1H, d, *J*=5.3 Hz), 7.57 (1H, dd, *J*=9.0, 2.1 Hz), 7.97 (1H, d, *J*=2.1 Hz), 8.07 (1H, d, *J*=9.0 Hz), 8.75 (1H, d, *J*=5.3 Hz). ($CDCl_3$) (prisms) δ: 2.06 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 4.15 (1H, ddd, *J*=10.0, 5.2, 2.3 Hz), 4.24 (1H, dd, *J*=12.6, 2.3 Hz), 4.35 (1H, dd, *J*=12.6, 5.2 Hz), 5.24 (1H, dd, *J*=10.0, 9.5 Hz), 5.45 (1H, dd, *J*=9.5, 9.2 Hz), 5.54 (1H, dd, *J*=9.2, 7.6 Hz), 5.62 (1H, d, *J*=7.6 Hz), 7.32 (1H, d, *J*=6.2 Hz), 7.73 (1H, dd, *J*=9.0, 1.9 Hz), 8.17 (1H, d, *J*=9.0 Hz), 8.65 (1H, d, *J*=1.9 Hz), 8.94 (1H, d, *J*=6.2 Hz). ($CDCl_3$) (needles) δ: 2.06 (3H, s), 2.08 (3H, s), 2.09 (3H, s), 2.10 (3H, s), 4.13 (1H, ddd, *J*=10.0, 5.2, 2.2 Hz), 4.23 (1H, dd, *J*=12.6, 2.2 Hz), 4.34 (1H, dd, *J*=12.6, 5.2 Hz), 5.24 (1H, dd, *J*=10.0, 9.5 Hz), 5.44 (1H, dd, *J*=9.5, 8.9 Hz), 5.54 (1H, dd, *J*=8.9, 7.7 Hz), 5.58 (1H, d, *J*=7.7 Hz), 7.24 (1H, d, *J*=6.1 Hz), 7.69 (1H, dd, *J*=9.0, 1.6 Hz), 8.14 (1H, d, *J*=9.0 Hz), 8.55 (1H, d, *J*=1.6 Hz), 8.92 (1H, d, *J*=6.1 Hz). IR (KBr) cm^{-1}: 1746 (prisms), 1752 (needles). ESI-MS *m/z*: 510.1144 ($C_{23}H_{25}ClNO_{10}$ $(M+H)^+$: 510.1162).

Preparation of 5 Typical Procedure: A suspension of **4a** (500 mg, 1.02 mmol) and Amberlyst® A-26 (OH) (1.50 g) in methanol (50 ml) was stirred for 1 h at room temperature. Insoluble materials were filtered and the colorless powder was extracted with a large excess of methanol. The extract was combined with the filtrate and evaporated *in vacuo* yielding compound **5a** (337 mg, q.).

4-(β-D-Glucosyloxy)-6-methylquinoline (**5a**) as a Colorless Crystalline Powder (Ethanol) (q.): mp 180—182 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 2.55 (3H, s), 3.46 (1H, dd, *J*=9.5, 9.2 Hz), 3.55 (1H, dd, *J*=9.2, 9.2 Hz), 3.59 (1H, ddd, *J*=9.5, 5.7, 2.1 Hz), 3.68 (1H, dd, *J*=9.2, 7.7 Hz), 3.72 (1H, dd, 12.2, 5.7 Hz), 3.93 (1H, dd, *J*=12.2, 2.1 Hz), 5.28 (1H, d, *J*=7.7 Hz), 7.15 (1H, d, *J*=5.3 Hz), 7.61 (1H, dd, *J*=8.6, 1.9 Hz), 7.85 (1H, d, *J*=8.6 Hz), 8.16 (1H, d, *J*=1.9 Hz), 8.61 (1H, d, *J*=5.3 Hz). IR (KBr) cm^{-1}: 3357 (br). ESI-MS *m/z*: 322.1268 (Calcd for $C_{16}H_{20}NO_6$ $(M+H)^+$: 322.1291).

4-(β-D-Glucosyloxy)-6-methoxyquinoline (**5b**) as Colorless Needles (Ethanol) (74%): mp 175—177 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.46 (1H, dd, *J*=9.2, 9.2 Hz), 3.56 (1H, dd, *J*=9.2, 9.2 Hz), 3.59 (1H, ddd, *J*=9.2, 5.7, 2.3 Hz), 3.69 (1H, dd, *J*=9.2, 7.4 Hz), 3.73 (1H, dd, *J*=12.6, 5.7 Hz), 3.93 (1H, dd, *J*=12.6, 2.3 Hz), 3.96 (3H, s), 5.29 (1H, d, *J*=7.4 Hz), 7.17 (1H, d, *J*=5.2 Hz), 7.39 (1H, dd, *J*=9.2, 2.9 Hz), 7.65 (1H, d, *J*=2.9 Hz), 7.85 (1H, d, *J*=9.2 Hz), 8.54 (1H, d, *J*=5.2 Hz). (KBr) cm^{-1}: 3356 (br). ESI-MS *m/z*: 360.1065 (Calcd for $C_{16}H_{19}NNaO_7$ $(M+Na)^+$: 360.1059).

4-(β-D-Glucosyloxy)-7-methoxyquinoline (**5c**) as Colorless Needles (Ethanol) (q.): mp 177—181 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.45 (1H, dd, *J*=9.5, 9.1 Hz), 3.54 (1H, dd, *J*=9.1, 9.1 Hz), 3.57 (1H, ddd, *J*=9.5, 5.7, 2.1 Hz), 3.65 (1H, dd, *J*=9.1, 7.8 Hz), 3.72 (1H, dd, *J*=12.1, 5.7 Hz), 3.92 (1H, dd, *J*=12.1, 2.1 Hz), 3.95 (3H, s), 5.29 (1H, d, *J*=7.8 Hz), 7.07 (1H, d, *J*=5.4 Hz), 7.20 (1H, dd, *J*=9.2, 2.4 Hz), 7.29 (1H, d, *J*=2.4 Hz), 8.25 (1H, d, *J*=9.2 Hz), 8.60 (1H, d, *J*=5.4 Hz). (KBr) cm^{-1}: 3376 (br). ESI-MS *m/z*: 360.1066 (Calcd for $C_{16}H_{19}NNaO_7$ $(M+Na)^+$: 360.1059).

4-(β-D-Glucosyloxy)-6-phenoxyquinoline (**5d**) as Colorless Needles (Ethyl Acetate) (74%): mp 170—173 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.43 (1H, dd, *J*=9.6, 9.0 Hz), 3.52 (1H, dd, *J*=9.1, 9.0 Hz), 3.57 (1H, ddd, *J*=9.6, 5.7, 2.2 Hz), 3.58 (1H, dd, *J*=9.1, 7.7 Hz), 3.71 (1H, dd, *J*=12.1, 5.7 Hz), 3.91 (1H, dd, *J*=12.1, 2.2 Hz), 5.29 (1H, d, *J*=7.7 Hz), 7.06 (2H, ddd, *J*=8.7, 2.1, 1.1 Hz), 7.16 (1H, tt, *J*=7.4, 2.1 Hz), 7.19 (1H, d, *J*=5.4 Hz), 7.39 (2H, ddd, *J*=8.7, 7.4, 1.1 Hz), 7.48 (1H, dd, *J*=9.2, 2.8 Hz), 7.87 (1H, d, *J*=2.8 Hz), 7.97 (1H, d, *J*=9.2 Hz), 8.63 (1H, d, *J*=5.4 Hz). (KBr) cm^{-1}: 3255 (br). ESI-MS *m/z*: 422.1217 (Calcd for $C_{21}H_{21}NNaO_7$ $(M+Na)^+$: 422.1216).

4-(β-D-Glucosyloxy)-6-*N*,*N*-dimethylaminoquinoline (**5e**) as an Ochre Crystalline Powder (Ethyl Acetate) (85%): mp 179—184 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.09 (6H, s), 3.46 (1H, dd, *J*=9.7, 9.0 Hz), 3.55 (1H, dd, *J*=9.1, 9.0 Hz), 3.56 (1H, ddd, *J*=9.7, 5.6, 2.3 Hz), 3.68 (1H, dd, *J*=9.1, 7.8 Hz), 3.73 (1H, dd, *J*=12.1, 5.6 Hz), 3.92 (1H, dd, *J*=12.1, 2.3 Hz), 5.27 (1H, d, *J*=7.8 Hz), 7.06 (1H, d, *J*=5.3 Hz), 7.31 (1H, d, *J*=2.9 Hz), 7.44 (1H, dd, *J*=9.4, 2.9 Hz), 7.79 (1H, d, *J*=9.4 Hz), 8.38 (1H, d, *J*=5.3 Hz). (KBr) cm^{-1}: 3357 (br). ESI-MS *m/z*: 351.1561 (Calcd for $C_{17}H_{23}N_2O_6$ $(M+H)^+$: 351.1556).

4-(β-D-Glucosyloxy)-6-*N*-phenylaminoquinoline (**5f**) as Brown Needles (Ethyl Acetate) (74%): mp 168—175 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.45 (1H, dd, *J*=9.6, 9.0 Hz), 3.52 (1H, dd, *J*=9.0, 9.0 Hz) 3.58 (1H, ddd, *J*=9.6, 5.6, 2.3 Hz), 3.62 (1H, dd, *J*=9.0, 7.7 Hz), 3.72 (1H, dd, *J*=12.2, 5.6 Hz), 3.91 (1H, dd, *J*=12.2, 2.3 Hz), 5.29 (1H, d, *J*=7.7 Hz), 6.93 (1H, tt, *J*=7.3, 1.2 Hz), 7.08 (1H, d, *J*=5.3 Hz), 7.23 (2H, ddd, *J*=8.7, 2.0, 1.2 Hz), 7.29 (2H, ddd, *J*=8.7, 7.3, 2.0 Hz), 7.51 (1H, dd, *J*=9.1, 2.6 Hz), 7.80 (1H, d, *J*=9.1 Hz), 7.87 (1H, d, *J*=2.6 Hz), 8.44 (1H, d, *J*=5.3 Hz). (KBr) cm^{-1}: 3323 (br). ESI-MS *m/z*: 421.1356 (Calcd for $C_{21}H_{22}N_2NaO_6$ $(M+Na)^+$: 421.1376).

4-(β-D-Glucosyloxy)-quinoline (**5g**) as a Colorless Crystalline Powder (Ethanol) (99%): mp 178—179 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.46 (1H, dd, *J*=9.7, 9.1 Hz), 3.56 (1H, dd, *J*=9.2, 9.1 Hz), 3.60 (1H, ddd, *J*=9.7, 5.7, 2.2 Hz), 3.69 (1H, dd, *J*=9.2, 7.8 Hz), 3.73 (1H, dd, *J*=12.2, 5.7 Hz), 3.93 (1H, dd, *J*=12.2, 2.2 Hz), 5.31 (1H, d, *J*=7.8 Hz), 7.20 (1H, d, *J*=5.4 Hz), 7.58 (1H, ddd, *J*=8.4, 7.0 1.3 Hz), 7.76 (1H, ddd, *J*=8.6, 7.0, 1.5 Hz), 7.96 (1H, dd, *J*=8.6, 1.3 Hz), 8.38 (1H, dd, *J*=8.4, 1.5 Hz)), 8.70 (1H, d, *J*=5.4 Hz). (KBr) cm^{-1}: 3348 (br). ESI-MS *m/z*: 308.1129 (Calcd for $C_{15}H_{18}NO_6$ $(M+H)^+$: 308.1129).

4-(β-D-Glucosyloxy)-7-chloroquinoline (**5h**) as a Colorless Crystalline Powder (Ethyl Acetate) (58%): mp 167—172 °C (decomp.). ^{1}H-NMR (CD_3OD) δ: 3.45 (1H, dd, *J*=9.7, 9.0 Hz), 3.55 (1H, dd, *J*=9.2, 9.0 Hz), 3.60 (1H, ddd, *J*=9.7, 5.7, 2.2 Hz), 3.67 (1H, dd, *J*=9.2, 7.7 Hz), 3.72 (1H, dd, *J*=12.2, 5.7 Hz), 3.93 (1H, dd, *J*=12.2, 2.2 Hz), 5.30 (1H, d, *J*=7.7 Hz), 7.21 (1H, d, *J*=5.5 Hz), 7.56 (1H, dd, *J*=9.0, 2.1 Hz), 7.95 (1H, d, *J*=2.1 Hz), 8.36 (1H, d, *J*=9.0 Hz), 8.72 (1H, d, *J*=5.5 Hz). (KBr) cm^{-1}: 3347 (br). ESI-MS *m/z*: 342.0715 (Calcd for $C_{15}H_{17}ClNO_6$ $(M+H)^+$: 342.0739).

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ORIGINAL PAPER

Proteome analysis of new antimalarial endoperoxide against *Plasmodium falciparum*

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Abstract N-89, a new antimalarial endoperoxide, was selected as a promising antimalarial compound showing high activity and selectivity. To study the mechanism of N-89 action, N-89 resistant strain (NRC10) was obtained by intermittent drug pressure. NRC10 had a tenfold increase in the EC_{50} value of N-89. No cross-resistance was obtained with other antimalarial compounds. Comparative proteome analysis of N-89 sensitive and NRC10 strains revealed over-expression of 12 spots and down-regulation of 14 spots in NRC10. Fifteen proteins were identified of *Plasmodium falciparum* origin. The identified proteins representing several functions, mainly related to the glycolytic pathway, and metabolism of protein and lipid. Our results suggest that identified proteins may be candidates of antimalarial endoperoxide targets.

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Introduction

Malaria, caused by infection with apicomplexan parasites of the genus *Plasmodium*, remains a global health problem (Spielmann et al. 2006). Despite decades of effort to control malaria, it is still the most devastating parasitic disease of humans, killing millions of children each year (Silva-Neto et al. 2002). *Plasmodium falciparum* has become resistant to nearly all currently employed antimalarial drugs used for prophylaxis and treatment (White 2004; Pessi et al. 2005). As drug resistance is an important limit in the control of *P. falciparum* (Peters 1988; Walker et al. 2000; Daher et al. 2006), there is an immediate need for new antimalarial drugs and a need to understand their mechanism of action and possible mechanism of resistance so that appropriate measures can be taken in their use to delay possible eventual ineffectiveness.

The completion of the *P. falciparum* genome sequence has given rise to the post-genomic era in malaria research (Gelhaus et al. 2005). The ability to accurately measure comparative levels of protein expression after drug challenge, metabolic stress or other perturbations represents one of the most important goals in post-genomic malaria research (Nirmalan et al. 2004).

1,2,6,7-Tetraoxaspiro [7,11] nonadecane (N-89, Fig. 1) contains an endoperoxide structure. It is a promising antimalarial endoperoxide, which showed high antimalarial activity and selectivity in our previous study (Kim et al.

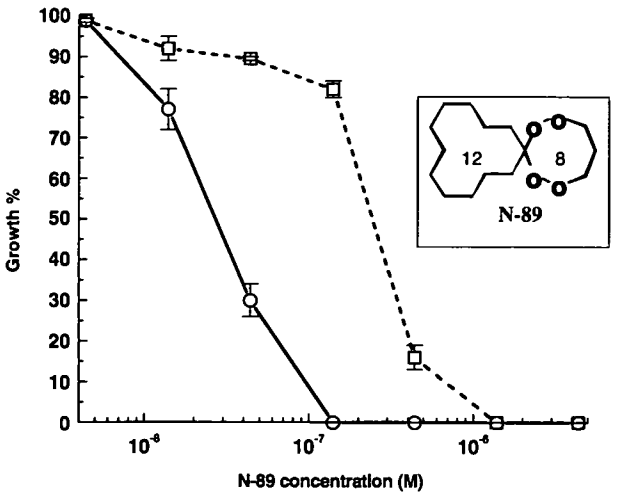

Fig. 1 In vitro antimalarial activity of the N-89 compound on the FCR-3 (*circles*) and NRC10 (*squares*) strains. The EC_{50} value was calculated from a semilog graph of the percentage of parasite growth against the N-89 concentration

2001). This compound is structurally unrelated to classical quinoline and antifolate drugs.

In this paper, we studied the drug sensitivity test and proteome analysis to understand the mode of action of endoperoxide using N-89 sensitive and resistant strains.

Materials and methods

Antimalarial compounds

N-89 was dissolved in dimethyl sulphoxide and stored at 20°C. Subsequent dilutions were determined by the magnitude of dilutions needed to reach the desired concentration. Artemisinin, quinine and chloroquine were supplied from Sigma (St. Louis, MO). Mefloquine was supplied from Hoffmann-La Roche (Basel, Switzerland).

Parasite cultivation

P. falciparum FCR-3 strain (ATCC 30932) was used in our study. The FCR-3 strain was cultivated by a modification of the method of Trager and Jensen (1976) using 5% hematocrit of type A human red blood cells suspended in RPMI 1640 (Gibco, NY) and supplemented with heat-inactivated 10% type A human serum. The plates were placed in a CO_2–O_2–N_2 incubator (5% O_2, 5% CO_2, and 90% N_2) at 37°C.

Development of resistant strain

Intermittent drug exposure The FCR-3 strain was exposed to a gradual increase in N-89 concentrations: 1×10^{-7}, 4×10^{-7}, 6.8×10^{-7}, and 1×10^{-6} M for 2 years. When the parasites could sustain a certain concentration, the next higher concentration was used. When parasitemia reached less than 50% of the control strain, the medium containing N-89 was then replaced with a drug-free medium and the parasites were cultured in the absence of drug until parasitemia increased. Parasitemia was monitored daily with Giemsa-stained (Merck, Germany) thin blood smears. N-89 resistant parasites were obtained after 2 years of intermittent drug pressure.

Parasite cloning To obtain a pure homogenous and resistant clone, a single erythrocyte microdilution cloning technique was used (Trager et al. 1981; Oduola et al. 1988). Suspension was adjusted to 2% hematocrit and 0.5–0.7 parasites per well. N-89 was added at 5×10^{-7} M. Parasites were maintained in 96-well microtiter plates to which 150 µl of the cloning suspension had been added to each well. Plates were incubated at 37°C, 5% O_2, 5% CO_2, and 90% N_2. We checked parasitemia and changed the medium with fresh red blood cells and N-89 every 5 days. When parasite growth increased, the medium was changed every day. Thin blood smears were prepared and detected with Giemsa stain. More than 100 fields were examined under microscopy and up to 1 month; if no parasite was detected then the plates were discarded. When parasites were observed in a certain well, its contents were transferred individually to another new plate for continuous follow up.

Drug susceptibility tests

In vitro drug susceptibility tests were performed according to method described by Kim et al. (1998). Briefly, parasitized erythrocyte suspension (995 µl) with 3% hematocrit and 0.3% parasitemia in 24-well plates were exposed for 72 h to 5 µl of drug at various concentrations without a daily change of medium. The results were recorded from microscopic observations of thin blood smears and expressed as the effective concentration that inhibits 50% of parasites relative to the control (EC_{50}). All data points represented the mean of at least three experiments. The EC_{50} of N-89 was estimated to evaluate the sensitivity of the obtained clones.

Cell extract preparation

Parasites were synchronized to the ring stage by D-sorbitol treatment (Lambros and Vanderberg 1979; Kumar et al. 2003). Synchronized parasites were harvested by centrifugation, lysed in a saponin solution, then washed twice with PBS. The cell pellet was rehydrated in a rehydration sample buffer (8 M urea, 2% CHAPS, 50 mM DDT, 0.001%

Table 1 EC_{50} values of different antimalarial compounds on FCR-3 and NRC10 strains

Compounds	EC_{50} (M)	
	FCR-3[a]	NRC10[b]
N-89	2.5×10^{-8}	2.5×10^{-7}
Artemisinin	1.5×10^{-8}	1.6×10^{-8}
Quinin	4.1×10^{-7}	4.4×10^{-7}
Chloroquine	1.4×10^{-8}	1.6×10^{-8}
Mefloquine	1.2×10^{-8}	1.5×10^{-8}

[a, b] All points represent the mean of triplicate experiments.

bromophenol blue, and 0.2% *w/v* Bio-Lyte 3/10 ampholytes [BioRad, Hercules, CA, USA]) at room temperature for 30 min, added with protease inhibitor cocktail for plant cells (1:100 *v/v*, Sigma). Cell lysates were sonicated on ice (Branson sonifier 250) for 6 cycles with 30 s of cooling in between. Samples were centrifuged at 10,000 rpm for 10 min at 20°C to remove insoluble materials such as nucleic acids and hemozoin crystals (Nirmalan et al. 2004). Protein concentration was assayed by RCDC protein assay kits (BioRad). Proteins were stored at −80°C until use in electrophoresis.

Two-dimensional electrophoresis (2-DE)

The first dimension (IEF) separation was performed at 20°C using IPG strips (linear pH range 3–10). IPG strips were rehydrated for 12 h in a rehydration sample buffer and focused according to the manufacturer's recommendations using PROTEAN II CELL (BioRad). A parasite protein sample of 50–200 μg was applied to the gel. After IEF, strips were equilibrated in 2 steps of 20 and 10 min each in equilibration sample buffers (BioRad) supplemented with 2% (*w/v*) DDT and 2.5% (*w/v*) iodoacetamide, respectively. Proteins were separated in the second dimension electrophoresis by 12.5% polyacrylamide gels, using 25 mM Tris base, 192 mM glycine, and 0.1% SDS as the running buffer. SDS-PAGE protein marker was used as the standard in the second dimension. After 2-DE, gels were stained with SYPRO-Ruby (BioRad) and silver stains (Wako Pure Chemical, Osaka, Japan). Gels were imaged with the MOLECULAR IMAGER FX pro. Images were analyzed using the PDQest software (BioRad). In these experiments, all samples were run in at least triplicate.

Protein identification

Silver-stained protein spots were excised and subjected to in-gel reduction, alkylation, and tryptic digestion as described previously (Shevchenko et al. 1996; Wilm et al. 1996). The peptide mixtures were analyzed by nano LC-MS/MS using Agilent 1100 LC/MSD Trap XCT Ultra series system (Agilent Technologies, Santa Clara, CA, USA). The ionization system was a Chip Cube using HPLC-Chip-MS (Agilent).

Database searches were performed against the NCBInr database for the malaria parasite and the Swissprot database for *Homosapiens* using a spectrum Mill MS proteomic workbench offered by Agilent (Software version 3.3). The parameters for the search were as follows: carbamidomethylation of cysteine was set as a fixed modification and methionine oxidation was set as a variable modification. Identification of proteins was validated when at least two peptide sequences were matched with the database sequences.

Results

N-89 resistant strain

Fifteen N-89 resistant clones were obtained by cloning the parasite under N-89 pressure. The growth rate of the pressured cloned strains was better than the pressured pre-

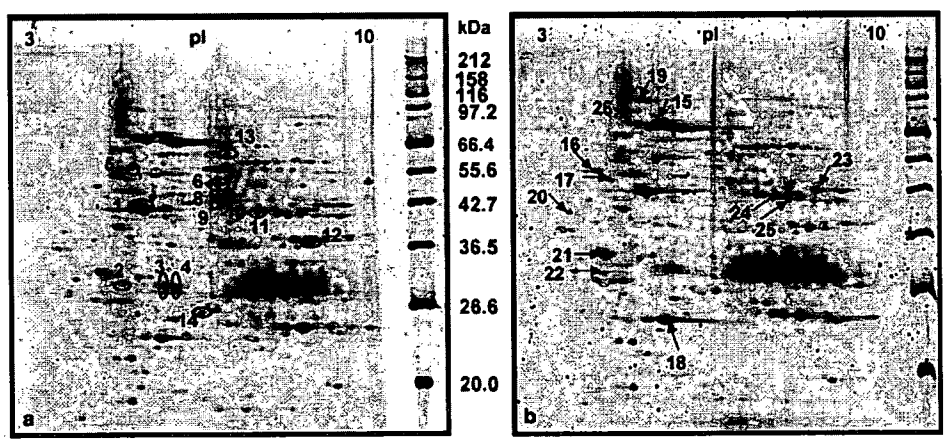

Fig. 2 2-D electrophoretic pattern of the FCR-3 and NRC10 strains. SYPRO-Ruby stained, 2-D gel protein profiles of *P. falciparum*. **a** FCR-3 strain. **b** NRC10 strain. *Circles* indicate the down-regulated spots in the NRC10 strain. *Arrows* indicate the up-regulated spots in the NRC10 strain. *Spot numbers* refer to the identification listed in Table 2

Table 2 Protein identification and expression levels between FCR-3 and NRC10 strains

Spot no.	Protein identification	Expression in resistant strain	Folds exp[a]	Species	NCBI accession no.	pI[b]	MW[c]	Matched peptides	% AA coverage[d]
1	Actin	Down-regulated	2.2	*P. falciparum*	23509135	5.21	41,870.9	14	39
2	14-3-3 protein homologue	Down-regulated	1.6	*P. falciparum*	23612834	4.96	29,476.2	4	18
3	Phosphoethanolamine *N*-methyltransferase	Down-regulated	1.4	*P. falciparum*	23615568	5.43	31,043.3	8	36
4	Phosphoethanolamine *N*-methyltransferase, putative	Down-regulated	2.0	*P. falciparum*	23615568	5.43	31,043.3	7	30
5	Vimentin	Down-regulated	1.7	Human	P08670	5.06	53,520.7	17	42
6	Enolase	Down-regulated	1.5	*P. falciparum*	23495020	6.21	48,678.0	18	54
7	Enolase	Down-regulated	2.6	*P. falciparum*	23495020	6.21	48,678.0	25	64
8	Ornithin aminotransferase	Down-regulated	1.7	*P. falciparum*	1200566	6.75	46,103.5	10	28
	26S protease regulatory subunit, putative			*Pf*	23615260	6.30	46,891.4	7	17
9	Ornithine aminotransferase	Down-regulated	3	*P. falciparum*	1200566	6.75	46,103.5	9	26
	S-adenosylmethionine synthetase			*P. falciparum*	23505122	6.28	44,844.2	10	29
10	Ornithine aminotransferase	Down-regulated	1.5	*P. falciparum*	46361043	6.47	46,055.4	13	34
11	Ornithine aminotransferase	Down-regulated	1.5	*P. falciparum*	1200566	6.75	46,103.5	4	13
12	Glyceraldhyde-3-phosphate dehydrogenase	Down-regulated	1.7	*P. falciparum*	23497672	7.59	36,635.4	14	45
13	Chaperonin cpn 60, mitochondrial precursor	Down-regulated	2.0	*P. falciparum*	407859	4.91	79,445.1	3	5
14	Uridine phosphorylase, putative	Down-regulated	2.1	*P. falciparum*	23504618	6.07	26,858.3	8	34
15	Acylamino-acid-releasing enzyme	Up-regulated	2.5	Human	P13798	5.29	81,225.0	9	14
	Transitional endoplasmic reticulum ATPase			Human	P55072	5.14	89,191.1	8	12
16	Ribonuclease inhibitor	Up-regulated	2.5	Human	P13489	4.71	49,842.6	14	43
	26S proteasome non-ATPase regulatory subunit			Human	P55036	4.68	40,736.9	7	22
17	Merozoite surface protein 7 precursor	Up-regulated	1.3	*P. falciparum*	15808773	4.72	41,363.7	3	9
18	Peroxiredoxin 2	Up-regulated	1.6	Human	P32119	5.67	21,760.8	10	41
19	Transitional endoplasmic reticulum ATPase	Up-regulated	1.8	Human	P55072	5.14	89,191.1	29	39
20	Endoplasmic reticulum-resident calcium binding protein	Up-regulated	1.5	*P. falciparum*	23508293	4.49	39,374.3	3	13
21	14-3-3 protein epsilon	Up-regulated	1.8	Human	P62258	4.63	29,174	9	36
22	14-3-3 proteinβ/α	[e]	[e]	Human	P31946	4.76	2,787.3	7	29
23	Phosphoglycerate kinase 1	Up-regulated	2.0	Human	P00558	8.30	44,483.8	19	51
24	DNA J domain protein, putative	Up-regulated	1.7	*P. falciparum*	46361243	8.21	44,669.5	4	10
	26S proteasome regulatory subunit S10B			Human	P62333	7.09	44,173.2	17	41
	Carbonic anhydrase1			Human	P00915	6.64	29,739.1	6	27

Table 2 (continued)

Spot no.	Protein identification	Expression in resistant strain	Folds exp[a]	Species	NCBI accession no.	pI[b]	MW[c]	Matched peptides	% AA coverage[d]
25	Fructose-biphosphate aldolase	Up-regulated	1.7	*P. falciparum*	23497496	8.33	40,105.2	6	17
	Carbonic anhydrase1			Human	P00915	6.64	29,739.1	6	27
26	Heat shock protein	Up-regulated	1.8	*P. falciparum*	23505079	5.18	72,387.9	39	51

All of these differentially expressed proteins are presented in Fig. 2.
[a] The ratio of expression of proteins in NRC10 compared to the FCR-3 strain. It indicates the average level of triplicate experiments.
[b] Calculated isoelectric point (pI) as recorded in the NCBI protein database.
[c] Calculated molecular weight (MW) as recorded in the NCBI protein database.
[d] The percentage of total protein sequence covered by identified peptides. Matched peptides and % AA coverage represent the best measured values.
[e] Spot was detected only in the NRC10 gels.

cloned heterogeneous strain. NRC10 (N-89 resistant clone number 10) showed higher growth rate with N-89 pressure and EC_{50} value of N-89 than other clones (data not shown). Figure 1 shows the antimalarial activity of N-89 against the NRC10 and FCR-3 strains. Drug susceptibility tests showed that there was a tenfold increase in the EC_{50} value of N-89 on the NRC10 strain (2.5×10^{-7} M) compared to the original FCR-3 strain (2.5×10^{-8} M). NRC10 showed marked decrease in the sensitivity of NRC10 to N-89 at a certain concentration (1.4×10^{-7} M) compared to the FCR-3 strain. The growth rates at this point reached 80% for the NRC10 strain and 0% for the FCR-3 strain (Fig. 1). No cross-resistance was obtained to either a structurally related drug (artemisinin) or to structurally unrelated drugs (quinine, chloroquine and mefloquine), as shown by the EC_{50} values, which were similar to the FCR-3 strain (Table 1).

Differentially expressed proteins between N-89 sensitive and resistant strains

Figure 2 represents the two-dimensional electrophoresis pattern of the FCR-3 and NRC10 strains. Approximately 200 spots were separated by 2-DE using 7 cm IPG strips within the pH range 3–10. By matching the different gel patterns of both strains, there was over-expression of 12 spots and down-regulation of 14 spots in NRC10. It is interesting to note that one spot was expressed in the NRC10 strain only, human 14-3-3 protein β/α (spot no. 22). Table 2 shows the protein identification and expression level of the FCR-3 and NRC10 strains. The values of expression ratio were ranged from 1.3 to 2.6. Eleven proteins were identified of human origin and 15 proteins were identified of *P. falciparum* origin. The up-regulated parasite proteins were identified as merozoite surface protein 7 (MSP 7) precursor, endoplasmic reticulum-resident calcium binding protein (PfERC), DNA J domain protein, fructose-biphosphate (FBP) aldolase, and heat shock protein. The down-regulated parasite proteins were identified as actin, 14-3-3 protein homologue, phosphoethanolamine *N*-methyl transferase (PfPEMT), enolase, ornithine aminotransferase (OAT), *S*-adenosylmethionine synthetase (SAMS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), chaperonin cpn 60, and uridine phosphorylase.

Discussion

N-89 was developed to be an effective antimalarial compound to overcome the multidrug resistant *P. falciparum*. In this study, a N-89 resistant clone was obtained after 2 years of drug pressure and it was used to investigate the antimalarial mechanism of N-89. Comparative analysis of 2-D gels of N-89 sensitive (FCR-3) and resistant (NRC10) strains revealed a number of differentially expressed protein spots, as shown in Fig. 2. The identified proteins of *P. falciparum* are shown in Table 2. Three glycolytic enzymes such as enolase, GAPDH, and FBP aldolase were detected. Because the energy production of *P. falciparum* depends entirely on the glycolytic pathway as a parasite and because erythrocytes lack a complete Krebs cycle, glycolytic enzymes seem to be promising drug candidates (Pal-Bhowmick et al. 2004). Endoplasmic reticulum related proteins were identified (Nirmalan et al. 2004), i.e., 14-3-3 protein and PfERC. 14-3-3 Proteins are cytosolic adaptor proteins that modulate intracellular signaling, cell cycle control, transcriptional control, and apoptosis in humans (Towbin et al. 2003), while its function in *Plasmodium* is unknown (Al-Khedery et al. 1999). PfERC was reported to be of unknown function in malaria parasites (La Greca et al. 1997). PfPEMT is an important enzyme in phospholipids

synthesis and it could be possible targets for new antimalarial drugs (Witola et al. 2006). Proteins known from the merozoite stage of the parasites (MSP7 precursor) plays an important role in merozoite binding and invasion (O'Donnell et al. 2000), but it might also play another role rather than invasion, as it was observed in the trophozoite stage in our experiment. OAT plays a central role in ornithine homeostasis and biosynthesis (Gafan et al. 2001).

Our data demonstrate the feasibility of proteomics as an approach to identify novel endoperoxide-related proteins. Resistance to endoperoxide may be multifactorial due to the affection of more than one protein and enzyme. The role of the identified proteins in the mechanism of endoperoxides remains unclear and further studies are in progress, such as the knockout technique, to identify the nature of parasite proteins and to test whether these changes mediate the antimalarial effect of N-89.

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ORIGINAL ARTICLE

Polymorphisms of cytochrome *b* gene in *Leishmania* parasites and their relation to types of cutaneous leishmaniasis lesions in Pakistan

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ABSTRACT

The exact species and/or strains of *Leishmania* parasites involved strongly influence the clinical and epidemiological features of leishmaniasis, and current knowledge of those influences and relationships is inadequate. We report that cytochrome *b* (*cyt* b) gene sequencing identified causal *Leishmania* parasites of 69 cutaneous leishmaniasis cases in Pakistan over a 3-year period. Of 21 cases in highland areas (Quetta city, Balochistan province), 16 (76.2%) were identified as *Leishmania (L.) tropica* and five (23.8%) as *Leishmania (L.) major.* Of 48 cases from lowland areas, cities/villages in Indus valley in Sindh and Balochistan provinces, 47 (97.9%) were identified as *L. (L.) major* and one (2.1%) as *L. (L.) tropica*. Statistical analysis (Fisher's exact test) revealed a significant difference ($P < 0.0001$) in the distribution of the two species by altitude; *L. (L.) major* is predominant in lowland and *L. (L.) tropica* at highland areas. The present result enriched our earlier finding, based on the first year's cultured parasite data, that only *L. (L.) tropica* was found in highland areas and only *L. (L.) major* in lowland areas. Among *Leishmania* samples analyzed, three types of *cyt* b polymorphism of *L. (L.) major* were found, including 45 (86.5%) cases of type I, six (11.5%) of type II and one (2%) of type III. We report for the first time on the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing from clinical samples. Moreover, we found no correlation between clinical presentation (wet-, dry- and/or mixed-types of cutaneous lesions) and causal *Leishmania* parasites.

Key words: cutaneous leishmaniasis lesions, *cyt* b gene sequencing, *Leishmania (L.) major* polymorphisms, *Leishmania (L.) tropica*, Pakistan.

INTRODUCTION

Leishmaniasis is the result of infection with intracellular protozoan parasites belonging to the genus *Leishmania*.[1] It affects more than 12 million people in 88 countries, with 350 million people at risk; every year there are 1–1.5 million new cases of cutaneous leishmaniasis (CL) and 0.5 million cases of visceral

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leishmaniasis (VL).[2] The appearance of clinical features depends on the complex interactions resulting from the invasiveness, tropism and pathogenicity of the parasite and the immune response of the host.[3] The epidemiology of leishmaniases is extremely diverse and far from fully elucidated.[4] More than 20 species of *Leishmania* parasites are known to infect humans and approximately 30 species of sandfly to transmit the disease.[2]

Pakistan, a tropical and subtropical country located in the northwest of South Asia, is an endemic area for leishmaniasis. The country is divided into four provinces, namely the North West Frontier Province (NWFP), Punjab, Sindh and Balochistan.[5] VL and CL are more common in Pakistan than are mucocutaneous and diffuse cutaneous leishmaniasis.[5–10] VL, considered deadly if untreated, mainly occurs in the northern region of the country, in areas such as Baltistan district, Chilas district, Azad Jammu and Kashmir, although reported sporadically in other areas of NWFP, Balochistan and Punjab provinces.[11]

In Pakistan, CL is popularly known as oriental sore, Delhi boil, Baghdad boil and Quetta sore.[5–7] Most of the skin lesions are of the wet-type and caused by *Leishmania (L.) major* which is endemic in NWFP and Balochistan province.[7] Some patients from the city of Multan in Punjab province presented with dry-type lesions, which was taken to indicate that only *Leishmania (L.) tropica* was present in that area.[8] Certain districts (Jacobabad, Larkana and Dadu) in Sindh province were reported to be endemic areas for CL, with the presence of both wet- and dry-type lesions taken to indicate clinically the presence of both *L. (L.) tropica* and *L. (L.) major* in that region.[5]

The identification of *Leishmania* species is important not only from an epidemiological perspective but also on clinical grounds in order to select diagnostic methods, plan treatment, define patient prognosis and monitor clinical outcomes. Accurate identification of the parasites must be based on molecular approaches because parasitological, clinical or epidemiological features by themselves are insufficient for the task.[4,12–14] One molecular technique, polymerase chain reaction (PCR), can quickly give exact diagnoses, which can reduce working hour losses, costs and social suffering of the patients.[14]

Among the molecular methods applied in *Leishmania* species identification, DNA-based techniques have been used increasingly. One of them, PCR amplification and sequencing of cytochrome *b* gene method (*cyt* b gene sequencing) has recently been established as a useful tool for the identification and phylogenetic study of the genus *Leishmania*, able to differentiate among human-infecting species and from other trypanosomatids.[15] Previous comparison of *cyt* b gene sequencing results for the cultured parasites with the split-specimen findings of other molecular techniques such as multi-locus enzyme electrophoresis (MLEE) analysis and polymorphism-specific (PS)-PCR showed total agreement among the results.[12,16] Those findings led us to use *cyt* b procedures for exploration of polymorphism in CL-causing *Leishmania* species from Pakistan.

In this study, we report for the first time the presence of polymorphisms in *L. (L.) major* (types I, II and III) based on species identification using *cyt* b gene sequencing in two different altitudes of Pakistan over a 3-year period. Moreover, we report an association between species and/or types of *Leishmania* and clinical presentation (dry-, wet- and mixed-types) of the skin lesions.

METHODS

Study areas and collection of sample

A total of 69 *Leishmania* biopsy specimens were obtained from 69 different subjects with cutaneous leishmaniasis in Pakistan. Individuals were residing in areas around Quetta city (Balochistan province), located 1600–1800 m a.s.l. in highland or mountainous regions, and in Sukkur city, Jacobabad district, Larkana district (Sindh province) and Jhal Magsi district, Jafar Abad district, Sibi city (Balochistan province) which are approximately 100 m a.s.l. and located in lowland regions of Pakistan. We diagnosed the clinical lesions according to textbook[1,3,17,18] criteria which were compiled and are described in Table 1. Thirty-one biopsy samples were collected in 2003, 28 in 2004, and 10 in 2005. The patients involved in the surveys were treated with meglumine antimonate and/or antibiotics by local physicians, depending on clinical diagnosis and clinical indications.[5]

Table 1. Characterization of types of cutaneous leishmaniasis lesions

	Dry type	Wet type
Lesion	Single, grow slowly and persist for a year or more.	Multiple, mature more rapidly, lasting a few months.
Course	A small, brownish nodule appears which becomes a slowly expanding plaque, reaching 1–2 cm in about 6 months.	– Red furuncle-like nodule. – After 2 weeks, a central crust forms. – Crust may fall away to reveal the underlying ulcer. The raised, red margin enlarges in 2–3 months.
Healing	After 8–12 months, lesions regress and ulcers heals.	Heal in 2–6 months, leaving scars.

Mixed-type lesions combine or mix the features mentioned.

Each lesion was examined by a dermatologist, and those found to be consistent with CL were cleaned with soap and water and swabbed with ethanol before samples were taken; each patient gave informed consent for surgical biopsy. A sterile scalpel was used to make an incision in the border of the lesion; and each biopsy specimen was put immediately into a sterile screw-capped 2-mL tube containing 70% ethanol; the tubes were stored at room temperature and brought to Japan by the dermatologist for PCR and *cyt* b sequencing analysis.

Extraction of DNA from the clinical samples

From the above biopsy samples, DNA extractions were performed according to the protocol for extraction of DNA from the tissue which was described by the company (GenomicPrep Cells and Tissue DNA Isolation Kit, Amersham Biosciences, USA).

Polymerase chain reaction amplification

From the above genomic DNA extract, PCR amplification and *cyt* b gene sequencing-based identification of *Leishmania* parasites were carried out as follows. We performed PCR using 0.2 μL DNA polymerase Ex Taq (Takara, Japan) in a total PCR solution volume of 50 μL. Each PCR solution contained 1 μL of 100 to 200 ng/μL DNA template, *Leishmania cyt* b gene consensus primer (0.25 μL of LCBF1 forward primer [100 μM], 5′-taatacgactcactataGGTGTAGGTTTTAGTYTAGG-3′; and 0.25 μL of LCBR2 reverse primer [100 μM], 5′-gggttttcccagtcacgacgCTACAATAAACAAATCATAATATRCAATT-3′), 4 μL of 2.5 mM deoxyribonucleotide triphosphate (dNTP), 5 μL of 10X buffer and distilled water 39.3 μL. PCR conditions were initial denaturation at 94°C for 1 min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min, followed by a final extension of 72°C for 5 min.

For the samples which were not amplified by DNA polymerase Ex Taq, PCR using 0.5 μL DNA polymerase Phusion (High-Fidelity DNA Polymerase; Finnzymes, Espoo, Finland) in a total PCR solution volume of 50 μL were performed. Each PCR solution contained 1 μL of 100–200 ng/μL DNA sample template, 0.5 μL *Leishmania cyt* b gene consensus primer, 4 μL of 2.5 mM dNTP, 10 μL of 10X buffer and 34 μL distilled water. PCR conditions were initial denaturation at 98°C for 30 s, followed by 39 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 45 s, followed by a final extension of 72°C for 5 min.

For the samples which were not amplified by means of the above, we performed nested PCR by amplification with COIIIF (5′-taatacgactcactataGTTTATATTGACATTTTTGTWGATT-3′) and MURF4R (5′-gggttttcccagtcacgacgAATCTCTCTCTCCCTT-3′) primers, following which the resulting 1 μL of PCR products were amplified again with *Leishmania cyt* b gene consensus primers as mentioned for Ex Taq PCR. All PCR products were visualized by gel electrophoresis with 0.7% Agarose-LE, Classic type (Nacalai Tesque, Kyoto, Japan).

DNA purification and sequencing

The amplified DNA products were harvested by gel electrophoresis on 0.4% Seakam GTG agarose gel (FMC BioProduct, Foster City, CA, USA). The visualized DNA products were excised carefully and then purified by QIA Quick Gel Extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation. Concentration of each purified DNA product was measured by Gene Spec

Figure 1. Clinical appearance of cutaneous leishmaniasis cases in Pakistan. (a) Dry-type (RU-51 in Table 2), (b) wet-type (RU-22 in Table 2), and (c) mixed-type (RU-12 in Table 2).

III (Naka Instruments, Ibaraki, Japan) and checked again with gel electrophoresis (0.7%) by comparing with a concentration-known DNA sample as positive control. DNA sequencing was carried out on an ABI PRISM-301 automated sequencer (Applied Biosystems, Foster City, CA, USA) by using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The following primers were used for sequencing: T7 (17 mer) 5′-AATACGACTCACTATAG-3′, U19 (19 mer) 5′-GGTTTTCCCAGTCACGACG-3′, LCYTB F4L (22 mer) 5′-TGTTATTGAATATGAGGTAGTG-3′ and LCYTBR4 (26 mer) 5′-GAACTCATAAAATAATGTAAACAAAA-3′.

Analysis of sequenced result

The sequenced results were assembled and edited by using Genetyx-Mac software ver. 11.0. (Software Development, Tokyo, Japan). They were compared with previously published reference strains, available from EMBL/DDBJ/GenBank, *L. (L.) major*, accession number AB 095970 and *L. (L.) tropica*, accession number AB 095960.

RESULTS

Clinical and epidemiological profiles of study patients

The patients were 45 males and 24 females (M : F, 2:1), ranging 1–50 years in age (mean age, 20 years). Thirteen patients were under 9 years old, 26 were 10–19 years old, 14 were 20–29 years old, six were 30–39 years, seven were 40–49 years and three were 50 years old. According to the patients' own statements, the evolution of their disease processes (in terms of the time since they had first noticed a lesion) ranged from 2 weeks to 3 years. Characteristics of the CL-patients and their clinico-epidemiological data are summarized in Table 2. Examples of the dry-, wet- and mixed-type clinical lesions of cutaneous leishmaniasis in cases included in the present study are shown in Figure 1.

Findings based on *cyt* b gene sequencing

The *cyt* b analysis showed 17 cases of *L. (L.) tropica* (16, 94.1%, in highland areas and one, 5.9%, in lowland areas) and 52 cases of *L. (L.) major* (47, 90.4%, in lowland and five, 9.6%, in highland), showing a significant difference ($P < 0.0001$, Fisher's exact test) in distribution of the two species by altitude; *L. (L.) major* is predominant in the lowlands, and *L. (L.) tropica* in highland areas (Fig. 2).

In this study, three types of *cyt* b polymorphism of *L. (L.) major* were identified. In the sequencing result of *L. (L.) major cyt* b, in what we have called *L. (L.) major* type I, cytosine (C) is replaced by thymine (T) at nucleotide positions 416 and 799. In what we call *L. (L.) major* type II, at nucleotide positions 416 and 799, C is replaced by T, and in nucleotide position 436,

Table 2. Detailed characteristics of cutaneous leishmaniasis samples and clinico-epidemiological profiles of the patients

Sample name	Species	Altitude	Skin Lesion site	Lesion no.	Lesion type	Lesion evolution (days)	Age (years)	Sex	Geographical origin of patient
RU 1	*L. (L.) mj* type I	L	Buttock	1	Dry	90	30	M	Sukkur, Arore
RU 2	*L. (L.) mj* type II	L	Rt arm	1	Dry	90	45	F	Sukkur, Khairpur
RU 3	*L. (L.) mj* type III	L	Rt arm	1	Mixed	180	38	M	Larkana, Sono Khan
RU 4	*L. (L.) mj* type I	L	Both arm	4	Dry	30	10	F	Larkana, Sono Khan
RU 5	*L. (L.) mj* type I	L	Lt arm, buttock, rt leg	3	Dry	30	12	F	Larkana, Warah
RU 6	*L. (L.) mj* type I	L	Rt arm	1	Dry	60	15	M	Larkana, Warah
RU 7	*L. (L.) mj* type I	L	Lt arm	1	Dry	NA	18	M	Sukkur, Pano Aqil
RU 8	*L. (L.) mj* type I	L	Rt leg	1	Dry	60	25	M	Jacobabad
RU 9	*L. (L.) mj* type I	L	Lt leg	1	Dry	NA	40	F	Sukkur, Khairpur
RU 10	*L. (L.) mj* type I	L	Lt upper arm	1	Dry	30	45	F	Larkana, Warah
RU 11	*L. (L.) mj* type I	L	Arm	1	Mixed	30	3	F	Larkana, Shahdadkot
RU 12	*L. (L.) mj* type I	L	Face	1	Mixed	60	7	F	Larkana, Shahdadkot
RU 13	*L. (L.) mj* type I	L	Face, rt leg	2	Mixed	60	10	M	Larkana, Kambar
RU 14	*L. (L.) mj* type I	L	Rt leg	1	Mixed	90	28	M	Larkana, Sono Khan
RU 15	*L. (L.) mj* type I	L	Both legs	3	Mixed	180	50	M	Larkana, Kambar
RU 16	*L. (L.) mj* type I	L	Ear, rt upper arms	2	Mixed	45	1	M	Larkana, Sono Khan
RU 17	*L. (L.) mj* type I	L	Face	2	Mixed	45	20	M	Larkana, Sono Khan
RU 18	*L. (L.) mj* type I	L	Both legs	3	Mixed	60	30	M	Larkana, Shahdadkot
RU 19	*L. (L.) mj* type I	L	Buttock	4	Dry	NA	7	M	Sukkur, Pano Aqil
RU 20	*L. (L.) mj* type I	L	Rt leg	1	Wet	30	2	M	Larkana, Sono Khan
RU 21	*L. (L.) mj* type I	L	Lt arm	1	Wet	60	2	M	Baluchistan, Jhal
RU 22	*L. (L.) mj* type I	H	Lt arm	2	Wet	60	10	F	Quetta city
RU 23	*L. (L.) mj* type I	H	Face	1	Wet	30	10	M	Quetta, Mari Abad
RU 24	*L. (L.) mj* type I	H	Lt leg	2	Wet	75	12	F	Quetta, Mari Abad
RU 25	*L. (L.) mj* type I	L	Both arms and trunk	3	Wet	60	15	M	Larkana, Warah
RU 26	*L. (L.) mj* type I	L	Both arms	2	Wet	15	15	M	Jacobabad
RU 27	*L. (L.) mj* type I	L	Lt arm	1	Wet	30	15	M	Jacobabad
RU 28	*L. (L.) mj* type I	L	Lt leg	1	Wet	60	20	F	Sibi City
RU 29	*L. (L.) mj* type I	L	Lt leg, buttock	4	Wet	60	20	M	Larkana, Warah
RU 30	*L. (L.) mj* type I	L	Face, rt arm	2	Wet	75	20	M	Larkana, Kambar
RU 31	*L. (L.) mj* type I	L	Both legs	2	Wet	60	20	M	Sukkur, Shikarpur
RU 32	*L. (L.) mj* type I	L	Lt leg	1	Wet	30	25	F	Baluchistan, Jhal
RU 33	*L. (L.) mj* type I	L	NA	1	Wet	90	25	M	Sukkur, Khairpur
RU 34	*L. (L.) mj* type I	L	Lt arm, both legs	4	Wet	30	28	M	Jacobabad
RU 35	*L. (L.) mj* type I	L	Chest, lt arm	3	Wet	30	40	M	Sukkur City
RU 36	*L. (L.) mj* type I	L	Lt arm, rt leg	3	Wet	60	50	F	Sukkur, Khairpur
RU 37	*L. (L.) mj* type I	L	Rt leg	1	Wet	30	8	M	Larkana, Warah
RU 38	*L. (L.) mj* type I	L	Lt arm	1	Wet	NA	10	F	Sukkur, Khairpur
RU 39	*L. (L.) mj* type I	L	Rt arm, both legs	3	Wet	NA	19	M	Sukkur, Khairpur
RU 40	*L. (L.) mj* type I	L	Lt leg	2	Wet	90	24	M	Larkana, Warah
RU 41	*L. (L.) mj* type I	L	Rt leg	4	Wet	60	25	M	Larkana, Warah
RU 42	*L. (L.) mj* type I	L	Rt leg	2	Wet	30	40	F	Larkana, Warah
RU 43	*L. (L.) mj* type II	L	Lt arm	1	Wet	NA	14	M	Baluchistan, Jhal
RU 44	*L. (L.) mj* type II	L	Both legs	4	Wet	60	18	M	Jacobabad
RU 45	*L. (L.) mj* type II	L	Both legs	4	Wet	60	19	M	Sukkur, Khairpur
RU 46	*L. (L.) mj* type II	L	Lt arm	1	Wet	NA	29	M	Sukkur, Shikapur
RU 47	*L. (L.) tr*	H	Face	2	Dry	360	3	F	Quetta, Mari Abad
RU 48	*L. (L.) tr*	H	Face	2	Dry	180	7	F	Quetta city
RU 49	*L. (L.) tr*	H	Rt ear	1	Dry	30	9	F	Quetta, Mari Abad
RU 50	*L. (L.) tr*	H	Lt leg	1	Dry	360	14	M	Quetta city
RU 51	*L. (L.) tr*	H	Both arms	3	Dry	1080	21	M	Quetta, Mari Abad
RU 52	*L. (L.) tr*	L	Lt arm	4	Dry	30	30	M	Baluchistan, Jhal
RU 53	*L. (L.) tr*	H	Lt arm	2	Mixed	180	18	M	Quetta city
RU 54	*L. (L.) tr*	H	Face	1	Wet	360	7	F	Quetta city
RU 55	*L. (L.) tr*	H	Face	2	Wet	60	14	M	Quetta, Samagali
RU 56	*L. (L.) tr*	H	Face, lt leg	2	Wet	60	16	F	Quetta, Mari Abad

Table 2. *Continued.*

Sample name	Species	Altitude	Skin Lesion site	Lesion no.	Lesion type	Lesion evolution (days)	Age (years)	Sex	Geographical origin of patient
RU 57	*L. (L.) tr*	H	Rt arm, rt leg	3	Wet	60	16	M	Quetta, Gorabad
RU 58	*L. (L.) tr*	H	Rt upper arm	1	Wet	60	30	M	Quetta, Mari Abad
RU 59	*L. (L.) tr*	H	Face, lt arm	2	Wet	360	50	M	Quetta, Mari Abad
RU 60	*L. (L.) mj* type II	L	Lt leg	4	Wet	75	10	F	Larkana, Gul Mohd
RU 61	*L. (L.) mj* type I	L	Rt leg	1	Wet	90	40	M	Larkana, Sarang Kumar
RU 62	*L. (L.) mj* type I	L	Lt arm, lt leg	2	Wet	60	12	F	Jacobabad, Dera Murad
RU 63	*L. (L.) mj* type I	H	Face, rt leg	2	Wet	360	12	M	Quetta, Mari Abad
RU 64	*L. (L.) mj* type I	L	Lt leg	1	Wet	60	17	M	Larkana, Mehar
RU 65	*L. (L.) mj* type I	H	Lt arm	1	Wet	30	30	F	Quetta city
RU 66	*L. (L.) tr*	H	Rt leg	1	Wet	360	6	M	Quetta, Mari Abad
RU 67	*L. (L.) tr*	H	Lt leg	1	Wet	360	10	M	Quetta, Mari Abad
RU 68	*L. (L.) tr*	H	Rt arm	1	Wet	180	45	F	Quetta, Mari Abad
RU 69	*L. (L.) tr*	H	Face, lt leg	3	Mixed	1080	5	F	Quetta, Mari Abad

L. (L.) tr, Leishmania leishmania tropica; *L. (L.) mj, Leishmania leishmania major*; H, highland; L, lowland; lt, left; rt, right; NA, not available; M, male; F, female.

Table 3. Types of *Leishmania* parasites identified in highland and lowland areas of Pakistan

Type of *Leishmania*	No. of cases identified in Lowlands					Lowland total	Highland total
	Sukkur	Jacobabad	Larkana	Jafar Abad/Jhal Magsi	Sibi		
L. (L.) major type I	10	5	22	2	1	40	5
L. (L.) major type II	3	1	1	1	0	6	0
L. (L.) major type III	0	0	1	0	0	1	0
L. (L.) tropica	0	0	0	1	0	1	16

guanine (G) is replaced by adenine (A). In the sequence we call *L. (L.) major* type III, in corresponding nucleotide position number 265, A is replaced by G and C is replaced by T at nucleotide positions 416 and 799 (Fig. 3). We identified 40 cases of *L. (L.) major* type I, six of *L. (L.) major* type II, one of *L. (L.) major* type III and only one of *L. (L.) tropica* in cases from lowland areas of Pakistan. We identified 16 cases of *L. (L.) tropica* and five of *L. (L.) major* type I in patients from the highlands around Quetta city, in a mountainous region close to Afghanistan (Table 3).

In our *cyt* b gene analysis findings, the parasites from patients who presented with dry-type lesions were identified as *L. (L.) tropica* in six cases (38%) and as *L. (L.) major* in 10 cases (62%). In those clinically presenting with wet-type lesions, nine cases (21%) were identified as *L. (L.) tropica* and 33 (79%) as *L. (L.) major*. In those clinically presenting with mixed-type lesions, two cases (18%) were identified as *L. (L.) tropica* and the remaining nine (82%) as *L. (L.) major* (Table 4). Statistical analysis for relationship between types of skin lesions (wet-, dry- and/or mixed-types) and type of *Leishmania* parasite (i.e. *L. (L.) tropica* vs *L. (L.) major*) identified yielded a χ^2 test result at $P > 0.05$ (SPSS 11.0, Chicago, IL, USA), demonstrating that clinical features are not reliable indicators of the species of *Leishmania* parasite at the present study sites in Pakistan.

Table 4. Types of *Leishmania* parasite identified by cytochrome *b* gene sequencing and their presenting types of clinical lesions

Presenting features	*L. (L.) tropica*	*L. (L.) major*				Total
		Total	Type I	Type II	Type III	
Dry type	6	10	9	1	0	16
Wet type	9	33	28	5	0	42
Mixed type	2	9	8	0	1	11
Total	17	52	45	6	1	69

DISCUSSION

Cutaneous leishmaniasis is prevalent in Pakistan and has been reported in all provinces and almost all major cities.[5–9] The disease manifests not only in

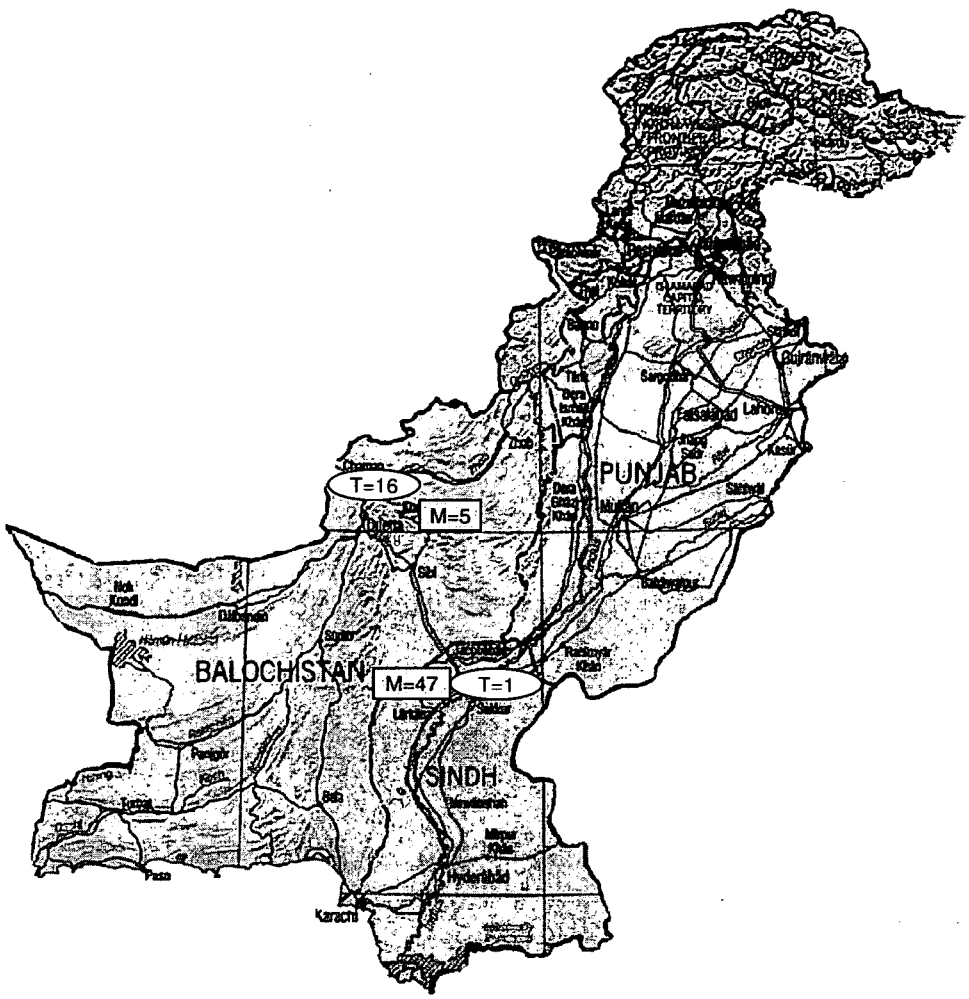

Figure 2. Types of cutaneous leishmaniasis cases identified in the two study areas. M, *Leishmania (L.) major*; T, *Leishmania (L.) tropica*.

classical presentation but also in various forms such as acute paronychial, chancriform, annular, palmoplantar, zosteriform and erysipeloid forms.[6,10] Molecular techniques are favored also for the identification of such non-classical cases.

Regarding the causative agents of CL in Pakistan, the most commonly mentioned parasites were *L. (L.) major* and *L. (L.) tropica*.[5,9,12] It had been believed that *L. (L.) major* initiates the wet or moist lesions of CL and that *L. (L.) tropica* triggers the dry lesions of CL.[1,3,17,18] However, our findings indicate that both *L. (L.) major* and *L. (L.) tropica* can present with either type of lesion and with mixed lesions, and no statistical association was found between the types of presenting lesions (dry-, wet- or mixed-types) and the *Leishmania* species identified. This study thus contradicts, for example, the previous assumption of complete absence of *L. (L.) major* in an area where patients present only with dry-type lesions.[8] Our findings show that it is not possible to determine the causal type of *Leishmania* parasite by clinical presentation alone.

In the lowland areas of Pakistan, *L. (L.) major* type II and type III were found in only seven of our cases, six of the former and one of the latter. Regarding *L. (L.) major* type I, the majority of patients (40 cases) were from the lowland areas, and five were from mountainous areas around Quetta city in Balochistan

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L.(L.)major/5ASKH          241 TTTTGTGATATATATATTTATAGTAATAATAGGTTTTATTGGCTATGTTTTACCATGTAC  300
L.(L.)major/Friedlin       241 ************************************************************  300
L.(L.)major/Type I         241 ************************************************************  300
L.(L.)major/Type II        241 ************************************************************  300
L.(L.)major/Type III       241 ************************G***********************************  300

L.(L.)major/5ASKH          361 TGGTACTTGACTTTGTTATTGAATATGAGGTAGTGAGTATATTAATGATTTTACACTGTT  420
L.(L.)major/Friedlin       361 ************************************************************  420
L.(L.)major/Type I         361 ************************************************************  420
L.(L.)major/Type II        361 ************************************************************  420
L.(L.)major/Type III       361 ************************************************************  420

L.(L.)major/5ASKH          421 AAAATTACATGTGTTGCATGTGCTATTACCTTTTGTATTAATACTTGTAATATTTATGCA  480
L.(L.)major/Friedlin       421 ************************************************************  480
L.(L.)major/Type I         421 ************************************************************  480
L.(L.)major/Type II        421 ****************A*******************************************  480
L.(L.)major/Type III       421 ************************************************************  480

L.(L.)major/5ASKH          781 GGTTATTTTATTATTTTCCTTATTTTTGTTTATATTA                        817
L.(L.)major/Friedlin       781 ************************************                        817
L.(L.)major/Type I         781 ******************T*******************                        817
L.(L.)major/Type II        781 ******************T*******************                        817
L.(L.)major/Type III       781 ******************T*******************                        817
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Figure 3. Cytochrome *b* gene alignment showing the sites of different nucleotide positions of *L. (L.) major* type I, *L. (L.) major* type II and *L. (L.) major* type III. *, sequence identities.

province. Only one case of *L. (L.) tropica* was identified in the lowland area (Jhal Magsi, Balochistan province), and all the remaining 16 cases were identified in highland areas. A significant difference ($P < 0.0001$, Fisher's exact test) was found in the distribution of the two species by altitude, with *L. (L.) major* predominant in lowland areas and *L. (L.) tropica* in highland areas. The present result enriched our earlier findings, based only on the first year's cultured parasite data, that only *L. (L.) tropica* was found in highland areas and only *L. (L.) major* in lowland areas.[12] The small discrepancy found between the previous and the present studies might be due to some unidentified factors such as increased sample size, migration of patients from lowland to highland and vice versa. Our finding from biopsy

samples of *cyt* b polymorphism in *L. (L.) major* and homology in *L. (L.) tropica* confirms the previous findings of polymorphism in the former species and homology in the latter identified by MLEE.[12]

In the field of PCR-based methods for detecting *Leishmania* species, it has been necessary to isolate the parasite in culture before using any of the existing high-resolution techniques such as isoenzymes, randomly amplified polymorphic DNA analysis and schizodemes. Also, recovery of parasites in culture is rarely more than approximately 70% efficient even with easily cultured *Leishmania* parasites.[19] In our study, we could explore the causal *Leishmania* parasite up to intraspecies level from the clinical samples. This finding can reduce the procedures which are necessary to isolate the parasite in culture. The technique we used, PCR followed by *cyt* b gene sequencing, will be useful for identifying *Leishmania* parasite strains from clinical samples.

Previous studies revealed genetic polymorphism in natural populations of different *Leishmania* species, which could explain the plasticity of these parasites and their ability to adapt the changing ecological conditions. One study reported finding three patterns of genetic and biological variation among *L. (L.) major* strains in rodent reservoir hosts (*Rhombomys opimus*, *Merioned libycus* and *Psammomys obesus*) from central Asia, the Middle East and Africa, explored by permissively primed intergenic polymorphic (PPIP)-PCR and single-stranded conformation polymorphism (SSCP)-PCR.[20] To link the above report and our findings, we would need to explore the role of the sandfly (vector) in polymorphism. However, our report fills a gap in the understanding of the epidemiological phenomena of cutaneous leishmaniasis.

In conclusion, we identified three types of *L. (L.) major* polymorphism in patients from two different altitude regions of Pakistan, one region in lowland areas (~100 m a.s.l.) of Sindh province (Sukkur city, Jacobabad district and Larkana district), Balochistan province (Jhal Magsi district, Jafar Abad district and Sibi city) and the other a highland area (1600–1800 m a.s.l.) of Balochistan province (Quetta city). For reasons of feasibility and accessibility, the study was confined to the above-mentioned areas. To explore the whole country's *Leishmania* parasite profile, we would need to extend the study into the other two provinces, Punjab and NWFP. To develop a more complete profile of the patterns of leishmaniasis, continual and vigilant surveillance is required. Several other pockets of infection and of vectors and reservoirs of leishmaniasis in Pakistan still need to be studied, and such studies are sure to contribute to needed knowledge concerning the clinical forms, causal agents and eco-epidemiological patterns of leishmaniasis in Pakistan.

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Anaerobic NADH-Fumarate Reductase System Is Predominant in the Respiratory Chain of *Echinococcus multilocularis*, Providing a Novel Target for the Chemotherapy of Alveolar Echinococcosis▽

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Alveolar echinococcosis, which is due to the massive growth of larval *Echinococcus multilocularis*, is a life-threatening parasitic zoonosis distributed widely across the northern hemisphere. Commercially available chemotherapeutic compounds have parasitostatic but not parasitocidal effects. Parasitic organisms use various energy metabolic pathways that differ greatly from those of their hosts and therefore could be promising targets for chemotherapy. The aim of this study was to characterize the mitochondrial respiratory chain of *E. multilocularis*, with the eventual goal of developing novel antiechinococcal compounds. Enzymatic analyses using enriched mitochondrial fractions from *E. multilocularis* protoscoleces revealed that the mitochondria exhibited NADH-fumarate reductase activity as the predominant enzyme activity, suggesting that the mitochondrial respiratory system of the parasite is highly adapted to anaerobic environments. High-performance liquid chromatography–mass spectrometry revealed that the primary quinone of the parasite mitochondria was rhodoquinone-10, which is commonly used as an electron mediator in anaerobic respiration by the NADH-fumarate reductase system of other eukaryotes. This also suggests that the mitochondria of *E. multilocularis* protoscoleces possess an anaerobic respiratory chain in which complex II of the parasite functions as a rhodoquinol-fumarate reductase. Furthermore, in vitro treatment assays using respiratory chain inhibitors against the NADH-quinone reductase activity of mitochondrial complex I demonstrated that they had a potent ability to kill protoscoleces. These results suggest that the mitochondrial respiratory chain of the parasite is a promising target for chemotherapy of alveolar echinococcosis.

Echinococcosis is a near-cosmopolitan zoonosis caused by helminthic parasites belonging to the genus *Echinococcus* (family Taeniidae) (18). The life cycle of *Echinococcus* spp. includes an egg-producing adult stage in the definitive hosts and a larval stage in intermediate hosts including humans. The larval stage of the parasite produces a large number of infective protoscoleces that develop to adult worms after being ingested by the definitive host, or they produce a new parasite mass when liberated inside the intermediate host, causing metastases of the parasite lesions. The two major species of medical and public health importance are *Echinococcus granulosus* and *E. multilocularis*, which cause cystic echinococcosis and alveolar echinococcosis (AE), respectively.

Human AE is a life-threatening disease, and without careful clinical management, it has a high fatality rate and poor prognosis. Humans acquire AE infection by ingesting eggs from adult parasitic worms. Early diagnosis and treatment (mainly by radical surgery) of human AE are difficult because the disease progresses slowly and usually takes more than several years before clinical symptoms become apparent. An efficient chemotherapeutic compound is still not available. The first choice for the chemotherapy of AE is benzimidazole derivatives (18), but they are parasitostatic rather than parasitocidal against larval *E. multilocularis*. Therefore, the development of highly effective antiechinococcal drugs is urgently needed.

Biological systems for energy metabolism are essential for the survival, continued growth, and reproduction of all living organisms. "Typical" mitochondria are usually considered to be oxygen-consuming, ATP-producing organelles. In fact, typical mitochondria, such as those found in mammalian cells, require oxygen to function. They use pyruvate dehydrogenase for oxidative decarboxylation of pyruvate to acetyl coenzyme A, which is then completely oxidized to CO_2 through the Krebs cycle. Most of the energy is produced by oxidative phosphorylation: the electrons from NADH and succinate are transferred to oxygen by the proton-pumping electron transfer respiratory chain in which ubiquinone (UQ) (Fig. 1A) is commonly used as an electron mediator. The backflow of the protons results in ATP formation by the mitochondrial ATP synthase.

In parasitic organisms, on the other hand, the carbohydrate and energy metabolic pathways of adult parasitic helminths differ greatly from those of their vertebrate hosts. The most important factors in this respect are the nutrient and oxygen supply (reviewed in references 4, 12, and 13). Parasitic hel-

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