Isolation of Genes Mediating Resistance to Inhibitors of Nucleoside and Ergosterol Metabolism in Leishmania by Overexpression/Selection*

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Paulo C. Cotrim§§, Lynne K. Garrity§, and Stephen M. Beverley§§

From the §Department of Molecular Microbiology, Washington University Medical School, St. Louis, Missouri 63110, the §Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and the ¶Instituto de Medicina Tropical/DMIP, University of São Paulo Medical School, São Paulo-SP 05403-000, Brazil

We tested a general method for the identification of drug resistance loci in the trypanosomatid protozoan parasite Leishmania major. Genomic libraries in a multicopy episomal cosmid vector were transfected into susceptible parasites, and drug selections of these transfecant libraries yielded parasites bearing cosmids mediating resistance. Tests with two antifolates led to the recovery of cosmids encoding DHFR-TS or PRTI, two known resistance genes. Overexpression/selection using the toxic nucleoside tubercidin similarly yielded the TOR (toxic nucleoside resistance) locus, as well as a new locus (TUB2) conferring collateral hypersensitivity to allopurinol. Leishmania synthesize ergosterol rather than cholesterol, making this pathway attractive as a chemotherapeutic target. Overexpression/selection using the sterol synthesis inhibitors terbinafine (TBF, targeting squalene epoxidase) and itraconazole (ITZ, targeting lanosterol C14-demethylase) yielded nine new resistance loci. Several conferred resistance to both drugs; several were drug-specific, and two TBF-resistant cosmids induced hypersensitivity to ITZ. One TBF-resistant cosmid encoded squalene synthase (SQS1), which is located upstream of the sites of TBF and ITZ action in the ergosterol biosynthetic pathway. This suggests that resistance to “downstream” inhibitors can be mediated by increased expression of ergosterol biosynthetic intermediates. Our studies establish the feasibility of overexpression/selection in parasites and suggest that many Leishmania drug resistance loci are amenable to identification in this manner.

Trypanosomatid protozoans of the genus Leishmania are the causative agent of leishmaniasis, a parasitic disease with a prevalence of 12 million cases in 88 countries and a worldwide incidence of 1.5–2 million cases per year (1). Pentavalent antimonial remains the primary drug used for treatment of clinical disease, although it has several drawbacks. Antimonial treatments are expensive, inactive when administered orally, require long courses of high dose treatment to be effective, and exhibit toxicity in proportion to dose and duration of treatment, and there are reports of the emergence of drug-resistant parasites (2). Thus, there is an urgent need for improved methods of chemotherapy.

One approach for the identification of prospective drug targets in Leishmania has been the study of drug-resistant parasites generated in the laboratory. As in other organisms, drug resistance frequently involves modifications of the gene encoding the primary drug target, such as mutations, rearrangements, or amplifications. In Leishmania, gene amplification is observed following stepwise selection for drug resistance, and amplification of the gene encoding methylene-tetrahydrofolate reductase-thymidylate synthase (DHFR-TS) and pteridine reductase (PTRI), members of the P-glycoprotein superfamilly (PGPA and MDRI), ornithine decarboxylase, and N-acetylglucosaminyltransferase, among others (see Refs. 4–8 for reviews and references). In addition to amplifications, cells obtained by stepwise drug selection frequently exhibit multiple resistance mechanisms. For example, Leishmania selected for resistance to the antifolate methotrexate (MTX) usually exhibit alterations in MTX uptake and less commonly amplification of DHFR-TS and PTRI, and rarely, point mutations in DHFR-TS, singly or in various combinations (4–6, 8–13). Even when drug resistance via gene amplification is a viable resistance mechanism, its frequency of occurrence is relatively low in Leishmania (less than 10−7) (4). Once induced, amplifications can be displaced by other mechanisms, depending on experimental variables such as the length of time in culture and selective drug concentration. Thus, the stochastic and occasionally transient occurrence of gene amplification limits its general utility in recovering potential drug resistance loci.

In this report we describe a more directed approach toward the identification of genes whose overexpression leads to drug resistance in Leishmania. This takes advantage of recent advances in our ability to manipulate genetically this parasite by transfection of functional multicopy episomal DNAs (14–16). We applied a “multicopy suppression” technique to the identification of drug resistance genes in Leishmania, similar to those previously performed in yeast and prokaryotes (17). In this approach, a library of transfected parasites is created, each bearing a different 30–40-kb segment of the parasite genome inserted into the Escherichia coli-Leishmania shuttle cosmid.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U30455.

†To whom correspondence should be addressed: Dept. of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-747-2630; Fax: 314-747-2634; E-mail: beverley@borcim.wustl.edu.

‡The abbreviations used are: MTX, methotrexate; TUB, tubercidin; ITZ, itraconazole; TBF, terbinafine; HygB, hygromycin B; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; kb, kilobase pair.

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vector cLHYG (18). Since expression of the passenger Leishmania sequences from episomes occurs autonomously and is related to the copy number, genes carried by cLHYG should be overexpressed. In effect, these segments of Leishmania DNA are “pre-amplified,” and drug pressure on such transfected parasite libraries should yield cosmids directly conferring drug resistance.

Here we tested the overexpression/selection approach with two drugs known to yield resistance via gene amplification in Leishmania, MTX, and tubercidin, and we showed that overexpression/selection successfully yielded loci previously detected. We then probed a parasite metabolic pathway not previously studied molecularly in Leishmania, that of sterol biosynthesis. As in fungi, Leishmania synthesizes ergosterol rather than cholesterol as its bulk membrane sterol (19), and this shift similarly offers great potential for selective chemotherapy as well as the study of the evolution of biochemical pathways.

EXPERIMENTAL PROCEDURES

Parasites, Culture, and Transfection—Leishmania major strain Friedlin V1 is a virulent clonal derivative of the Friedlin line (MHOM/IL/80/Friedlin), which was Friedlin A1 (A1) a clonal avirulent line derived from the Friedlin line after multiple passages in vitro (20); both were obtained from D. L. Sacks (National Institutes of Health). L. major strain LV39 clone 5 (LV39cl5) is a virulent clonal derivative of the LV39 strain (MHKO/SU/89/P), whereas LV39 clone 79 (LV39cl79) is an avirulent clonal derivative obtained after chemical mutagenesis; both were obtained from R. Titus (Colorado State University). Cells were grown in M199 medium, which contained 10% heat-inactivated fetal bovine serum (14). Parasites were transfected by electroporation, and clonal lines were obtained by plating on M199 semisolid media (14) containing 40 μg/ml hygromycin B (HygB) for recovery of cosmid transfecteds. For transfection of cosmid library DNA we used 14–40 μg DNA per transfection, and colonies from multiple transfactions were pooled (21).

To identify cosmid-bearing lines exhibiting drug resistance, 10⁶ control or cosmid library-transfected cells were plated on 100-mm M199 plates, containing increasing concentrations of the selective drug of interest. Macroscopic colonies were counted after 10–15 days of incubation and recovered into M199 medium.

Selective Drugs—Allopurinol, inosine dialdehyde, MTX, and tubercidin, and the pteridine O129 (2,4-diamino-6,7-diisopropylpteridine) were obtained from Sigma. Sandoz Pharmaceutical generously provided TBF. ITZ was purchased from the Jansen Research Foundation.

Cosmid Libraries—Three libraries containing 30–40-kb inserts of L. major genomic DNA were constructed in the E. coli/Leishmania shuttle vector cLHYG, using either shear (V1) or Sau3A partial digestion (LV39cl79), to prepare the genomic DNA inserts (18). Cosmid library DNA was prepared by SDS/alkali lysis followed by polyethylene glycol precipitation (18, 21). Transfection of the V1 cosmid library into Friedlin A1 line yielded 17,900 independent transfecteds (10,600 from Friedlin A1, 2,100 from Friedlin V1 Sau3A partial-digestion library and 7,300 from Friedlin V1 shear library). Transfection of LV39cl5 cosmid library DNAs into LV39cl79 yielded 3,600 independent transfecteds.

Analysis of Drug Resistance—Prior to tests of drug resistance, we increased the cosmid copy number within transfecteds with elevated aminoglycoside treatment (14). Primary transfecteds were selected in a stepwise manner, beginning at 125 μg of HygB/ml and progressing through four 1:10 passages into 250 and 500 μg/ml and, in some cases, to 1 mg/ml. Transfectants containing fragments cloned in pSNBR (22) were similarly selected for resistance to 32 μg/ml G418.

For determination of drug sensitivity, parasites were inoculated into M199 media (1 ml in 24-well microtiter plates or 10 ml in T25 culture flasks) lacking HygB and containing the test drug, at a starting concentration of 2 × 10⁵ cells/ml. Parasite numbers were determined using a Coulter Counter (model ZBI) after 2–3 days of incubation at 26 °C. The effective concentration for 50% inhibition (EC₅₀) was defined as that drug concentration that resulted in a 50% decrease in cell number, measured at the time when control cultures lacking drug had reached late log phase (typically less than 10³/ml; Ref. 11). Statistical tests for drug resistance utilized the parameter fold resistance, defined as the average ratio of the experimental cell line EC₅₀ to that of the parental control line measured in the same experiment, over n independent experiments (11).

Molecular Techniques—General molecular methods were performed as described (10, 14, 16). Genomic DNA enriched for cosmid DNA was recovered from 3 × 10⁶ cultured Leishmania cells by an alkaline/SDS lysis protocol followed by polyethylene glycol precipitation (18, 21), and cosmids were recovered by transformation into E. coli DH5α. Cosmid transfecteds were cloned into pUC19/pUc18 (18) or pSMB76 (5'-GACAG/C/GCT/GT/GC/GA/A/GAT/C/GC/TTC/GA/TG/CT/GC/GC/CA/G/AT/GA/TG/TA/GT/GT/TG/CA/GC/G/AT/GG/GC/G) and pHFR-TS, a 0.3-kg PCR amplification product containing the primers 5'-CTGGCGGCGGCGGGGCGGGAG and 5'-ctgacgagctcggcggagtgccgag and served as an internal reference standard.

PCR—Standard PCR was performed using a “hot start” protocol of 10 min at 98 °C, followed by 10 min at 90 °C, the addition of 1 unit of Taq polymerase (Boche Molecular Biochemicals), and 30 cycles consisting of 1 min at 94 °C, 2 min at 55 °C, and 3 min at 72 °C, followed by one 7-min elongation at 72 °C. A “touchdown” PCR (25) used a varying annealing temperature, decreasing 1 °C every third cycle from 60 °C to a touchdown at 50 °C. The protocol consisted of a total of 30 cycles of 1 min at 94 °C, 2 min at temperatures decreasing from 60 °C to 50 °C, and 3 min at 72 °C, followed by 10 cycles with an annealing temperature fixed at 50 °C. Degenerate primers corresponding to conserved regions of squalene cyclase (ERG7), squalene epoxidase (ERG1), acetyl-CoA thiolase (ERG10), and lanosterol C₁₄-demethylase (ERG11) were used in these studies; details are available from the authors. With the squalene cyclase primers, a product was obtained with cTbf5 template. However its sequence showed high similarity (p < 1 × 10⁻³) to a large family of squalene protein phosphorylases (PfTs, Laboratory strain 2783; GenBank™ AF068751).

Sequence of SQS1—A 3-kb Pol1 fragment of cosmids cItz4 was identified by Southern blot analysis with the SQS1 probe, and this was subcloned into the pUC vector for sequencing using Taque 2.0 (Amersham Pharmacia Biotech) and gene-specific primers. The SQS1 sequence was deposited in GenBank™ (U30455).

RESULTS

A Test of Overexpression/Selection: MTX Resistance—Methotrexate-resistant Leishmania frequently exhibit amplification of either the DHFR-TS or PTR1 genes (4–8). We asked whether selection of cosmid-transfected L. major libraries would yield these two loci, and/or perhaps others. Two Leishmania cosmid transfected libraries were used: a V1 library in Friedlin strain A1, containing 17,900 independent cosmid transfecteds, and an LV39cl5 library in strain LV39cl79, containing 3,600 independent transfecteds (approximately 1000 cosmids constitute a “1-hit” library for Leishmania; Ref. 18). 10⁶ cells from either transfected library were plated on drug containing 0.2 μg/ml MTX but not on 6 μg/ml or higher concentrations of MTX (Table I, part A). In contrast, the A1 cosmid library transfected pool yielded 250 colonies on 6 μg/ml MTX and continued to yield colonies up to 24 μg/ml MTX.
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Table I
Differential recovery of colonies from cosmid transfectant libraries after plating on MTX

|                     | A. Friedlin A1 | B. LV39cI79 |
|---------------------|---------------|-------------|
| MTX                 | Control       | Cosmid library transfectants | MTX | Control | Cosmid library transfectants |
|                     | EC₅₀ | Fold resistance | EC₅₀ | Fold resistance |
| MTX                 |       |                 |       |                 |
| μM                  |       |                 | μM   |                 |
| 48                  | 0    | 0.003           | 0.003| 1               |
| 24                  | 0    | 8               | 0.32 | 0               |
| 12                  | 0    | 10              | 0.18 | 0               |
| 6                   | 0    | 250             | 0.08 | 3               |
| 3                   | 30   | >1000           | 0.04 | 500             |
| 1.5                 | 500  | >1000           | 0.02 | >1000           |
| 0                   | >1000| >1000           | 0    | >1000           |

Table II
MTX resistance conferred by PTR1 and DHFR-TS cosmids

The values ± S.D. of 3 independent experiments are given. The values for the three V1 cPTR1 cosmids were averaged together.

| Cell line          | Cosmid transfected | EC₅₀ | Fold resistance |
|--------------------|--------------------|------|-----------------|
| Friedlin A1        | Control            | 0.18 | ± 0.01          |
| V1 cPTR1a,b,c      | 1.05 ± 0.03        | 5.9  | ± 0.46          |
| V1 cDHFR-TSsa      | 4.53 ± 0.58        | 23.5 | ± 1.5           |
| LV39cI79 cDHFR-TSc | 1.36 ± 0.51        | 7.4  | ± 2.6           |
| LV39cI79 Control   | 0.003 ± 0.0002     | 1    |                 |
| V1 cPTR1a,b,c      | 4.3 ± 2.5          | 1,510| ± 640           |
| LV39cI79 cPTR1f    | 0.005 ± 0.0005     | 2.49 | ± 0.26          |
| V1 cDHFR-TSsa      | 0.97 ± 0.06        | 420  | ± 190           |
| LV39cI79 cDHFR-TSc | 0.7 ± 0.3          | 280  | ± 190           |

These data showed that the overexpression/selection strategy recovered the two loci known to confer MTX resistance following gene amplification in Leishmania. Encouraged by these findings, we proceeded to test the overexpression/selection strategy for several other classes of drugs with known or potential utility in Leishmania chemotherapy.

Toxic Nucleosides—Tubercidin (7-deaza-adenosine; TUB) is a toxic purine nucleoside used previously to generate drug-resistant Leishmania. TUB’ mutants of L. donovani show loss of adenosine kinase or decreased tubercidin uptake (26, 27), whereas TUB’ mutants in L. mexicana show a dramatic decrease in nucleoside uptake, induced by amplification of the gene TOR (toxic nucleoside resistance) (24, 28).

In platings of the A1 cosmid transfectant library on increasing concentrations of tubercidin, 39 colonies showing differential survival were obtained, from which three different cosmids were recovered (Table III). Southern blot analysis showed that the cTub1a and cTub1b cosmids were related and contained TOR, whereas the remaining cosmids did not contain TOR nor any other locus studied in this work. Following transfection into A1, the two TOR cosmid transfectants exhibited modest increases in TUB resistance, from 2- to 3.4-fold, and much higher levels of resistance to inosine dialdehyde (15–22-fold) and allopurinol (44–89-fold; Table IV). In L. mexicana, amplification or overexpression of TOR yielded high levels of resistance to both TUB and inosine dialdehyde (500- and 75-fold respectively; Ref. 24).

The cTub1a and cTub1b cosmids each were recovered from only a single colony, whereas cTub2 was recovered many times (Table III). cTub2 cosmid transfectants showed a different resistance profile from that of the TOR cosmid transfectants, exhibiting only a modest level of TUB and inosine dialdehyde resistance (1.6–1.8-fold) and hypersensitivity to allopurinol (about 0.7-fold; Table IV).

Sterol Metabolism—We used two inhibitors of ergosterol biosynthesis to identify prospective resistance loci (Fig. 1). Terbinafine (TBF) is an allylamine that inhibits ergosterol biosynthesis in fungi and Leishmania by targeting squaene epoxidase (29–31), and itraconazole (ITZ) is an azole that inhibits a subsequent step, the P450-dependent lanosterol C₁₄-demethylase (31–33).

Thirty-nine colonies were obtained differentially after plating the A1 cosmid transfectant library on increasing concentrations of TBF, yielding seven cosmids. Restriction mapping, Southern blot, and PCR analysis showed that these corresponded to different loci unrelated to each other or to DHFR-TS, PTR1, TOR, cTub2, or MDR1 (Table III; data not shown). Twenty-eight colonies were obtained differentially from the ITZ selections, yielding four different cosmids unrelated to each other or the other loci mentioned above (Table III; data not shown). These 11 cosmids were transfected back into A1 cells to confirm their role in drug resistance.

For the cosmids arising from TBF selection, a low level of resistance was observed in most transfectants, ranging from 1.4- to 2.5-fold (Table V). For all but cTbf5, this low level of resistance was statistically significant. Transfectants were also tested for cross-resistance to the “downstream” inhibitor ITZ (Fig. 1). The cTbf1, cTbf3, cTbf6, and cTbf7 transfectants showed higher resistance to ITZ than to TBF (2.7–6.5-fold), whereas the cTbf2 and cTbf4 transfectants showed modest hypersensitivity (0.3-fold), and cTbf5 showed no significant resistance. These results implicated six of these loci in resistance and/or susceptibility to sterol synthesis inhibitors.

For the cosmids arising from ITZ selection, low level resistance was observed in the transfectants, ranging from 1.3 to
*Procedures* for details).

- **P450-dependent lanosterol C14-demethylase** (squalene epoxidase (the expected target of TBF; designed degenerate primers based on conserved regions of isolates from fungi and mammals (34), we used a heterologous 1.9-fold).

- **ERG7** has been 6.1-fold; the resistance conferred by cItz3 was not significant (Table V). Tests with the “upstream” inhibitor TBF showed a low level of cross-resistance for transfectants bearing cItz3 (1.9-fold).

Since many genes involved in sterol biosynthesis have been isolated from fungi and mammals (34), we used a heterologous PCR approach based on evolutionarily conserved sequences to search for several of these in the cItz or cTbf cosmids. We designed degenerate primers based on conserved regions of squalene epoxidase (the expected target of TBF; ERG1), the P450-dependent lanosterol C14-demethylase (ERG11, encoding the expected target of ITZ), squalene synthase (ERG9), squalene cyclase (ERG7), and acetyl-CoA thiolase (ERG10). These were tested using standard or touchdown PCR protocols, using individual cosm id DNAs as **L. major** genomic DNA as templates. Most primer pairs failed to yield any specific amplification product with any template.

With squalene synthase primers, an amplification product was obtained with both genomic DNA and cItz4 templates. Its sequence showed good homology with squalene synthase genes of humans and fungi, with conservation of several regions associated with substrate recognition (Fig. 2). This included two aspartate-rich motifs im-

### Table III

| Selective drug | EC₅₀ | Cosmid | Relevant gene | Strain no. | Chromosome Size | Concentrations recovered | Na. colonies obtained |
|---------------|-----|--------|--------------|------------|----------------|--------------------------|----------------------|
| MTX          | 0.18 | cDHFR-TSa | DHFR-TS | B1666 | 0.5 | 6, 12, 24 µM | 31 |
| MTX (LV39)   | 0.003 | cDHFR-TSc | DHFR-TS | B1667 | 0.5 | 0.16, 0.32 µM | 15 |
| O/129        | 0.2  | cDHFR-TSe | DHFR-TS | B1670 | 0.5 | 1.5 µg/ml | 2 |
| TUB          | 0.056 | cDHFR-TSf | DHFR-TS | B1671 | 0.5 | 15, 250 µM | 1 |
| TBF          | 17   | cTub1a | TOR | B1670 | 2.2 | 1.8 µM | 1 |
| ITZ          | 20   | cItz1 | SQSI | B1680 | 1.15 | 15, 30, 60, 250, 500 µM | 23 |

* The following cosmid pairs were indistinguishable in restriction digestions with several enzymes: cDHFR-TSa.f; cDHFR-TSc.g; and cPTR1d.h.

* The sizes of the DHFR-TS and PTR1 chromosomes were determined previously (11).

* LV39 marks selections performed using the cosmids derived from strain LV39cl5 transfected into strain LV39cl79 (see “Experimental Procedures” for details).

* Indicates the gene is unknown.

### Table IV

| Selective drug | EC₅₀ | Tubercul | Inosine dihydro | Allopurinol |
|---------------|-----|----------|----------------|-------------|
|               | µM  | µM      | µM            | µM         |
| A1 control    | 105 ± 6.4 | 36.5 ± 12 | 0.6 ± 0.1 | 1 |
| cTub1a (TOR)  | 350 ± 50 | 575 ± 318 | 26.0 ± 4.0 | 4 |
| cTub1b (TOR)  | 295 ± 52 | 800 ± 283 | 52.0 ± 11.0 | 3 |
| cTub2         | 172 ± 32 | 67 ± 32 | 0.4 ± 0.1 | 4 |

* All cell lines are derivatives of Friedlin A1. The mean ± S.D. of (n) independent experiments is given.

* Values significantly different from A1 are shown (Student’s t test).

* Values are p < 0.01.

* Values are p < 0.05.

![Image](https://via.placeholder.com/150)
Acetyl-CoA

Farnesyl-P-P

Squalene Synthase

Squalene

TERBINAFINE
(TBF)

Squalene Epoxydase

Lanosterol

ITRACONAZOLE
(ITZ)

C_{14}-demethylase

Ergosterol

Fig. 1. Abbreviated summary of the ergosterol biosynthetic pathway and the sites of action of ITZ and TBF.

...plicated as the binding site for the Mg$^{2+}$/Mn$^{2+}$-diphosphate moiety of prenyl substrates (Regions I/II and III/IV), and the hydrophobicity of a region implicated in membrane binding (Region VI; Refs. 35, 36). The Leishmania sequence showed a much closer relationship to squalene synthases than to phytoene synthases in data base searches, and we termed this gene squalene synthase 1 (SQS1).

To test whether SQS1 was responsible for ITZ resistance, clt4z deletions were made, transfected into A1, and scored for ITZ resistance (Fig. 3). These studies mapped the ITZ resistance locus to the 3-kb ApaI fragment bearing SQS1 (deletions clt4z-I and -H4), whereas loss or disruption of SQS1 resulted in a loss of ITZ resistance (clt4z-H1 and -H5). As the size of the cosmid DNA insert was progressively reduced, the level of ITZ resistance rose, from about 4-fold with clt4z-4 to about 13-fold with deletion clt4z-H4 (Fig. 3). A similar phenomenon was noted with other drug resistance cosmids, perhaps arising from an increased copy number and/or level of expression from smaller constructs. The clt4z-H4 transfectants remained susceptible to TBF (data not shown). Thus, clt4z encodes an enzyme of the sterol biosynthetic pathway, squalene synthase, whose expression from a multicopy episome confers resistance to ITZ but not TBF.

Assignment of Cosmid Drug Resistance Loci to Leishmania Chromosomes—Radiolabeled cosmids were hybridized to chromosomes of the Friedlin A1 strain of L. major, separated by pulsed-field gel electrophoresis. All cosmids identified single chromosomes (Table III). Several different cosmids hybridized to chromosomes of similar size, such as cTbf1 and cTbf7 (0.8 Mb), clt4z and clt4z (1.15 Mb), or cTbf5 and clt4z (1.12 Mb). We used Southern blot hybridization of the cosmid probes to restriction enzyme-digested chromosomal DNA to ask whether they represented the same locus. In all cases, the patterns differed considerably (data not shown), suggesting that all arose from different loci.

DISCUSSION

In this study we showed that an overexpression/selection approach for the identification of drug resistance genes works well in the protozoan parasite Leishmania. We tested this approach by selecting for antifolate resistance, which when applied to wild-type parasites frequently leads to the recovery of lines bearing amplification of either (or both) of two loci, PTR1 and DHFR-TS (9, 13, 37–39). Our strategy similarly led to recovery of multiple cosmids bearing these two genes, attesting to their efficacy and efficiency. No new loci were identified in the MTX selection experiments, such as the Leishmania genes FTL and BT1, which encode the folate/MTX and biotin/MTX folate transporter, respectively (40, 41). Similar results were obtained for the antifolate O/129, which inhibits the pteridine reductase activities of both PTR1 and DHFR-TS (42) and shows good activity against both developmental stages of L. major.

These data provide additional genetic evidence for the view that for both MTX and O/129, the primary targets are the cellular enzymes DHFR-TS and PTR1.

Selection with the toxic nucleoside TUB yielded two different loci. One encoded TOR, a gene known to be associated with TUB resistance in Leishmania amazonensis (24, 28). The mechanism by which TOR overexpression mediates decreased purine nucleoside uptake is unknown; the predicted TOR protein does not encode a hydrophobic transport protein, and it could function by down-regulating transporter function (28). Transfection of the two TOR-containing cosmids yielded a low level of TUB resistance but much higher cross-resistance to the toxic nucleoside inosine dialdehyde and toxic nucleobase allopurinol. This pattern of cross-resistance differs considerably from that seen by TOR overexpression in Leishmania mexicana and points to differences among Leishmania species in purine uptake pathways, a phenomenon noted in earlier studies (44, 45). Despite the relatively low level of TUB resistance conferred by the TOR cosmids in L. major, it was sufficient to lead to their recovery.

Recovery of the cosmid cTub2 identified a second locus for TUB resistance, mediating resistance to both TUB and inosine dialdehyde and hypersensitivity to allopurinol. Although the level of drug resistance was modest, cTub2 was recovered in 37 colonies, whereas TOR was recovered in only 2 colonies (Table III). Although there are several possibilities for the mode of action, we favor one where cTub2 encodes (or up-regulates) a purine nucleoside transporter activity described previously in Leishmania (28, 46). This model posits that increased purine uptake from the culture media (containing 100 μM adenine) would decrease the potency of toxic purine nucleosides and increase the potency of toxic purine nucleobases, as seen in the TUB2 cosmid transfectant (Table IV).

Leishmania, unlike mammalian cells, are unable to synthesize purines de novo (47). As purine salvage and interconversions are essential to parasite survival, they are attractive targets for selective chemotherapy. Study of the TOR and TUB2 loci may provide information about the mechanisms used by Leishmania for essential purine uptake.

Ergosterol Biosynthetic Genes and Drug Resistance—Bulk sterol biosynthesis in Leishmania and fungi generates ergosterol instead of cholesterol (19), and several antifungal inhibitors targeting this pathway show good activity against Leishmania species (48, 49). In fungi, the genes encoding the enzymes involved ergosterol biosynthesis have been identified (31). Previously no molecular analysis of this pathway had been undertaken in Leishmania.

Selection experiments with the sterol biosynthesis inhibitors TBF and ITZ yielded a total of 11 unrelated cosmids, 9 of which showed activity in subsequent tests. These conferred a variety of drug resistance.
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TABLE V

Resistance conferred by cosmids recovered from TBF and ITZ selections

| Cell line | Terbinafine | | Itraconazole | |
|-----------|-------------|---|-------------|---|
|           | EC₅₀ (µM) | Fold resistance | n | EC₅₀ (µM) | Fold resistance | n |
| A Friedlin A1 | 13.6 ± 4.7 | 1 | 6 | 1.58 ± 0.25 | 1 | 5 |
| cTFb1 | 26.0 ± 17.6 | 1.9 ± 0.70 | 6 | 9.50 ± 4.51 | 6 | 5.62 ± 3.22 | 4 |
| cTFb2 | 22.8 ± 6.5 | 1.8 ± 0.66 | 5 | 1.05 ± 0.11 | 3 | 0.37 ± 0.12 | 6 |
| cTFb3 | 34.6 ± 25.5 | 2.4 ± 0.83 | 8 | 4.64 ± 0.61 | 5 | 2.98 ± 0.78 | 5 |
| cTFb4 | 36.0 ± 23.0 | 2.5 ± 0.53 | 5 | 0.57 ± 0.19 | 3 | 0.36 ± 0.14 | 5 |
| cTFb5 | 19.0 ± 5.7 | 1.4 ± 0.55 | 5 | 1.35 ± 0.28 | 3 | 1.49 ± 0.39 | 4 |
| cTFb6 | 20.2 ± 11.9 | 1.5 ± 0.40 | 6 | 5.62 ± 2.28 | 3 | 3.80 ± 1.59 | 4 |
| cTFb7 | 27.0 ± 3.9 | 1.9 ± 0.56 | 5 | 4.42 ± 2.03 | 2 | 2.75 ± 1.00 | 5 |
| B Friedlin A1 | 11 ± 11.5 | 1 | 4 | 0.81 ± 0.19 | 1 | 4 |
| cItz1 | 14.6 ± 7.56 | 1.32 ± 0.60 | 4 | 1.37 ± 0.51 | 1 | 1.65 ± 0.26 | 6 |
| cItz2 | 21.2 ± 7.18 | 1.90 ± 0.50 | 4 | 4.77 ± 2.46 | 6 | 6.15 ± 3.84 | 4 |
| cItz3 | 12.0 ± 10.0 | 1.13 ± 0.05 | 3 | 1.07 ± 0.38 | 1 | 1.27 ± 0.31 | 3 |
| cItz4 | 16.5 ± 4.8 | 1.47 ± 0.52 | 4 | 3.37 ± 1.20 | 4 | 4.07 ± 0.57 | 4 |

a Values are p < 0.05.
b Values are p < 0.01.

Fig. 2. Comparison of amino acid sequences of squalene synthases. The sequences for L. major SQS1, human, and S. cerevisiae squalene synthases and the phytene synthase (PS) from Erwinia herbicola are shown. Amino acids identical in three sequences have been shaded, and the locations of several conserved domains discussed in the text or previously are numbered (36). Regions I/II and III/IV bear an aspartate-rich motif proposed to represent the binding site for diphosphate moiety of the prenyl substrates, whereas region VI is hydrophobic and may function as a membrane-binding domain. The SQS1 nucleic acid sequence has been deposited in GenBank (U30455).

of resistance patterns (Table V) as follows: several conferred cross-resistance to both TBF and ITZ (cTFb1, cTFb3, cTFb6, cTFb7, and cItz2), two conferred resistance only to ITZ (cItz1 and cItz4), and remarkably, two conferred TBF resistance and ITZ hypersensitivity (cTFb2 and cTFb4).

Although the level of resistance conferred by the cTFb and cItz cosmids was modest, there are several reasons to be confident that these encode bona fide resistance genes. First, the level of resistance for nine cosmids was statistically significant (Table V). Second, the magnitude of resistance conferred by transfection of known resistance genes on transfected cosmids or from endogenous amplifications is often similarly modest. This was seen for vinblastine-selected MDR1 amplifications (50, 51), primaquine-selected PTR1 (H-region) amplifications (11, 13), and the TOR and SQS1 loci identified here. This may represent a limitation of the cosmid-based approach, as resistance often increases greatly as irrelevant regions of the transfected construct are removed (Fig. 3). Third, several of the cosmids were recovered multiply (cTFb3, cTFb4, cItz1, and cItz2), as noted earlier for TUB2. Finally, several cosmids selected for TBF resistance showed collateral effects with ITZ, which would be highly unlikely for irrelevant loci. Thus, it is probable that most of the cosmids identified here contain active resistance genes, as confirmed for the SQS1 gene borne by cosmid cItz4. Progress in sequencing the Leishmania genome will aid this effort greatly in the future (52).

A candidate gene approach led to the identification of SQS1 on cosmid cItz4, and deletional studies confirmed that SQS1 mediated ITZ resistance. Squalene synthase is an attractive target for chemotherapy (36), with many potential inhibitors under investigation. The availability of the Leishmania SQS1 will permit validation of this enzyme target by a gene knockout approach (53) and the generation of quantities of active enzyme sufficient for detailed biochemical and structural studies.

Our PCR-based screen for other ergosterol biosynthetic enzymes was largely unsuccessful, with both genomic and cosmid DNA as templates. Barring technical complications, the simplest explanation is that sequence divergence was responsible, as trypanosomatid protozoans are quite divergent from fungi and mammals (54). Possibly, these genes do not occur in our set...
Deletions of the 37-kb elevated C14-demethylase activity through gene amplification, (possibly through the action of multidrug efflux transporters), inhibitors has been associated with decreased accumulation biosynthetic pathway (Fig. 1). In fungi, resistance to azole which is several steps “downstream” of SQS1 in the ergosterol way, perhaps acting through inactivation or transport mechanisms, may contain loci unrelated to the ergosterol biosynthetic pathway (Fig. 1). In fungi, resistance to azole inhibitors has been associated with decreased accumulation (possibly through the action of multidrug efflux transporters), elevated C14-demethylase activity through gene amplification, and significantly, increased squalene epoxidase activity (34). Thus, an overall elevation of upstream sterol intermediates may serve to overcome inhibition of the demethylase. This would greatly expand the pool of prospective genes recoverable with overexpression/selection methods with these drugs and may account for the cross-resistance patterns seen with the cosmids cTbf1, cTbf3, cTbf6, cTbf7, and cItz2.

Squalene synthase is also “upstream” of the presumptive target of TBF, squalene epoxidase (Fig. 1). However, SQS1 overexpression did not lead to TBF resistance (Table V), even with the most active cItz4-H4 deletion (Fig. 3). Conceivably, TBF and ITZ may act on other targets in Leishmania, which is several steps “downstream” of SQS1 in the ergosterol biosynthetic pathway (Fig. 1). In fungi, resistance to azole inhibitors has been associated with decreased accumulation (possibly through the action of multidrug efflux transporters), elevated C14-demethylase activity through gene amplification, and significantly, increased squalene epoxidase activity (34). Thus, an overall elevation of upstream sterol intermediates may serve to overcome inhibition of the demethylase. This would greatly expand the pool of prospective genes recoverable with overexpression/selection methods with these drugs and may account for the cross-resistance patterns seen with the cosmids cTbf1, cTbf3, cTbf6, cTbf7, and cItz2.

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