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

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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 transfectant libraries yielded parasites bearing cosmids mediating resistance. Tests with two antifolates led to the recovery of cosmids encoding *DHFR-TS* or *PTR1*, 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 C_{14}-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 antimony 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 often is observed following stepwise selection for drug resistance, as the small size of the parasite genome facilitates the visualization of amplified DNA (3–5). Analysis of amplified DNAs has led to the identification of the genes encoding dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) and pteridine reductase (*PTR1*), members of the P-glycoprotein superfamily (*PGPA* and *MDR1*), 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)[1] usually exhibit alterations in MTX uptake and less commonly amplification of *DHFR-TS* and *PTR1*, 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 GenBank™/EBI Data Bank with accession number(s) U30455.

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[1] 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.

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 (referred to as V1) is a virulent clonal derivative of the Friedlin line (MHOM/IL/80/Friedlin), whereas Friedlin A1 (A1) is 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 (MRHO/SU/59/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 transfectants. For transfection of cosmid library DNA we used 14–40 μg DNA per transfection, and colonies from multiple transfections were pooled (21).

To identify cosmid-bearing lines exhibiting drug resistance, 10^6 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, tubercidin, and the pteridine O/129 (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 *Sau*3A partial digestion (LV39cl5, V1) 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 transfectants (10,600 from Friedlin V1 *Sau*3A partial-digestion library and 7,300 from Friedlin V1 shear library). Transfection of LV39cl5 cosmid library DNAs into LV39cl79 yielded 3,600 independent transfectants.

Analysis of Drug Resistance—Prior to tests of drug resistance, we increased the cosmid copy number within transfectants with elevated aminoglycoside treatment (14). Primary transfectants 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 2×10^5 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 (EC_{50}) 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 10^7/ml; Ref. 11). Statistical tests for drug resistance utilized the parameter fold resistance, defined as the average ratio of the experimental cell line EC_{50} 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 3×10^7 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 fragments were cloned in the *Leishmania* shuttle vector pSNBR (22), and PCR products were cloned in the vector pGEM-T (Promega).

Leishmania chromosomes were prepared in agarose plugs, separated by pulse field gel electrophoresis using a Bio-Rad CHEF Mapper, stained with ethidium bromide, and transferred to nylon membranes (23). Gene-specific hybridization probes were made by PCR using *L. major* DNA template and the following primers: *SQS1*, a 0.3-kb PCR product obtained with primers SMB75 (5′-GACAC(G/C)(G/C)T(G/C)GA(A/G)GA(T/C)GA(T/C)ATG) and SMB76 (5′-CC(G/C)GCGTA(A/G)TA(A/G)TG(A/G)CA(A/G)TA); *DHFR-TS*, a 0.3-kb PCR amplification product obtained with the primers 5′-CTGGCGCCGCTGCCGGAGG and 5′-ctctagaggtaccatATGTCCAGGGCAGCTGCGA (lowercase letters represent bases not present in the genome); *PTR1*, a 0.9-kb PCR product obtained with primers 5′-gcggatcccatATGACTGCTCCGACC and 5′-ggcggatccTCAGGCCCGGGTAAGGCTGTA; and *MDR1*, A 0.4-kb product obtained with the primers PC01 (5′-TTCTC(T/C)GG(G/C)(G/T)C(G/T)TC(T/C)GGGTGCGGCAAG) and PC02 (5′-GTC(G/C)AG(G/C)GC(G/T)CT(G/C)GT(T/C)GCCTC(G/C)TC). The *TOR* probe was the *Leishmania* DNA insert from plasmid pTUB/SB9 (24). Fragments were excised from agarose gels following electrophoresis, purified by glass milk (GeneClean II; Bio 101, Inc.), labeled with [α-^{32}P]dCTP by random priming, and used as hybridization probes. With cosmid probes, weak hybridization to the 500-kb chromosome recognized by *DHFR-TS* flanking sequences present in the cLHYG vector occurred (18) 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 (Roche 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 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 C_{14}-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 ($p < 10^{-28}$) to a large family of serine-threonine protein phosphatases (PP1s; Laboratory strain 2783; GenBank™ AF068751).

Sequence of SQS1—A 3-kb *Pst*I fragment of cosmid cItz4 was identified by Southern blot analysis with the *SQS1* probe, and this was subcloned into the pUCπ vector for sequencing using Taquenase 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 transfectant libraries were used: a V1 library in Friedlin strain A1, containing 17,900 independent cosmid transfectants, and an LV39cl5 library in strain LV39cl79, containing 3,600 independent transfectants (approximately 1000 cosmids constitute a "1-hit" library for *Leishmania*; Ref. 18). 10^6 cells from either transfectant library were plated on progressively increasing concentrations of MTX, and the number of colonies was compared with that of a control consisting of parental or cLHYG-transfected A1 or LV39cl79 (Table I).

The A1 line was inhibited by MTX with an EC_{50} of 0.2 μM in M199 culture medium (Table II). Platings with A1 control parasites yielded colonies up to 3 μM MTX but not on 6 μM or higher concentrations of MTX (Table I, part A). In contrast, the A1 cosmid library transfectant pool yielded 250 colonies on 6 μM MTX and continued to yield colonies up to 24 μM MTX

TABLE I
Differential recovery of colonies from cosmid transfectant libraries after plating on MTX

A. Friedlin A1			B. LV39cl79		
MTX	Control	Cosmid library transfectants	MTX	Control	Cosmid library transfectants
μM			μM		
48	0	0	0.64	0	0
24	0	8	0.32	0	13
12	0	10	0.16	0	6
6	0	250	0.08	3	200
3	30	>1000	0.04	500	>1000
1.5	500	>1000	0.02	>1000	>1000
0	>1000	>1000	0	>1000	>1000

TABLE II
MTX resistance conferred by PTR1 and DHFR-TS cosmids
The means ± S.D. of 3 independent experiments are given. The values for the three V1 cPTR1 cosmids were averaged together.

Cell line	Cosmid transfected	EC_{50}	Fold resistance
Friedlin A1	Control	0.18 ± 0.01	1
	V1 cPTR1a,b,c	1.08 ± 0.03	5.9 ± 0.46
	LV39cl5 cPTR1f	1.18 ± 0.03	6.3 ± 2.6
	V1 cDHFR-TSa	4.33 ± 0.58	23.5 ± 1.5
	LV39cl5 cDHFR-TSc	1.36 ± 0.51	7.4 ± 2.6
LV39cl79	Control	0.003 ± 0.0002	1
	V1 cPTR1a,b,c	4.3 ± 2.5	1,510 ± 640
	LV39cl5 cPTR1f	0.008 ± 0.0005	2.49 ± 0.26
	V1 cDHFR-TSa	0.97 ± 0.06	420 ± 190
	LV39cl5 cDHFR-TSc	0.7 ± 0.3	280 ± 190

(Table I, part A). The LV39cl79 line of *L. major* was more susceptible to inhibition by MTX, with an EC_{50} of 0.003 μM (Table II). The basis for this difference with the Friedlin A1 line, or the CC-1 line studied previously (10), is unknown. Platings with LV39cl79 control parasites yielded colonies up to a concentration of 0.08 μM but not thereafter, whereas the LV39cl79 cosmid library transfectant pool yielded colonies at concentrations up to 0.32 μM (Table I, part B).

The cosmids within colonies showing differential MTX survival in the A1 and LV39cl79 transfectant library platings were recovered and analyzed by restriction enzyme digestion, PCR with specific primers, and Southern blot hybridization with *DHFR-TS* or *PTR1* probes. Ten different cosmids were obtained, four containing *DHFR-TS,* and six containing *PTR1* (Table III). Both loci were recovered from each library, and there was no correlation between the locus recovered and the selective drug concentration applied.

To confirm the functionality of the recovered cosmids, several were transfected into A1 and LV39cl79 (Table II). In A1, all cosmids gave MTX resistance, ranging from 6-fold for the *PTR1* cosmids to 7–23-fold for the *DHFR-TS* cosmids. The variation observed probably reflects differences in the flanking sequences, which could affect expression and/or copy number. Similarly, all cosmids gave MTX resistance when transfected into LV39cl79, although the fold resistance values were elevated (1500-fold for most PTR1 cosmids and 350-fold for the DHFR-TS cosmids) due to the sensitivity of the parental line. The EC_{50} values for the LV39cl79 transfectants were to those seen with the A1 transfectants, suggesting that they were comparably active.

We performed a similar selection with another antifolate, O/129 (2,4-diamino-6,7-diisopropylpteridine). From these experiments, a total of 31 colonies showing differential survival were analyzed, from which 5 different cosmids were obtained (Table III). As for MTX, all cosmids contained either *PTR1* or *DHFR-TS*, and several of these cosmids were indistinguishable from those recovered with MTX (Table III).

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^r mutants of *L. donovani* show loss of adenosine kinase or decreased tubercidin uptake (26, 27), whereas TUB^r 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 cosmid did not contain *TOR* nor any other locus studied in this work. Following transfection into A1, the two *TOR* cosmid transfectants showed 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 squalene epoxidase (29–31), and itraconazole (ITZ) is an azole that inhibits a subsequent step, the P450-dependent lanosterol C_{14}-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

TABLE III
Loci identified from Leishmania cosmid-transfectant library selections

Selective drug	EC_{50}	Cosmid	Relevant gene	Strain no.	Chromosome Size	Concentrations recovered	No. colonies obtained
	μM				Mb		
MTX	0.18	cDHFR-TSa[a]	*DHFR-TS*	B1666	0.5[b]	6, 12, 24 μM	31
		cDHFR-TSb	*DHFR-TS*	B1668	0.5[b]	12, 24 μM	2
		cPTR1a	*PTR1*	B1660	0.8[b]	6, 12 μM	11
		cPTR1b	*PTR1*	B1661	0.8[b]	6, 12, 24 μM	21
		cPTR1c	*PTR1*	B1662	0.8[b]	24 μM	1
		cPTR1d[a]	*PTR1*	B1663	0.8[b]	6, 12, 24 μM	8
		cPTR1e	*PTR1*	B1664	0.8[b]	6, 12, 24 μM	3
MTX (LV39)[c]	0.003	cDHFR-TSc[a]	*DHFR-TS*	B1667	0.5[b]	0.16, 0.32 μM	15
		cDHFR-TSd	*DHFR-TS*	B1669	0.5[b]	0.08, 0.16 μM	7
		cPTR1f	*PTR1*	B1665	0.8[b]	0.16, 0.32 μM	3
O/129	0.2	cDHFR-TSe[a]	*DHFR-TS*	B1707	0.5[b]	1.5 μg/ml	2
		cDHFR-TSf[a]	*DHFR-TS*	B1705	0.5[b]	1.5, 3 μg/ml	15
		cDHFR-TSg[a]	*DHFR-TS*	B1706	0.5[b]	1.5, 3 μg/ml	4
		cPTR1g	*PTR1*	B1704	0.8[b]	1.5 μg/ml	2
		cPTR1h[a]	*PTR1*	B1703	0.8[b]	1.5, 3 μg/ml	8
TUB	0.056	cTub1a	*TOR*	B1670	2.2	1.8 μM	1
		cTub1b	*TOR*	B1671	2.2	0.9 μM	1
		cTub2	—[d]	B1672	2.0	0.9, 1.8 μM	37
TBF	17	cTbf1	—	B1673	0.8	17 μM	1
		cTbf2	—	B1674	1.05	17 μM	1
		cTbf3	—	B1675	0.5	17, 34 μM	23
		cTbf4	—	B1676	0.7	17, 34 μM	10
		cTbf5	—	B1677	1.125	17 μM	1
		cTbf6	—	B1678	0.9	17 μM	1
		cTbf7	—	B1679	0.8	17 μM	2
ITZ	20	cItz1	—	B1680	1.15	15, 30, 60, 250, 500 μM	23
		cItz2	—	B1681	1.2	15, 250 μM	3
		cItz3	—	B1682	1.125	15 μM	1
		cItz4	*SQS1*	B1683	1.15	250 μM	1

[a] The following cosmid pairs were indistinguishable in restriction digestions with several enzymes: cDHFR-TSa,f; cDHFR-TSc,g; and cPTR1d,h.
[b] The sizes of the *DHFR-TS* and *PTR1* chromosomes were determined previously (11).
[c] LV39 marks selections performed using the cosmids derived from strain LV39cl5 transfected into strain LV39cl79 (see "Experimental Procedures" for details).
[d] — indicates the gene is unknown.

TABLE IV
Resistance conferred by cosmids obtained from tubercidin selection to toxic purines and nucleosides

Cell line[a]	Tubercidin			Inosine dialdehyde			Allopurinol		
	EC_{50}	-Fold resistance[b]	*n*	EC_{50}	-Fold resistance[b]	*n*	EC_{50}	-Fold resistance[b]	*n*
	μM			μM			μM		
A1 control	105 ± 6.4	1	4	36.5 ± 12	1	2	0.6 ± 0.1	1	4
cTub1a (*TOR*)	350 ± 50	3.36 ± 0.3[c]	3	575 ± 318	15.1 ± 3.7[d]	2	26.0 ± 4.0	44.0 ± 14.0[d]	3
cTub1b (*TOR*)	205 ± 52	1.96 ± 0.5[d]	4	800 ± 283	21.8 ± 0.5[d]	2	52.0 ± 11.0	89.0 ± 28.0[d]	3
cTub2	172 ± 32	1.62 ± 0.2[c]	4	67 ± 32	1.8 ± 0.3[d]	2	0.4 ± 0.1	0.7 ± 0.1[d]	4

[a] All cell lines are derivatives of Friedlin A1. The mean ± S.D. of (*n*) independent experiments is given.
[b] Values significantly different from A1 are shown (Student's *t* test).
[c] Values are $p < 0.01$.
[d] Values are $p < 0.05$.

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 cItz2 (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 C_{14}-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 cosmid DNAs as well 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 from fungi and mammals, and we determined the sequence of the corresponding region of cItz4. An open reading frame of 414 amino acids emerged, whose prospective initiating AUG codon was located 3′ of a region that shows a high potential to serve as the *trans*-splice acceptor site required for the expression of functional mRNA in *Leishmania* (data not shown). The predicted *L. major* polypeptide showed 26% amino acid identity with the squalene synthase proteins of humans and fungi, with conservation of several regions associated with substrate recognition (Fig. 2). This included two aspartate-rich motifs im-

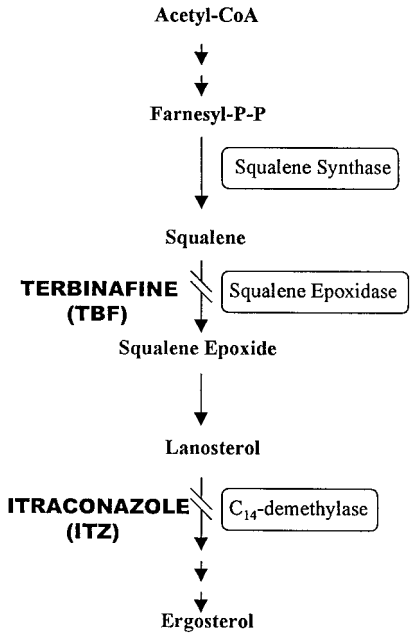

FIG. 1. **Abbreviated summary of the ergosterol biosynthetic pathway and the sites of action of ITZ and TBF.**

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, cItz4 deletions were made, transfected into A1, and scored for ITZ resistance (Fig. 3). These studies mapped the ITZ resistance locus to the 3-kb *Apa*I fragment bearing *SQS1* (deletions cItz4-H and -H4), whereas loss or disruption of *SQS1* resulted in a loss of ITZ resistance (cItz4-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 cItz-4 to about 13-fold with deletion cItz4-H4 (Fig. 3). A similar phenomenon was noted with other drug resistance cosmids,[2] perhaps arising from an increased copy number and/or level of expression from smaller constructs. The cItz4-H4 transfectants remained susceptible to TBF (data not shown). Thus, cItz4 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), cItz1 and cItz4 (1.15 Mb), or cTbf5 and cItz3 (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 its efficacy and efficiency. No new loci were identified in the MTX selection experiments, such as the *Leishmania* genes *FT1*[3] and *BT1*[3] which encode the folate/MTX and biopterin/folate transporters, 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.*[4] 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 (43).

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 nucleobase 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

[2] P. C. Cotrim and S. M. Beverley, unpublished observations.

[3] J. B. Moore and S. M. Beverley, manuscript in preparation.

[4] B. Nare, L. Hardy, and S. M. Beverley, manuscript in preparation.

TABLE V

Resistance conferred by cosmids recovered from TBF and ITZ selections

The mean ± S.D. of n independent experiments is given. Values significantly different from A1 are shown (Student's t test).

	Cell line	Terbinafine			Itraconazole		
		EC_{50}	-Fold resistance	n	EC_{50}	-Fold resistance	n
		μM			μM		
A	Friedlin A1	13.6 ± 4.7	1	6	1.58 ± 0.25	1	5
	cTbf1	26.0 ± 17.6	1.9 ± 0.7[a]	6	9.50 ± 4.51	6.56 ± 3.22[a]	4
	cTbf2	22.8 ± 6.5	1.8 ± 0.6[a]	5	0.56 ± 0.11	0.37 ± 0.12[b]	5
	cTbf3	34.6 ± 25.5	2.4 ± 0.8[b]	6	4.64 ± 0.61	2.98 ± 0.78[b]	5
	cTbf4	36.0 ± 23.0	2.5 ± 0.5[b]	5	0.57 ± 0.19	0.36 ± 0.14[b]	5
	cTbf5	19.0 ± 5.7	1.4 ± 0.5	5	1.35 ± 0.38	1.49 ± 0.39	4
	cTbf6	20.2 ± 11.9	1.5 ± 0.4[a]	6	5.62 ± 2.28	3.80 ± 1.59[a]	4
	cTbf7	27.0 ± 3.9	1.9 ± 0.5[a]	5	4.42 ± 2.03	2.75 ± 1.00[a]	5
B	Friedlin A1	11 ± 1.15	1	4	0.81 ± 0.19	1	4
	cItz1	14.6 ± 7.56	1.32 ± 0.60	4	1.37 ± 0.51	1.65 ± 0.26[b]	4
	cItz2	21.2 ± 7.18	1.90 ± 0.50[a]	4	4.77 ± 2.46	6.15 ± 3.84[a]	4
	cItz3	12.0 ± 1.0	1.13 ± 0.05	3	1.07 ± 0.38	1.27 ± 0.31	4
	cItz4 (SQS1)	16.5 ± 4.8	1.47 ± 0.52	4	3.37 ± 1.20	4.07 ± 0.57[a]	4

[a] Values are $p < 0.05$.
[b] Values are $p < 0.01$.

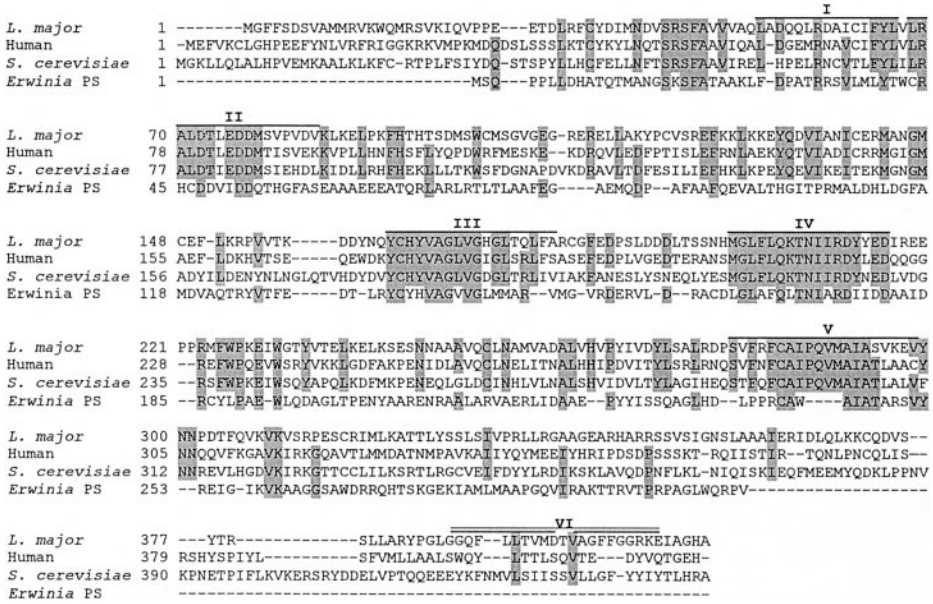

FIG. 2. **Comparison of amino acid sequences of squalene synthases.** The sequences for *L. major* SQS1, human, and *S. cerevisiae* squalene synthases and the phytoene synthase (*PS*) from *Erwinia herbicola* are shown. Amino acids identical in three sequences have been *shaded*, and the locations of 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 (cTbf1, cTbf3, cTbf6, cTbf7, and cItz2), two conferred resistance only to ITZ (cItz1 and cItz4), and remarkably, two conferred TBF resistance and ITZ hypersensitivity (cTbf2 and cTbf4).

Although the level of resistance conferred by the cTbf 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).[5] Third, several of the cosmids were recovered multiply (cTbf3, cTbf4, 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

[5] P. C. Cotrim, L. K. Garrity, and S. M. Beverley, unpublished data.

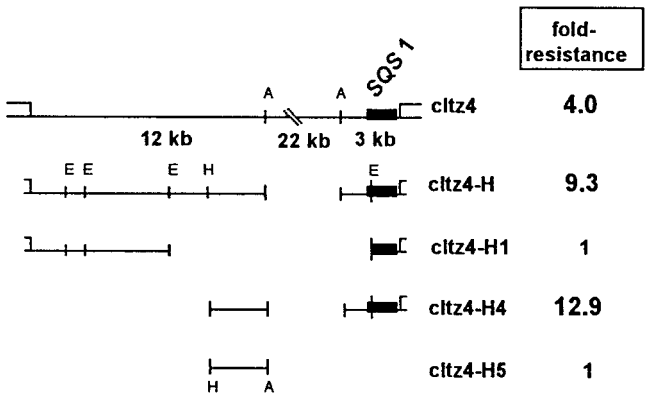

FIG. 3. **Map and ITZ resistance conferred by cItz4 cosmid deletions.** Deletions of the 37-kb *Leishmania* DNA insert of cItz4 were obtained by partial digestion with *Apa*I, yielding a 15-kb ITZ-resistant cItz4-H deletion. A second round of deletions from cItz4-H was produced by partial digestion with *Eco*RV or subcloning of fragments into the shuttle vector pSNBR (cItz4-H5). The *white segments* represent vector sequences and the *black segment* represents the 1.5-kb *SQS1* open reading frame. *A, Apa*I; *H, Hin*dIII; *E, Eco*RV. The fold resistance to ITZ is shown to the *right* of each construct.

of cosmids, perhaps because the selection is not yet saturated or because they are not capable of conferring ITZ and TBF resistance when carried on our cosmid vector. Some cosmids may contain loci unrelated to the ergosterol biosynthetic pathway, perhaps acting through inactivation or transport mechanisms. In this regard, we can exclude the *Leishmania* multidrug resistance gene *MDR1*, as our PCR primers successfully yielded a product with genomic but not cosmid DNA templates. Finally, resistance may occur by unexpected mechanisms in *Leishmania*. Together, these factors emphasize the value of the forward genetic approach taken here.

The recovery of *SQS1* in an ITZ resistance screen was unanticipated, since ITZ is an inhibitor of the P450-dependent lanosterol C_{14}-demethylase of both yeast and *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 C_{14}-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*, although current data do not support this view (29, 32, 48). The differences in response to elevated substrate levels (presumably arising from squalene synthase overexpression) suggest that the mode of inhibition may differ for ITZ and lanosterol C_{14}-demethylase, and TBF and squalene epoxidase. Both TBF and ITZ inhibit their respective fungal target enzymes in a noncompetitive manner (55, 56), but the properties of the *Leishmania* enzymes may differ. Differences between fungal and *Leishmania* sterol metabolism are also evident in the complexity of the resistance patterns exhibited by the cItz and cTbf cosmids. Chief among these is collateral hypersensitivity, which has not been reported previously in allylamine-resistant organisms. Our studies with sterol synthesis inhibitors in *Leishmania* have thus yielded an unexpected variety of novel loci and phenomena.

Strengths and Limitations of Overexpression/Selection Approach in Leishmania—Our data show that selection from multicopy cosmid transfectant libraries is an effective way to identify drug resistance loci, with many advantages over *de novo* mutant-based approaches such as gene amplification. It is faster, as long periods of stepwise selection are not required. Candidate resistance loci are recovered immediately in a genetically manipulable form, permitting identification of the active locus by deletional analysis and sequencing (Fig. 3). It minimizes the recovery of lines exhibiting resistance through "loss of function" mechanisms, as these occur at a much lower frequency than seen in cosmid selections (Table I). Drawbacks of this methodology are that the level of resistance is often low (albeit comparable to several known gene amplifications), although by using constructs with smaller *Leishmania* DNA inserts, increased levels of drug resistance can be obtained (Fig. 3). The limitation here will be the ability to completely cover the genome, given current transfection efficiencies. Nonetheless, despite the relatively modest level of drug resistance conferred, those cosmids recovered from overexpression/selection experiments that were studied in detail yielded *bona fide* drug resistance loci. These include *PTR1* and *DHFR-TS* in the case of two antifolates, *TOR* in the case of toxic nucleosides, and *SQS1* in the case of the sterol synthesis inhibitor ITZ.

For the antifolates, a large number of colonies bearing multiple cosmids with overlapping genomic DNA inserts encoding *PTR1* or *DHFR-TS* were obtained, in numbers consistent with prior functional rescue studies using *Leishmania* cosmid libraries (16, 57). Thus, the efficiency of recovery of resistance loci by the overexpression/selection approach was good. For TUB only two overlapping cosmids encoding *TOR* were identified, and for ITZ and TBF all loci were identified by a single cosmid (Table III). This suggests that these genetic selections have not yet saturated and that additional resistance genes may be found in future selections.

We recently have extended these studies to a number of other drugs, and in every case we have recovered one or more cosmids capable of mediating resistance. As the active genes within these and other cosmids are identified, hypotheses about their biochemical mode of action can be formulated. We expect these studies to lead to a better understanding of mechanisms of drug action and resistance and ultimately to improved anti-*Leishmania* chemotherapy.

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