CHEMOTHERAPY
FOR TRYPANOSOMIASIS

PROCEEDINGS OF A WORKSHOP HELD AT ILRAD
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A.S. Peregrine

THE INTERNATIONAL LABORATORY FOR RESEARCH ON ANIMAL DISEASES
BOX 30709 • NAIROBI • KENYA

The International Laboratory for Research on Animal Diseases (ILRAD) was established in 1973 with a mandate to develop effective control measures for livestock diseases that seriously limit world food production. ILRAD's research program focuses on African animal trypanosomiasis and East Coast fever, a form of theileriosis.

ILRAD is one of 13 centres in a worldwide agricultural research network sponsored by the Consultative Group on International Agricultural Research. In 1989 ILRAD received funding from the African Development Bank, the European Economic Community, the Rockefeller Foundation, the United Nations Development Programme, the World Bank and the governments of Australia, Belgium, Canada, Denmark, Finland, France, India, Italy, Japan, The Netherlands, Norway, Sweden, Switzerland, the United Kingdom, the United States of America and West Germany.

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ABBREVIATIONS, ACRONYMS AND SYMBOLS USED

ADP	adenosine diphosphate
ALA	alanine
Arg	arginine
ASN	asparagine
ASP	aspartic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AZT	azidothymidine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cGAPDH	cytosolic glyceraldehydephosphate dehydrogenase
CoA	coenzyme A
CoQ	coenzyme Q
CO_2	carbon dioxide
cPGK	cytosolic phosphoglycerate kinase
C.T.V.M.	Centre for Tropical Veterinary Medicine (Scotland)
C.V.L.	Central Veterinary Laboratory (Tanzania)
DFMO	DL-α-difluoromethylornithine
DHAP	dihydroxyacetone phosphate
DHFR	dihydrofolate reductase
DHFR-TS	dihydrofolate reductase-thymidylate synthase
DNA	deoxyribonucleic acid
EC_{50}	the concentration of drug in culture that reduces parasite growth by 50% compared to control cultures
FAD	flavin adenine dinucleotide
g	gram
GAPDH	glyceraldehydephosphate dehydrogenase
gGAPDH	glycosomal glyceraldehydephosphate dehydrogenase
GLN	glutamine
GLU	glutamic acid
Gly	glycine
GP	glycerol-3-phosphate
GPO	glycerophosphate oxidase
GSH	γ-glutamyl-cysteinylglycine
gTIM	glycosomal triosephosphate isomerase
H_2O_2	hydrogen peroxide
HA	haemagglutinin
HCl	hydrochloric acid
HIS	histidine
HPLC	high-performance liquid chromatograph(y)
Ig	immunoglobulin

ILRAD	International Laboratory for Research on Animal Diseases (Nairobi)
kcal	kilocalorie
K_d	dissociation constant
kDNA	kinetoplast deoxyribonucleic acid
KETRI	Kenya Trypanosomiasis Research Institute (Nairobi)
kg	kilogram
K_i	inhibitor constant
K_m	Michaelis constant
LD_{50}	the dose at which 50% of animals are killed (50% Lethal Dose)
LEU	leucine
LPHChO	1-acyl-*sn*-lyso phosphatidylcholine
M	molar
mdr	a transcribed gene whose increased expression is specific to multidrug resistant cell lines
MDR	multidrug resistance
Mel B	melarsoprol
mg	milligram
MgATP	magnesium adenosine triphosphate
min	minute
ml	millilitre
mM	millimolar
mol	mole
mRNA	messenger ribonucleic acid
MTX	methotrexate
NAD	nicotinamide-adenine dinucleotide
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
$NADP^+$	oxidized nicotinamide-adenine dinucleotide phosphate
NAD[P]H	reduced nicotinamide-adenine dinucleotide phosphate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
nmole	nanomole (10^{-9} of a mole)
NMR	nuclear magnetic resonance
ODA	Overseas Development Administration (British Government)
ODC	ornithine decarboxylase
$OH^{\cdot}$	hydroxyl radical
PEP	phosphoenolpyruvate
PGK	phosphoglycerate kinase
pH	hydrogen ion concentration
PHE	phenylalanine
pI	isoelectric point
pK	dissociation constant, negative logarithm of

pmole	picomole (10^{-12} of a mole)
PQ	primaquine
Pyr^R	pyrimethamine-resistant
Pyr^S	pyrimethamine-sensitive
RES	reticuloendothelial system
RNA	ribonucleic acid
SAM	S-adenosyl-methionine
SAM DC	S-adenosyl-methionine decarboxylase
TAO	trypanosome alternative oxidase
TB	terbinafine
TCA	tricarboxylic acid
TIM	triosephosphate isomerase
tRNA	transfer ribonucleic acid
Trp	tryptophan
$T[SH]_2$	trypanothione
TYR	tyrosine
VAL	valine
V/cm	volts per centimetre
V_{max}	maximum velocity
v/v	volume per volume
μ	micro (10^{-6}x)
μg	microgram (10^{-6} of a gram)
μM	micromolar
α	alpha
β	beta
γ	gamma
λ	lambda

Preface

In 1982 the world's human population was estimated to be 4.2 billion and increasing at a rate of approximately 2% per year. The highest growth rate is in Africa, where the population is estimated to be growing at 3.2% per year. There is, therefore, a continually increasing requirement for food on that continent. Although Africa's total food production is growing to meet the increasing demand, production has not kept pace with the expanding population (see Figure 1). Between 1965 and 1982, food production on the continent per person fell by approximately 12%, and even in the most productive regions total food production fell by 8% between 1969/71 and 1981/82. By contrast, from 1965 to 1982, food production rose by 6% in the Near East and by at least 49% in Asia. While Latin America and Asia have become almost self-sufficient in cereal production, Africa has become more and more dependent on imports and food aid. In 1963 Africa grew 96% of its cereal requirements; in 1985 it grew only 78%.

One disease in particular, trypanosomiasis, has had profound adverse effects on the whole pattern of agriculture in Africa. In sub-Saharan Africa the disease is transmitted to domestic livestock by the tsetse fly and impedes livestock production directly and crop production indirectly. The tsetse fly occurs in most of the humid and sub-humid zones, an area totalling approximately 10 million square kilometres, or about half the non-desert area of Africa (Figure 2).

Agriculture in Europe and most of Asia is based on mixed arable and livestock farming, with cattle providing people with protein in the form of meat and dairy produce, draught power for ploughing and transport, and fertilizer. Fields requiring a rest from cereal production may be used to grow fodder. In sub-Saharan Africa, livestock are integrated into crop farming only in the highlands of East Africa, which are too cool for tsetse, and in a few semi-arid regions, which are too dry for the fly. In the humid zones, livestock other than dwarf sheep and goats are sparse. In the sub-humid zones, livestock are at greatest risk from trypanosomiasis in the rainy season, during which stock farmers often entrust their cattle to nomadic pastoralists who move the animals to tsetse-free areas. Integration of crop and livestock farming throughout the year is therefore impossible. This separation of livestock and arable farming over much of Africa is a major barrier to agricultural development. Furthermore, of the 10 million square kilometres now infested with tsetse, some 7 million are thought to be otherwise suitable for mixed agriculture and livestock farming. Thus, in 1963, Africa's annual loss in meat production due to tsetse was estimated to be US$5 billion.

Control of trypanosomiasis currently relies on protecting and treating livestock with trypanocidal drugs, control of the tsetse fly and, to a lesser extent, use of trypanotolerant livestock breeds, resistant to the pathogenic effects of the disease. These control strategies are frequently used in isolation. However, it is becoming increasingly apparent that sustainable control of trypanosomiasis is more likely to be achieved by integrating the different control strategies.

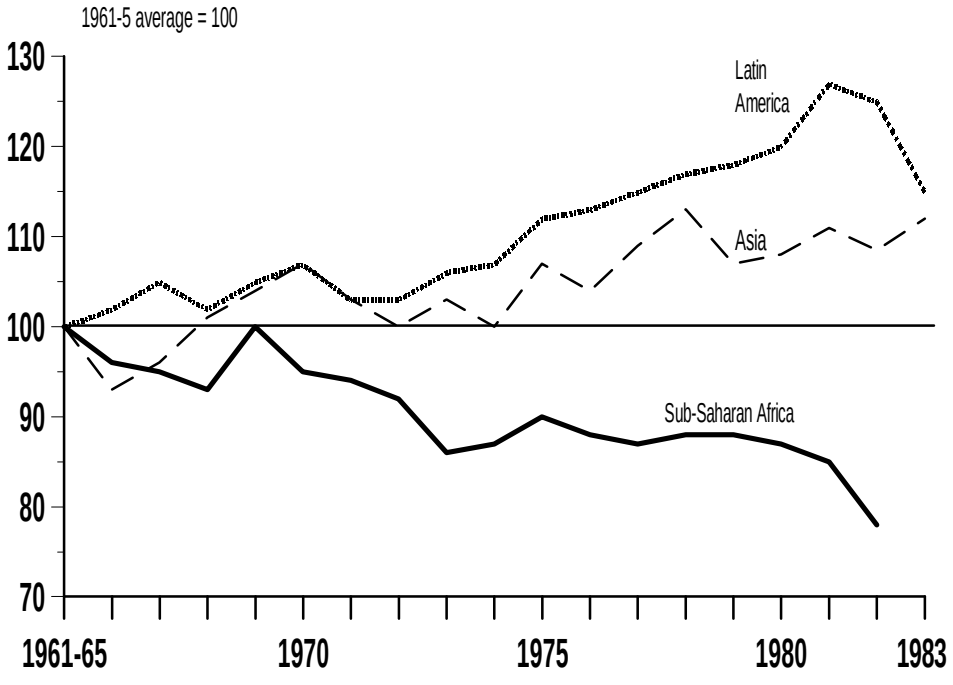

FIGURE 1. Graph showing per capita food production in Latin America, Asia and sub-Saharan Africa from 1961 to 1983. Source: *Financing Adjustment with growth in sub-Sahara Africa 1986–90,* World Bank, Washington D.C., 1986.

Chemotherapy for trypanosomiasis in cattle, sheep and goats currently relies on use of salts of three compounds: isometamidium, homidium and diminazene. All three compounds are closely related chemically and have been available for at least thirty years. Although these drugs have effectively controlled the disease in the field, the prevalence of resistance to each of the compounds appears to be increasing. There is, therefore, an urgent need to develop new compounds, chemically unrelated to those now is use. The development of new drugs, before drug resistance becomes a widespread problem, will help ensure the long-term productivity of domestic livestock in Africa.

Chemotherapy for trypanosomiasis in human beings is beset with similar problems: a limited repertoire of compounds, resistance to the drugs, drug toxicity and protracted treatment protocols.

In light of the current problems and limitations of chemotherapy for trypanosomiasis in domestic livestock and human beings, an international workshop was held at ILRAD in August 1989 to discuss research aimed at developing new trypanocides. Because of the scarcity of information on the phenotypic and genetic basis of drug resistance in trypanosomes, work on other protozoan parasites was also reviewed for its potential relevance to the development of new therapeutic agents and diagnostics for trypanocide resistance. The meeting highlighted areas of trypanosomal chemotherapy research in which developments are being made as well as areas where more research is required. The workshop underscored the necessity for collaboration between scientists of

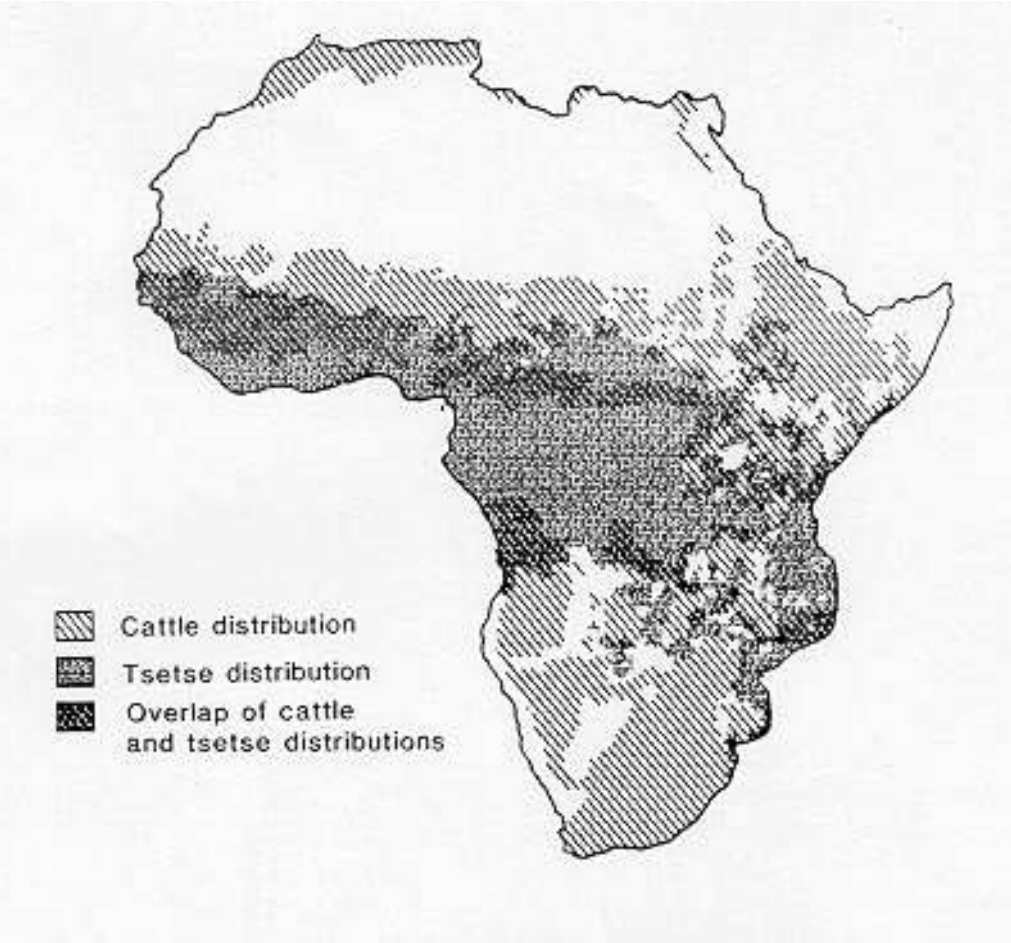

FIGURE 2. Major cattle-production areas and tsetse-infested zones in Africa. There is little overlap except in the regions of West and Central Africa where trypanotolerant livestock are kept.

different disciplines for complete development of a new compound to become a reality.

The editor thanks Ms Jyoti Sehmi and Ms Doris Lewa for their help in preparation of the manuscript. Drs. E.J. Bienen, O. ole-MoiYoi, P. Gardiner, J. Lonsdale-Eccles, P. Webster and D. McKeever read through parts of the manuscript and gave helpful advice. Mr R. Kruska kindly provided the cattle and tsetse distribution map used in this preface.

Andrew S. Peregrine
International Laboratory for
Research on Animal Diseases
Nairobi, July 1990

Opening address

A.R. Gray

Director General
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

ILRAD extends a warm welcome to you all. While here, I hope you will learn a little more about ILRAD and our research programs aimed at improved control of livestock diseases.

I would now like to say a few words to introduce the program for this workshop on `Chemotherapy for Trypanosomiasis'. Trypanosomiasis remains one of the most important diseases of domestic livestock in sub-Saharan Africa and constitutes a major impediment to livestock production on the African continent. Outside Africa, trypanosomiasis poses a significant constraint to livestock production in both the Middle and the Far East and in some areas of South America. Human trypanosomiasis, or sleeping sickness, is still a significant disease in Africa, where the World Health Organization estimates that approximately 20,000 new cases are reported each year. Trypanosomiasis in South America, in the form of Chagas' disease, is also an important disease of human beings.

On the African continent, in tsetse-infested areas, chemotherapy plays a vital role in maintaining and improving the production of domestic livestock. Chemotherapy ranks highly, alongside tsetse control and the use of trypanotolerant livestock, as a disease control measure. However, at present there are only a few drugs available for use as therapeutics and prophylactics against the disease and significant problems with drug resistance are being reported from many parts of Africa. Fortunately, the prevalence of significant resistance to some of the drugs appears to have remained low. The reasons for this are unclear. However, because there are so few drugs, there is an urgent need for development of new trypanocides, most especially drugs that are chemically unrelated to those currently on the market. Only this approach should overcome current problems with cross-resistance.

A similar problem exists with drugs for human trypanosomiasis: there is only a small repertoire of compounds and many of the drugs currently in use often produce severe toxic side effects. All is not gloom, though, as I am sure we will be hearing later in this workshop: field trials with the compound difluoromethylorinithine, or DFMO, have shown that it may be particularly efficacious in the treatment of late-stage *Trypanosoma brucei gambiense* infections, most particularly those cases that are refractory to treatment with organic arsenicals.

For these reasons, ILRAD two years ago established a chemotherapy research group. In addition to developing improved therapeutic strategies for combating drug resistance with the drugs currently available, the group has been

asked to develop techniques to identify and quantify problems with drug resistance within domestic livestock in the field. With such knowledge, it may be possible to introduce rational corrective therapeutic regimes at the field level. Studies are also being conducted to gain a clearer understanding of the phenotypic and genetic basis of drug resistance in different species of trypanosomes. During his presentation, Dr. Peregrine will enlarge on the areas of research in which ILRAD is involved.

You will see that our activities relate very much to aspects of managing chemotherapy and drug resistance as field problems. However, new leads in the control of trypanosomiasis by chemotherapy could have a significant impact on the productivity of livestock in many countries, and could improve the well-being of millions of people both directly and indirectly. That is why, with some additional funding from the United Nations Development Programme, we have invited you all here to participate in this workshop on `Chemotherapy for Trypanosomiasis'.

The objectives of the workshop are fivefold. First, the workshop will review the current state of research on aspects of biochemistry, molecular biology and therapeutics of pathogenic trypanosomes for their immediate and long-term relevance to the control of human and animal trypanosomiasis. Second, the workshop will review findings from research on *Plasmodium, Leishmania* and mammalian cells for their relevance and possible application to research programs on trypanosomiasis. Third, the meeting will review findings on new and potential drug-delivery systems for their possible contribution to the control of trypanosomiasis. Fourth, we hope that the workshop will identify areas of research where findings may offer new compounds or techniques for controlling trypanosomiasis. Fifth, and finally, it is hoped that this meeting will encourage the formation of links between scientists working in Africa and those working elsewhere. We particularly hope that such links will benefit and strengthen ILRAD's chemotherapy research program. The speakers at this workshop come from scientific institutions in Belgium, France, Kenya, The Netherlands, the United Kingdom and the United States of America. In addition, we also have present scientists from Ethiopia, Indonesia, Nigeria, Sudan, Tanzania and Zambia who are interested in trypanosomiasis chemotherapy. Unfortunately, there will not be time for everyone to give full-length presentations. However, I trust you will all feel that your participation in this workshop is equally important and useful.

I wish you well in your discussions this week and hope you enjoy your time at ILRAD and in Kenya.

INTRODUCTION

Overview of the chemotherapy research program at ILRAD

A.S. Peregrine*, I.A. Sutherland†, L. Mutharia§, R. Jamnadass*, M.A. Gray¶, V. Codjia*, Y.O. Aliu**, M. Mamman*, R. Silayo‡, G.D.M. d'Ieteren§§, S.K. Moloo* and N.B. Murphy*

*International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

†Department of Veterinary Parasitology
University of Glasgow Veterinary School
Bearsden, Glasgow G61 1QH, Scotland

§Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi, Kenya

¶Kenya Trypanosomiasis Research Institute
P.O. Box 362
Muguga, Kenya

**Department of Physiology and Pharmacology
Faculty of Veterinary Medicine
Ahmadu Bello University
Zaria, Nigeria

‡Faculty of Veterinary Medicine
Sokoine University of Agriculture
P.O. Box 3019
Morogoro, Tanzania

§§International Livestock Centre for Africa
P.O. Box 46847
Nairobi, Kenya

As a result of advice given in a review of its research program in 1986, the International Laboratory for Research on Animal Diseases (ILRAD) incorporated into its trypanosomiasis research program a chemotherapy component with a mandate to maintain the long-term efficacy of the three compounds currently marketed for use in cattle—diminazene, homidium and isometamidium. It is hoped that detailed information on the phenotypic and

genetic basis of resistance to these drugs, on their mode of action and on their pharmacokinetics will greatly enhance our knowledge about how the drugs should be used in the field and how problems with drug resistance should be combated. Concomitant with this research is the development of field-usable techniques that will rapidly quantify resistance to each of the drugs. Such `diagnostics' would enable one to monitor the prevalence and level of drug resistance in a given area and to decide rapidly on the most appropriate therapeutic intervention to use. ILRAD's research activities on chemotherapy for trypanosomiasis therefore fall under the following eight broad topics.

DEVELOPMENT OF *IN VITRO* CULTURE TECHNIQUES FOR IDENTIFYING AND QUANTIFYING DRUG RESISTANCE

At present the only method available for screening parasites for resistance to trypanocides is treatment of experimentally induced infections in cattle or mice. Neither is an ideal system: many field isolates will not grow in mice and it is costly to purchase cattle and to maintain them in fly-proof isolation. Dr. R. Kaminsky has been examining the suitability of *in vitro* cultivation systems for this purpose and his work is discussed elsewhere in this volume.

EFFICACY OF ISOMETAMIDIUM CHLORIDE AND DIMINAZENE ACETURATE IN ZEBU CATTLE

Earlier work has demonstrated that great variation exists in the period of prophylaxis afforded cattle by isometamidium chloride (Robson, 1962; Fairclough, 1963; Kirkby, 1964; Wiesenhutter *et al*., 1968). Results of studies undertaken to elucidate the reason(s) for this variation suggest that the period of prophylaxis afforded by isometamidium chloride is dose-dependent, independent of the level of metacyclic challenge, unaltered by the presence of infection at the time of drug administration, and principally dependent on variation in trypanosome drug sensitivity. The studies also showed that antigenic priming of the immune system did not occur in cattle on chemoprophylaxis. Therefore, there would appear to be no immunoprophylactic contribution to cattle maintained in such a manner (Peregrine *et al*., 1987, 1988).

Development of improved therapeutic regimes for the drugs currently available forms an equally important part of research within this area. Workers on the Kenya coast have recently claimed that if isometamidium chloride is given intravenously it can eliminate infections that do not respond to treatment by the recommended intramuscular route (Dowler *et al*., 1989). Since this strategy would benefit livestock owners who cannot readily obtain diminazene aceturate, studies have been carried out in cattle using three clones of *Trypanosoma congolense* that span the entire spectrum of resistance to isometamidum. Although intravenous administration did eliminate fully sensitive infections at doses up to 1.0 mg/kg it was not efficacious in eliminating infections that

expressed clinically significant levels of resistance. The apparent efficacy seen in the field may be due to other factors, such as acquired immunity.

Other studies are under way to determine whether repeat administration of diminazene aceturate enhances the drug's therapeutic activity. Since diminazene has a half-life in excess of 9 hours, it has been suggested that this approach may enhance the drug's therapeutic activity (Baggot, 1978).

MODE OF UPTAKE OF ISOMETAMIDIUM BY CLONES OF *T. CONGOLENSE*

ILRAD researchers are using well-characterized clones of *T. congolense* to determine the mode of uptake of isometamidium and the phenotypic basis of resistance to this drug. Uptake of isometamidium by sensitive clones of *T. congolense*, as determined by fluorescence spectrophotometry, has appeared to be an active process and to be energy dependent. Reduced uptake of isometamidium occurred in resistant clones and also appeared to be energy dependent. Fluorometry appears to be a reliable method for quantifying the level of isometamidium-resistance expressed by clones of *T. congolense*. Further studies are under way to determine the molecular basis of these observations.

EPIDEMIOLOGY OF DRUG RESISTANCE

This area of research endeavours to gain a clearer understanding of the dynamics of drug resistance in the field. When ILRAD's chemotherapy program began, it was uncertain whether homogeneous expression of drug resistance occurred in any given stock of trypanosomes. Nine clones were therefore derived from a *T. congolense* stock (IL 2856), obtained from Burkina Faso, which expresses a high level of resistance to both diminazene and isometamidium in mice, an unusual phenotype. All clones were characterized for their sensitivity to diminazene and isometamidium in mice. Significant clonal variation in resistance to isometamidium was observed. However, compared to sensitive clones, all nine clones derived from IL 2856 expressed a high level of resistance to isometamidium. Diminazene sensitivity studies produced similar results, with significant clonal variation in expression of resistance. In addition, all clones expressed a high level of resistance compared to reference sensitive clones. Finally, isoenzyme variants for eight enzymes were determined for each of the nine clones: all expressed the same variants. These studies therefore showed that *T. congolense* clones derived from one stock and with an apparent homogeneous background can, in the absence of drug selection, express significantly different levels of resistance to both diminazene and isometamidium, and express high levels of resistance to both drugs (Peregrine *et al.*, in press).

To determine a possible genetic basis for resistance to isometamidium, nine clones (subclones) were derived from one of the aforementioned clones (IL 2856 clone 1). The nine subclones were characterized in mice for their sensitivity to isometamidium (Table 1). Seven of the nine subclones expressed a

TABLE 1. Sensitivity of *T. congolense* IL 2856 clone 1 subclones to isometamidium chloride in mice

Population	CD_{50}(mg/kg)*	95% confidence interval (mg/kg)
IL 2856 clone 1	5.1	3.2–8.0
IL 2856 subclone 1.1	1.8	1.5–2.1
IL 2856 subclone 1.2	1.4	1.2–1.6
IL 2856 subclone 1.3	1.4	1.2–1.5
IL 2856 subclone 1.4	0.9	0.8–1.0
IL 2856 subclone 1.5	1.9	1.7–2.2
IL 2856 subclone 1.6	3.0	2.5–3.7
IL 2856 subclone 1.7	1.3	1.1–1.4
IL 2856 subclone 1.8	2.7	2.2–3.3
IL 2856 subclone 1.9	1.7	1.5–2.0

*Dose at which 50% of an infected population are cured.

significantly lower level of resistance to isometamidium than the parental clone. Furthermore, there was significant variation in resistance among the subclones. This suggests that genetic control of this phenotype in *T. congolense* is unstable. The latter hypothesis is consistent with recent studies on the instability of isometamidium-resistance in stocks of *T. congolense* (Nyeko *et al*., 1989) and is currently under investigation.

A final set of studies have focused on *T. congolense* IL 1180, a clone that expresses a high level of sensitivity to isometamidium. Over an 18-month period, a derivative has been produced *in vivo* that is 200-times more resistant to isometamidium than IL 1180. This derivative has been shown to be tsetse transmissible and to produce approximately the same infection rate in the gut, labrum and hypopharynx of *Glossina morsitans centralis* as IL 1180. The clone's tsetse transmissibility also appeared to be unaltered.

The aforementioned IL 1180 populations are currently being used in a study to look for biochemical markers that correlate with expression of resistance to isometamidium. Preliminary results from two-dimensional polyacrylamide gel electrophoresis analyses have shown that with increasing levels of resistance to isometamidium there are changes in the expression of certain proteins. Further work is under way to determine which, if any, of these proteins correlate with expression of resistance.

MOLECULAR BIOLOGY OF DRUG RESISTANCE

Drug-resistant *T. brucei brucei* isolates have been examined at the DNA level for evidence of gene amplification. Initial experiments involved isolation of DNA from resistant and sensitive trypanosomes, restriction endonuclease di-

gestion, gel electrophoresis, Southern blotting and probing with total genomic ^{32}P-labelled DNA from sensitive and resistant isolates. Hybridization to an apparent extrachromosomal element was detected in tracks of undigested DNA from the resistant isolate. Further work has shown that this element is DNA, that the element occurs in the genome of sensitive *T. b. brucei* but is only found as an extrachromosomal-type element in the resistant isolate and that clones from this isolate also contain the element. In addition, presence of the element appears to coincide with expression of resistance because trypanosomes passaged in the absence of selection appear to have a reduced copy number of the element. The copy number appears to increase when drug selection is reapplied.

DEVELOPMENT OF DRUG ASSAYS

Aliu and Odegaard (1983) described an ion-pair extraction and high-performance liquid chromatographic (HPLC) technique for measuring diminazene in the plasma of treated animals. Professor Aliu is currently at ILRAD to set up the technique and to determine the pharmacokinetics of diminazene in Zebu cattle. The data resulting from this study will be used in future experiments designed to examine whether repeat treatment regimes enhance diminazene's therapeutic activity.

ILRAD is also in the process of setting up a cold HPLC technique for assaying isometamidium levels in cattle sera (Kinabo and Bogan, 1988). It is also collaborating with the University of Glasgow, Scotland, in the evaluation of an isometamidium enzyme-linked immunosorbent assay that the University has developed. It is hoped that both techniques will be used to determine the pharmacokinetics of isometamidium in cattle.

THE TRYPANOTOLERANT LIVESTOCK NETWORK

Several years ago the International Livestock Centre for Africa (Addis Ababa) established the headquarters for a Trypanotolerant Livestock Network in Nairobi. Members of this network have been studying the productivity of trypanotolerant livestock in a number of field sites across Africa. Use of the group's extensive data sets is helping ILRAD researchers to determine the absolute requirements for trypanocides by cattle in the different projects, the associated increase in productivity and whether drug resistance is a significant problem at any of the sites. Information from a project in Ethiopia indicates that there may be a significant problem with resistance to diminazene at that site. For this reason, field isolates have been obtained from that site and are currently being characterized for their drug-resistance phenotypes at ILRAD so that the project may be advised about the most suitable intervention to eliminate the current problem. Preliminary results indicate a high prevalence of resistance to diminazene.

TRAINING AND OUTREACH

Training and Outreach form important parts of the chemotherapy program. Five students are currently working within the program. They are from Benin, Kenya, Nigeria, Sudan and Tanzania. Such training will help ILRAD form active collaborative programs with African universities and organizations involved in chemotherapy research. Such collaboration is of paramount importance because only an integrated pan-African trypanosomiasis chemotherapy program can ensure the long-term efficacy of the trypanocides currently in use.

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Workshop overview: recent developments in the chemotherapy of African trypanosomiasis

S.L. Croft

London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT, UK

The first-line drugs for the treatment of malaria, leishmaniasis and African trypanosomiasis were developed over 30 years ago, whilst chemotherapy for Chagas' disease has only become available in the last 15 years. Therapies for all these diseases are blighted by problems of resistance, variable efficacy between strains or species of the causative organisms, toxicity, difficulty of administration or a combination of these factors. The recommended drugs used for the treatment of human African trypanosomiasis remain melarsoprol, suramin and pentamidine (World Health Organization, 1979), and for animal trypanosomiasis, the salts of homidium, quinapyramine, diminazene, isometamidium and suramin (World Health Organization, 1979). However, the last decade has witnessed an enormous growth in our understanding of the biochemical and molecular processes of trypanosomes with the revelation of targets for the design and development of new drugs. Some of these will be described in this workshop along with recent developments in our understanding of drug resistance and new approaches to drug delivery.

METABOLIC PATHWAYS

Any strategy to identify novel anti-protozoal drugs must take into account the metabolic differences between stages of the life cycle and that there is often a reduction in the complexity of metabolic pathways as parasites are bathed in a nutritional environment (Fairlamb, 1989). An extensive number of anabolic, catabolic and regulatory pathways have been identified in parasitic protozoa that offer chemotherapeutic targets (Fairlamb, 1989).

Carbohydrate metabolism and energy production in the bloodstream forms of African trypanosomes are reliant upon glycolysis organized in a unique manner between a reduced mitochondrion, the cytoplasm and the glycosome (Opperdoes, 1987). This pathway is probably a target for suramin and forms the basis for several novel approaches (see A.B. Clarkson, P.A.M. Michels and F.M.D. Vellieux, this volume). The electron transport chain is a target in other protozoa (see J.F. Turrens, this volume), best illustrated by the naphthoquinones developed for the treatment of theileriosis and malaria (Hudson *et al.*, 1985). Nucleic acid synthesis in trypanosomatids also has unique characteristics. These

organisms, being unable to synthesize purines *de novo*, have evolved a special salvage pathway with enzymes that have a higher affinity for disruptive purine analogues than natural precursors. The bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS), an enzyme essential for the formation of tetrahydrofolate, a co-factor for the synthesis of pyrimidines, is the target for the antimalarials pyrimethamine and cycloguanil. Recent studies have characterized this enzyme in *Leishmania* (see S.M. Beverley, this volume), stimulating further drug design (Sirawaraporn *et al*., 1988). Nuclear and kinetoplast DNA-dependent processes are inhibited by many standard trypanocides that intercalate (phenanthridines) or bind externally to DNA (diamidines) (Williamson, 1979). This may interfere with the functioning of DNA and RNA polymerases as well as DNA topoisomerases I and II (see T.A. Shapiro, this volume). The metabolic pathways of polyamine synthesis have been most productive in the last decade for the identification of new anti-trypanosomal compounds, such as eflornithine (DL-α-difluoromethylornithine [DFMO]) which is now in extensive clinical trials (see C.J. Bacchi, this volume). Interference with polyamine synthesis may explain the mode of action of established drugs, e.g., diminazene (Bitonti *et al*., 1986). Polyamines have a wide number of regulatory functions in cells, including, in trypanosomatids, the synthesis of specific thiols (see A.H. Fairlamb, this volume). Eflornithine is also active against *Leishmania, Plasmodium* and *Pneumocystis carinii*. The predominance of ergosterol rather than cholesterol in the membrane is the basis for the selective toxicity of antifungal agents in *Leishmania* and *Trypanosoma cruzi* such as amphotericin B and the azole and allylamine sterol synthesis inhibitors (see L.J. Goad, this volume). Microtubules, an integral part of the trypanosomatid pellicle, flagellum and mitotic nucleus, are sensitive to phenothiazines and inhibitors of microtubular disassembly such as taxol. Cellular regulatory and protective processes, many unique to trypanosomatids, are also important targets. The transmembrane proton pump in *Leishmania*, which regulates internal pH and nutrient uptake, is sensitive to tricyclic antidepressants (Zilberstein and Dwyer, 1984). The susceptibility of trypanosomatids to oxidant stress has also been exploited either by using drugs that produce toxic metabolites or radicals, or by lowering the defence mechanisms of superoxide dismutase or glutathione (there is little or no catalase). Current interest is focused on the novel intracellular thiol trypanothione, which has a protective and regulatory role in trypanosomatids (see A.H. Fairlamb, this volume).

Biochemical studies have revealed many more potential targets, but there is one final area of cellular metabolism to be considered. The drug uptake process includes not only transport but also the intracellular metabolism, binding and distribution of a drug. This is critical for selective toxicity and for some trypanocides has been studied extensively (Hawking, 1963; Frommel and Balber, 1987). As far as is known, the antimalarials chloroquine and qinghaosu owe much of their selective toxicity to specific uptake by infected erythrocytes rather than any special disruption of a metabolic pathway (see S.R. Meshnick, this volume).

DRUG RESISTANCE

Resistance is a major problem in the treatment of many infectious diseases and for malaria it has been the major stimulus for the discovery of new drugs. The detection, origins and mechanisms of drug resistance in parasitic protozoa will be discussed in the Workshop. The term `drug resistance' covers a wide range of phenomena, including host-related and parasite-related factors.

Host-related factors that contribute to resistance include (1) *pharmacokinetics*, for example, poor distribution to infected tissues or intracellular sites or the variation in drug metabolism between individuals (e.g., mefloquine); and (2) *immunological status*, for example, the inactivity of pentavalent antimonials against diffuse cutaneous leishmaniasis and the diminished activity of suramin, quinapyramine and DFMO (Bitonti *et al*., 1986) in animals with a suppressed immune system.

Parasite-related factors that contribute to resistance include the following. (1) *reduction in drug accumulation*, described widely in trypanosomes for arsenicals (Hawking, 1963), diamidines, phenanthridines, acriflavine (Frommel and Balber, 1987) and DFMO in resistant *T. brucei* (Phillips and Wang, 1987), as well as for some anti-leishmanial compounds (see S.M. Beverley, this volume); (2) *change in enzyme target*, through either an increase in enzyme levels, shown by DHFR-TS in *Leishmania* resistance to methotrexate (see S.M. Beverley, this volume) and *Plasmodium* resistance to pyrimethamine (see J. Inselburg, this volume), or *changes in enzyme affinity*, as shown in *P. falciparum* DHFR-TS due to a single amino acid substitution (see J. Inselburg, this volume); (3) *alteration in drug metabolism*, that is, activities of cytochrome P450 and other detoxifying enzymes that have been reported to be higher in chloroquine-resistant strains of *P. berghei* (Ward, 1988), or, alternatively, failure to activate a prodrug, such as in metronidazole resistance; (4) *increased metabolite production or retention*, as reported in *T. brucei*, with increased levels of ornithine in resistant parasites competing with DFMO for ornithine decarboxylase (Bellofatto *et al*., 1987); and (5) *use of alternative pathways* to bypass the site of inhibition.

The origins of parasite-related resistance may be (1) natural, as in non-susceptible strains with no previous exposure to drugs, such as tryparsamide-resistant *T. rhodesiense* isolated from animals (Dukes, 1984); (2) due to drug pressure/exposure; (3) due to cross-resistance, as has been extensively reported for trypanocides (Hawking, 1963; Frommel and Balber, 1987); or (4) mutagen induced. The origin of the resistance is important to the field worker, as is the provision of rapid tests for the diagnosis of resistance in isolates (see R. Kaminsky, this volume). Strategies to combat resistance rely on (1) prevention through correct chemotherapeutic regimens based on knowledge of drug pharmacology; (2) combination therapy, most advanced in malaria prophylaxis; and (3) reversal of resistance, which has recently been experimentally demonstrated for chloroquine resistance in human and rodent malaria (see D.J. Krogstad, this volume) using the calcium channel blocker verapamil (Martin *et al*., 1987) or the tricyclic antidepressant desipramine (Bitonti *et al*., 1988), which reduces the drug efflux. This approach has recently been extended to trypanosomatids

and may be related to the presence of a multidrug-resistant gene (see I.B. Roninson, this volume).

DRUG DELIVERY SYSTEMS

Drug delivery systems are designed to improve the therapeutic index of a compound by increasing efficacy through improved delivery to the site(s) of infection, protecting the drug from host metabolism, and decreasing drug toxicity to the host. There are two different types of delivery systems: sustained- or slow-release systems, designed to maintain a therapeutic drug level, and site-specific targeting systems. Sustained-release systems have been used for the prophylaxis of animal trypanosomiasis to reduce both frequency of dosing and local toxicity. Early studies by Williamson (1970) used a depot of a precipitated complex of suramin with other trypanocides. More recently, trypanocides, in particular isometamidium, linked to dextran have given protection against infection in animal models (James, 1978) and reduced local toxicity in cattle. Studies with biodegradable polymers are under way. Other systems have been developed for therapy and prophylaxis of malaria, leishmaniasis and gastrointestinal helminths.

A large variety of delivery systems have been used in drug targeting, broadly classified as cellular, vesicular, particulate and molecular carriers. The presentations at this Workshop also discuss viral delivery (see S.J. Doxsey, this volume), more commonly associated with vaccines, and antisense oligodeoxynucleotides (see J.J. Toulmé and J. Goodchild, this volume), where the aim is intracellular targeting. The requirement for African trypanosomiasis is a carrier that can be retained in the circulation, avoiding the reticuloendothelial system, especially for *T. vivax* and *T. congolense* infections, and for the human disease, a carrier that can help a drug cross the blood-brain barrier. Studies initiated by Williamson *et al*. (1981) showed that daunorubicin required a covalently bound molecule carrier (bovine serum albumin) to show *in vivo* activity against *T. rhodesiense* (see J.B. Mitchell, this volume). A polyglutamic acid *cis*-platin complex was more effective and less toxic than free drug against *T. congolense* in mice. Drug targeting systems have been used for other protozoal infections, in particular visceral leishmaniasis where *L. donovani* parasites survive in the reticuloendothelial cells of the liver and spleen, cells which remove liposomes, niosomes and other larger particles from the circulation. Liposomes have been developed for other uses (see B.T. Rouse, this volume) and formulations containing amphotericin B and doxorubicin are in clinical trials for the treatment of systemic mycoses and cancer.

CONCLUSION

The identification of a novel compound with exquisite selective activity is only the first stage in drug development. The pharmacological properties of the compound also require consideration to ensure optimum delivery and therapeu-

tic levels at the site(s) of infection. New delivery systems can be designed to assist this purpose. The problem of resistance can be approached by a more complete understanding of the interactions between the drug, the parasite and the host, leading to better usage.

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TRYPANOSOMAL METABOLIC PATHWAYS: TARGETS FOR TRYPANOCIDES

Polyamines in chemotherapy for African trypanosomiasis

C.J. Bacchi*, N. Yarlett*, H. Nathan*, B. Goldberg*, J. Garofalo*, P.D. Sayer†, A. Njogu† and A.B. Clarkson, Jr.‡

*Haskins Laboratories and Biology Department
Pace University
New York, New York 10038, USA

†Kenya Trypanosomiasis Research Institute
P.O. Box 362
Muguga, Kenya

‡Department of Medical and Molecular Parasitology
New York University Medical Center
New York, New York 10016, USA

POLYAMINES AND THEIR SYNTHESIS

Polyamines are small molecules universally required for growth and division by prokaryotic and eukaryotic cells. The most important polyamines in eukaryotes are putrescine, spermidine and spermine, which are believed to have major roles in cell division, differentiation and the maintenance of essential cell functions (Pegg, 1986). The molecular underpinnings of polyamine action are still shrouded, but it is clear that their presence (especially spermidine) is important for conformational regulation of DNA, elongation and initiation of protein synthesis and fidelity of translation. Polyamines appear to be essential for ribosome and transfer RNA (tRNA) structure and function, and exert dramatic control over proteins such as topoisomerases (Marton and Morris, 1987).

Synthesis of polyamines is closely regulated, with the reactions catalysed by ornithine decarboxylase (Figure 1) and S-adenosyl-methionine decarboxylase emerging as the critical steps. Ornithine decarboxylase (ODC) produces putrescine from ornithine; S-adenosyl-methionine decarboxylase (SAM DC) synthesizes decarboxylated S-adenosyl-methionine (SAM), which is the aminopropyl group donor for spermidine and spermine synthesis. Aminopropyl groups are transferred to putrescine and spermidine by spermidine and spermine synthases, respectively (Figure 1).

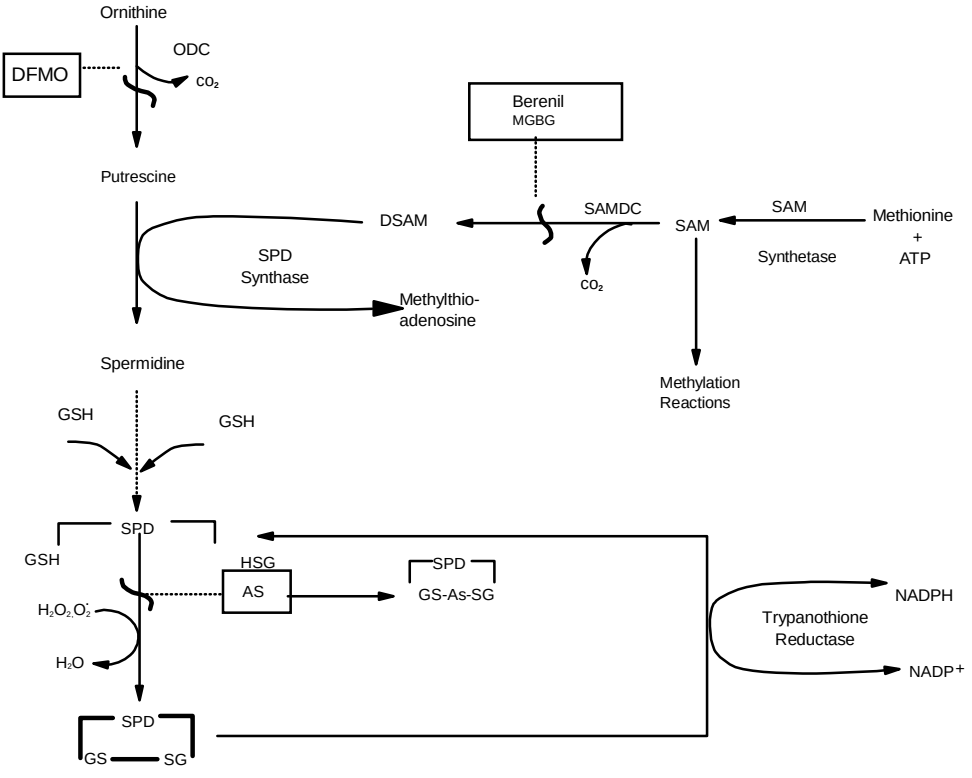

FIGURE 1. Polyamine biosynthesis and related reactions in African trypanosomes. Boxes indicate inhibitors. DFMO: DL-α-difluoromethylornithine, SAM: S-adenosylmethionine, SAM DC: SAM decarboxylase, SPD: spermidine, GSH: reduced glutathione, As: arsenical drug, MGBG: methylglyoxal *bis* (guanylhydrazone), ODC: ornithine decarboxylase, GSH-SPD-GSH: trypanothione.

NOVEL ASPECTS OF POLYAMINE SYNTHESIS IN TRYPANOSOMES

Despite apparent similarities between mammalian and trypanosome polyamine pathways, subtle differences exist. Trypanosomes synthesize putrescine from ornithine via ODC, and spermidine from methionine and putrescine by way of SAM synthase, SAM DC and spermidine synthase (summarized in Bacchi and McCann, 1987). The origin of ornithine is unclear since *Trypanosoma brucei brucei* lacks arginase (J. Garofalo and C.J. Bacchi, unpublished), a major source of ornithine in mammalian cells. African trypanosomes neither synthesize nor contain significant levels of spermine. Many cells avidly transport polyamines, but African trypanosomes do not, unless prior depletion of intracellular levels

has occurred (Bacchi *et al*., 1979, 1983). A key aspect of trypanosome polyamine metabolism is the presence of a unique spermidine-containing peptide, trypanothione (N^1N^8-*bis* [glutathionyl] spermidine), which serves as co-factor for glutathione reduction and hence is essential in protecting trypanosomes from free radical damage and other oxidant stress (Fairlamb, 1989).

INHIBITION OF POLYAMINE SYNTHESIS: RELATIONSHIP TO CHEMOTHERAPY

DL-α-difluoromethylornithine (DFMO; eflornithine) is an enzyme-activated inhibitor of ODC developed by the Merrell Dow Research Institute in the late 1970s. It is highly specific for ODC and exerts a time-dependent irreversible inhibition of the enzyme, becoming covalently bonded to the active site (Bey *et al*., 1987).

In bloodstream trypanosomes, DFMO rapidly blocks ODC activity, reduces polyamine content and blocks DNA, RNA and protein synthesis while causing alterations in morphology of long slender forms, producing cells similar to intermediate and stumpy forms (Bacchi and McCann, 1987; Giffin and McCann, 1989). Despite the broad-reaching biochemical consequences of DFMO treatment, the drug is not overtly cytotoxic.

In model *T. b. brucei* acute mouse infections, DFMO is curative when a 2% solution is administered continuously for three days (total dose: 5.3 g/kg/day [Bacchi *et al*., 1987]). Prolonged administration of DFMO (4% for four weeks) is curative in model central nervous system infections (Bacchi *et al*., 1987), but the total dose can be reduced significantly if combined with suramin (Clarkson *et al*., 1984).

Because of the extensive clinical experience with DFMO as an antitumour agent, clinical trials on Gambian sleeping sickness were begun in 1981. DFMO proved largely successful, with greater than 90% of more than 400 patients responding to the drug, as defined by clearance of trypanosomes from blood and cerebrospinal fluid and a low incidence of relapse (Schechter and Sjoerdsma, in press). Most patients had failed to respond to one or more courses of treatment with melarsoprol (Mel B), and most had involvement of the central nervous system at the time treatment was begun. DFMO has recently been administered intravenously for two weeks (400 mg/kg/day, total dose) followed by 300 mg/kg/day *per os* (4 × 75 mg/kg/day) for four additional weeks. This regimen has proven superior to previous oral dosing protocols, with most side effects being minor and disappearing on cessation of drug administration. These studies are detailed in several reviews (Sjoerdsma *et al*., 1984; Schechter *et al*., 1987; Schechter and Sjoerdsma, in press).

Despite the outstanding success of DFMO against disease caused by *T. b. gambiense*, the outlook for its use in treatment of East African sleeping sickness (caused by *T. b. rhodesiense*) is uncertain. In a limited number of trials in East Africa, various DFMO regimens, even those employing double the standard dose, have failed to cure some patients (S. Van Nieuwenhove, P. deRaadt, personal communications). It is becoming apparent from these initial studies

that alternatives to single drug therapy with DFMO will be needed for East African disease.

SPECIFICITY OF DFMO: THE REASON FOR ITS EFFICACY

The surprising activity of DFMO against African trypanosomes has been the subject of considerable study. DFMO efficacy appears to centre on the following.

(1) DFMO blocks antigenic variation, and a competent immune system is required to rid the parasites from the host (DeGee *et al*., 1983; Bitonti *et al*., 1988).

(2) In contrast to the mammalian host, in which ODC half-life is less than 1 hour (Pegg, 1986), *T. b. brucei* blood and midgut forms have ODC which has a long (greater than 2 hours) half-life (Phillips *et al*., 1987; Bacchi *et al*., 1989). The main structural difference between host and parasite ODC is the lack of a 36 amino acid sequence (`PEST' sequence) on the C-terminal end of the parasite ODC, which is one factor associated with a high turnover rate in the mammalian enzyme (Phillips *et al*., 1987). The low turnover rate of the parasite ODC implies a low synthesis rate and this allows more efficient reduction of enzyme activity by DFMO for a longer time (Bacchi *et al*., 1989).

(3) The spermidine-containing tripeptide trypanothione is unique to trypanosomatids (Fairlamb *et al*., 1985). It is the only substrate for trypanothione reductase and, in the reduced state, it functions to non-enzymatically reduce glutathione and maintain the redox balance of the cell. These reactions are detailed elsewhere in this volume (A.H. Fairlamb). DFMO treatment not only reduces spermidine content, but also reduces the levels of trypanothione and its intermediate, glutathionyl-spermidine (Fairlamb *et al*., 1987). The resulting stress on the capacity to detoxify free radicals should enhance parasite sensitivity to agents that produce free radicals, such as nifurtimox (Fairlamb, 1989).

(4) African trypanosomes lack a reservoir of spermine, and appear not to synthesize it. They also lack the oxidative pathway necessary to convert spermine to spermidine (J. Garofalo and C. Bacchi, unpublished). The persistence of spermine in DFMO-treated mammalian cells and its catalytic conversion to spermidine (Pegg *et al*., 1986) may be one reason why DFMO has been largely ineffective as an antitumour agent when used in single drug therapy (Schechter *et al*., 1987). As noted, uptake of exogenous polyamines in *T. b. brucei* occurs at a rate much reduced from that in mammalian cells (Bacchi *et al*., 1983). Although DFMO treatment enhances polyamine transport, these factors collectively signal the inability of trypanosomes to sequester physiologically significant amounts of exogenous polyamines.

RECENT STUDIES ON THE SUSCEPTIBILITY OF *TRYPANOSOMA B. RHODESIENSE* TO DFMO AND ARSENICALS

In view of recent clinical trials in East Africa with DFMO, we have examined l4 clinical isolates (Uganda, Kenya and Mozambique) from the Kenya Trypano-

somiasis Research Institute (KETRI) strain bank for susceptibility to trypanocides, using these strains as acute laboratory model infections (Bacchi *et al*., 1990). To summarize this work, seven isolates (50%) were moderately to highly refractory to DFMO in comparison to the standard test strain *T. b. brucei* Lab ll0/EATRO. Four of l4 isolates (21.4%) were moderately to highly refractory to melarsen oxide and Mel B. The levels of resistance varied greater than eightfold in some DFMO-refractory isolates and greater than threefold for the arsenicals. Since these strains had no prior contact with DFMO, the resistance attributed to DFMO is constitutive; two of the four arsenic-resistant strains were isolated from patients who had failed to respond to treatment with Mel B. Using the same experimental protocol, all the strains, with the possible exception of one, were susceptible to suramin, indicating that drug inefficacy was not due to sequestration of parasites in the central nervous system or other tissues inaccessible to the drug.

RESISTANCE TO DFMO

Studies have begun to assess potential differences in polyamine metabolism between DFMO-sensitive and -resistant strains. ODC activity in sensitive and resistant strains was found to be similar, with specific activities of 40–60 nmoles CO_2/mg protein/hour. Time-dependent K_i values for DFMO (Bitonti *et al*., 1985) were similar in both DFMO-sensitive *T. b. brucei* EATRO 110 and in DFMO-refractory *T. b. rhodesiense* isolates (25–50 μM), indicating that differences in the ODC active site are probably not responsible for the lack of response to DFMO.

Uptake of DFMO was similar in two sensitive and two refractory strains, in the range of 600 pmoles/mg protein/hour, when bloodstream trypomastigotes were incubated *in vitro* with (^{3}H)DFMO as in Bitonti *et al.* (1986). However, one refractory strain did exhibit a 60% reduction in DFMO uptake. Another potential mechanism accounting for resistance to DFMO may be the significantly increased accumulation of polyamines in bloodstream forms of two refractory isolates upon incubation *in vitro* with radiolabelled polyamines. Thus far, two of seven DFMO refractory isolates were found to accumulate polyamines at rates up to four times that of sensitive cells. These studies are preliminary and full details will be forthcoming in subsequent publications.

RESISTANCE TO ARSENICALS

As noted, four KETRI strains were refractory to melarsen oxide and Mel B. We began studies comparing sensitive and resistant strains in a trypanosome lysis assay based on that developed by Clarkson and Amole (1982): bloodstream forms are incubated in foetal bovine serum with various concentrations of arsenicals. Drug-sensitive isolates completely lysed within 15 minutes upon incubation in less than 20 μM melarsen oxide, while refractory strains withstood 30 minutes in 100 μM drug without lysing.

Since Fairlamb and co-workers have demonstrated that trypanothione may be a primary target of arsenical drugs in trypanosomes (Fairlamb, 1989; Fairlamb *et al.*, 1989), we began examination of total free thiol levels in resistant and susceptible strains using dithionitrobenzene. Refractory strains had a threefold increase in total thiol levels above those measured in sensitive strains (1–2 nmoles/10^8 cells), which indicates an increased ability to withstand arsenic-induced trypanothione loss and still maintain redox function (Fairlamb *et al.*, 1989). However, initial analyses of trypanothione, glutathionyl-spermidine and glutathione, using high-performance liquid chromatography methodology and monobromobimane as a fluorescing agent (Fairlamb *et al.*, 1989), indicated that the levels of these intermediates were similar in arsenic-resistant and arsenic-susceptible strains. These findings do not exclude trypanothione as a target for arsenical drugs, but do indicate the importance of further studies centring on spermidine content and the interconversion of oxidized and reduced trypanothione in arsenic-resistant strains.

CONCLUSIONS

In these studies we have begun to examine the basis for resistance to DFMO and arsenicals observed in clinical isolates. Possible mechanisms of resistance to DFMO include reduced uptake of the drug and increased uptake of polyamines. Overproduction of ODC or significant lack of response of the enzyme to DFMO does not appear to be a factor in resistance in these isolates.

Resistance to arsenicals is easily demonstrable by *in vivo* mouse testing and verification by lysis assay. The reason(s) for resistance is not yet apparent but may be based on the metabolism of trypanothione or the presence of additional thiol targets in these strains.

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Interaction of trypanocidal drugs with the metabolism and functions of trypanothione

A.H. Fairlamb

Department of Medical Parasitology
London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT, UK

In mammalian cells, one of the primary means of defence against damage by oxidants and other toxic substances involves the tripeptide glutathione (γ-glutamyl-cysteinylglycine [GSH]) and its ancillary enzymes glutathione reductase, glutathione peroxidase and glutathione-S-transferases. Trypanosomes and *Leishmania* are uniquely different from the mammalian host in that they do not contain glutathione reductase (Fairlamb and Cerami, 1985), an enzyme crucial to these processes. Instead, trypanothione (N^1,N^8-*bis* [glutathionyl] spermidine; $T[SH]_2$) (Fairlamb *et al*., 1985), which is the principal low-molecular weight thiol in logarithmically growing trypanosomatids (Fairlamb, 1989), acts in concert with a flavoprotein disulphide oxidoreductase, trypanothione reductase (Shames *et al*., 1986; Krauth-Siegel *et al*., 1987), subsuming the role of GSH and glutathione reductase in maintaining the correct intracellular redox balance in these organisms. Similarly, current evidence suggests that, at least in *Trypanosoma brucei* and *Crithidia fasciculata*, the role of GSH and glutathione peroxidase in the removal of hydrogen peroxide (H_2O_2) and other alkylperoxides has been replaced by $T[SH]_2$ and trypanothione peroxidase (Penketh and Klein, 1986; Henderson *et al*., 1987a). Indirect evidence suggests that this may be the case in *Leishmania* species and *T. cruzi* as well (Penketh *et al*., 1987). Trypanothione and its precursor metabolite N^1-glutathionylspermidine (Fairlamb *et al*., 1986) may also be involved in regulation of intracellular levels of unconjugated spermidine in *C. fasciculata* (Fairlamb, 1988b; Shim and Fairlamb, 1988). Whether this finding has any relevance to the medically important parasitic trypanosomatids is not clear at present. A summary of the overall metabolism and functions of trypanothione is illustrated in Figure 1 and the reader is referred to a number of reviews for more detailed information (Fairlamb and Henderson, 1987; Fairlamb, 1988a, 1989).

DRUGS INTERACTING WITH THE BIOSYNTHESIS OF TRYPANOTHIONE

Trypanothione is synthesized from its precursor amino acids (ornithine, methionine, glutamate, cysteine and glycine) as illustrated in Figure 1. The biosynthetic pathway as far as GSH and spermidine is identical to that of mammalian

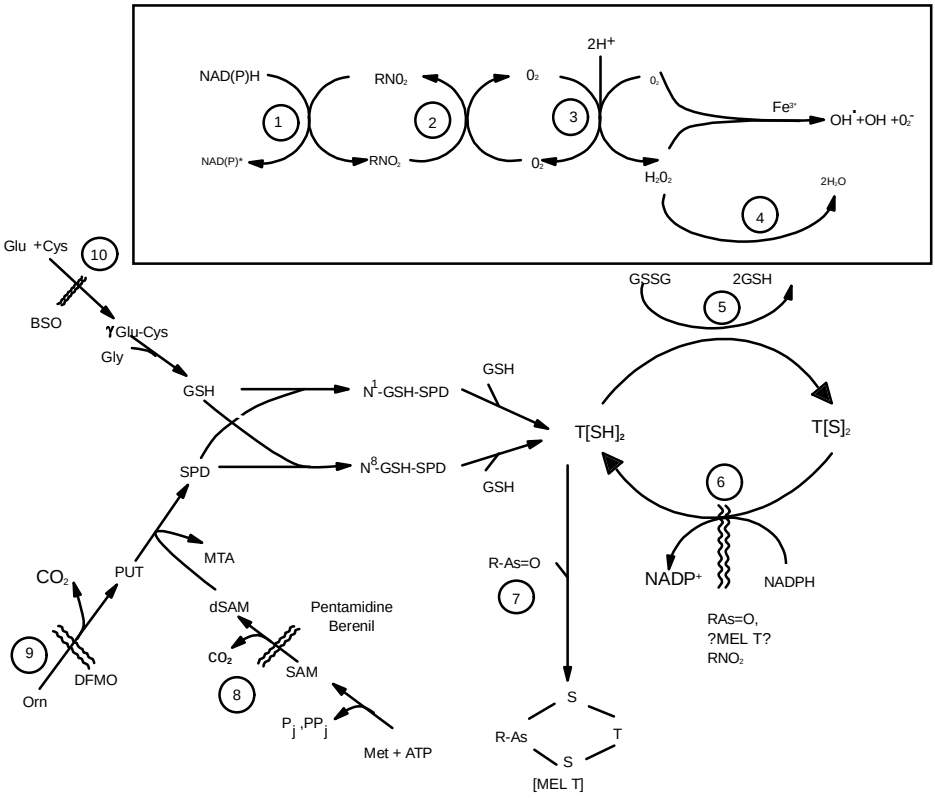

FIGURE 1. Metabolism and functions of trypanothione, showing possible sites of action of trypanocidal compounds. The boxed insert illustrates futile redox-cycling' by nitro compounds (RNO_2) ultimately forming hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). BSO: buthionine sulfoximine, DFMO: difluoromethylornithine, R–As=O: melarsen oxide, MEL T: trypanothione-melarsen adduct, PUT: putrescine, SPD: spermidine, GSH and GSSG: glutathione and its disulphide, GSH-SPD: glutathionylspermidine, $T(SH)_2$: dihydrotrypanothione, $T(S)_2$: trypanothione disulphide, SAM: S-adenosylmethionine, dSAM: decarboxylated S-adenosylmethionine, MTA: methylthioadenosine. Reactions 2, 5 and 7 are non-enzymatic; reaction 4, trypanothione peroxidase; reaction 6, trypanothione reductase. Reproduced from Fairlamb (1989).

cells, whereas the formation of trypanothione via the intermediate N^1-glutathionylspermidine is unique to trypanosomatids (Fairlamb *et al*., 1986). Although these latter reactions do not appear to be inhibited by any of the existing trypanocidal compounds (Fairlamb *et al*., 1986), they clearly represent a potentially novel target for drug development.

Two classes of trypanocidal drugs, the fluorinated analogues of ornithine and the diamidines, inhibit two rate-limiting enzymes in polyamine biosynthesis. Alpha-difluoromethylornithine (DFMO) selectively inhibits the first enzyme in the pathway (ornithine decarboxylase [ODC]; see C.J. Bacchi, this volume),

leading to a multiplicity of subsequent events, including: a decrease in trypanothione and other pathway intermediates (Fairlamb *et al*., 1987); an inhibition of macromolecular biosynthesis (Bacchi *et al*., 1983), including variant surface glycoprotein (Bitonti *et al*., 1988); and biochemical and morphological changes associated with differentiation to forms resembling the non-dividing short, stumpy form (De Gee *et al*., 1984; Giffin *et al*., 1986; Giffin and McCann, 1989). Two other factors have been implicated in the selective toxicity of DFMO to African trypanosomes: clearance by the host's immune system (De Gee *et al*., 1983; Bitonti *et al*., 1986b) and the prolonged half-life of *T. brucei* ODC (Phillips *et al*., 1987; Bacchi *et al*., 1989), with the presumption that *T. brucei* ODC turns over at considerably slower rates than the mammalian enzyme. Whatever the cause of selective toxicity, partial depletion of trypanothione *per se* seems unlikely to account for the rapid trypanocidal activity of DFMO *in vivo*. However, the pronounced synergism between DFMO and arsenical drugs (Jennings, 1988a, 1988b) is likely to occur by this mechanism (see below). Similarly, the report that Berenil® or pentamidine inhibits S-adenosyl-methionine decarboxylase in *T. brucei* (Bitonti *et al*., 1986a) could explain the synergism between DFMO and diamidines (McCann *et al*., 1983).

DRUGS INTERACTING WITH OXIDANT DEFENCE MECHANISMS OF THE PARASITE

A wide range of anti-parasitic compounds are thought to be selectively toxic by means of oxidant stress, that is, by promoting the formation of reactive species of oxygen (superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radicals) or other radical species (Docampo and Moreno, 1984a, 1984b, 1986). For example, the nitrofuran drugs, nifurtimox and nitrofurazone, are thought to undergo `futile-redox cycling' by enzymatic 1-electron reduction of the nitro group with subsequent reoxidation in the presence of molecular oxygen to regenerate the parent drug and form superoxide (O_2^-; see boxed area, Figure 1). Superoxide and H_2O_2 are maintained at low concentrations in trypanosomes by means of superoxide dismutase (Le Trant *et al*., 1983) and trypanothione peroxidase (Henderson *et al*., 1987a), respectively. However, if the parasite's ability to remove these compounds is exceeded, then hydroxyl ($OH^{\cdot}$) and other radical species are formed by the Haber-Weiss reaction. The sulphydryl groups of glutathione and trypanothione serve as a non-enzymatic second line of defence; if swamped, radical damage to other vital cellular components such as DNA and membrane lipids will ensue. Although the identity of the parasite enzymes catalysing redox-cycling of these drugs has not been established, trypanothione reductase has weak nitroreductase activity with nifurtimox or nitrofurazone (Henderson *et al*., 1988). This observation has been exploited to rationally design a series of `subversive-substrates' (Henderson *et al*., 1988), as will be described below.

DRUGS INTERACTING WITH TRYPANOTHIONE AND TRYPANOTHIONE REDUCTASE

The purification, properties and substrate specificity of trypanothione reductase have been reported for *C. fasciculata* (Shames *et al*., 1986; Henderson *et al*., 1987b) and *T. cruzi* (Krauth-Siegel *et al*., 1987), and recently, the gene for trypanothione reductase from *T. congolense* has been cloned, sequenced (Shames *et al*., 1988) and expressed in *Escherichia coli* (Sullivan *et al*., 1989). A partial gene sequence for trypanothione reductase from *L. donovani* has also been reported (Taylor *et al*., 1989). Comparison of the amino acid sequences for trypanothione reductase and glutathione reductase suggests a possible explanation for the pronounced difference in specificity for their disulphide substrates ($T[S]_2$ and GSSG, respectively). X-ray crystallographic studies on the binding of GSSG to glutathione reductase indicate that Arg_{37} is in Coulombic interaction with the glycine carboxylate group of the GS-I peptide (the half of GSSG covalently bound to glutathione reductase during catalysis) (Karplus *et al*., 1989). In trypanothione reductase, where the substrate contains no Gly-I carboxylate, Arg_{37} is exchanged for a Trp (Shames *et al*., 1988). This difference in substrate-specificity, together with the less discriminatory nature of substrate-binding in trypanothione reductase (Henderson *et al*., 1987b), has been exploited to develop a series of substituted naphthoquinones and nitrofurans. These `subversive substrates' not only inhibit reduction of trypanothione disulphide, but also act as redox-cycling agents producing O_2^- and H_2O_2, thus subverting the enzymes' normal anti-oxidant function (Henderson *et al*., 1988; Jockers-Scherubl *et al*., 1989). Computer-assisted molecular modelling will no doubt aid in further improvement of the trypanocidal activity of these and other inhibitors.

Trypanothione ($T[SH]_2$) has also been demonstrated to interact with the trivalent arsenical drug melarsen oxide to form a stable adduct (Mel T, Figure 1) (Fairlamb *et al*., 1989). When bloodstream forms of *T. brucei* are exposed to either melarsen oxide or melarsoprol *in vitro*, Mel T is the only arsenical derivative detectable in acid-soluble extracts of the cells. Thus, trypanothione would appear to be a primary target for these drugs, although the subsequent sequence of events leading to cell lysis is not yet understood. On the one hand, arsenicals could deplete $T[SH]_2$ by forming Mel T, which in turn competitively inhibits trypanothione reductase (K_i = 9 μM), interfering with the protective functions of $T[SH]_2$. On the other hand, $T[SH]_2$ could act to sequester arsenicals, thereby preventing them from binding to other target protein sulphydryl groups. Regardless of the correct interpretation of these observations, synergism between DFMO and arsenical drugs (Jennings, 1988a, 1988b) would appear to involve DFMO-induced depletion of $T[SH]_2$.

In summary, trypanothione and its related metabolism presents several novel and unique areas for drug development. A number of existing drugs, including DFMO, melarsoprol, the diamidines, nitrofurazone and nifurtimox, have inhibitory effects on different aspects of trypanothione metabolism and function, thereby offering a rational biochemical basis for understanding the synergistic activity between these apparently unrelated classes of drugs.

ACKNOWLEDGEMENTS

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Inhibitors of sterol biosynthesis in Trypanosomatidae and their potential for chemotherapy

L.J. Goad

Department of Biochemistry
University of Liverpool
P.O. Box 147
Liverpool L69 3BX, UK

The drugs in routine clinical use for the treatment of leishmaniasis and trypanosomiasis have undesirable toxicity and the disadvantage that they must be administered parenterally. The urgent need for new, orally active chemotherapeutic drugs against these diseases has been highlighted in reviews on the chemotherapy of leishmaniasis (Berman, 1988) and trypanosomiasis (Gutteridge, 1985). Ideally, a drug is required that is inhibitory to some specific biochemical process unique to the parasite. In the search for effective new chemotherapeutic agents, attention has focused recently on antifungal compounds. These drugs are attractive because they have already undergone extensive toxicity testing, many can be administered orally, they have favourable pharmacokinetic properties and they are already commercially available, thus reducing costs of development.

The antifungal drugs belonging to the azole and allylamine groups of compounds are effective because they are inhibitors of sterol biosynthesis. Cholesterol is biosynthesized by man, but in fungi the major sterol produced is the C_{28}-compound ergosterol. The antifungal compounds are effective chemotherapeutic agents because the sterol pathway in fungi is considerably more sensitive to inhibition by these drugs than is cholesterol production in human cells. The imidazoles and triazoles (e.g., ketoconazole and itraconazole) are inhibitors of a cytochrome P450-dependent sterol 14α-demethylase. This enzyme catalyses the oxidative removal of the 14α-methyl group, which is an essential step in the conversion of the precursor, lanosterol, into the major fungal sterol, ergosterol (Mercer, 1984; Vanden Bossche, 1988). Inhibition of this enzyme causes a depletion of ergosterol and an excessive accumulation of unusual 14α-methylsterols in the fungal cell. It is considered that this change in sterol composition has adverse effects on membrane integrity and function, with consequent suppression of growth. The allylamines (e.g., naftifine and terbinafine) block sterol synthesis at the squalene epoxidation step, which is a necessary prelude to squalene cyclization to produce lanosterol, which is the first cyclic precursor of ergosterol (Ryder, 1988). In this case, the depletion of ergosterol is accompanied by a build-up of a large quantity of squalene, which again is believed to result in membrane disruption and inhibition of fungal growth (Ryder, 1988).

Several studies have revealed that ketoconazole, an imidazole derivative, is active against *Leishmania* species. It inhibited proliferation of *L. mexicana mexicana* promastigotes (Berman *et al*., 1984), *L. mexicana mexicana*, *L. tropica* and *L. donovani* amastigotes in human and mouse macrophages (Berman, 1981, 1982; Berman and Lee, 1984) and *L. donovani* in golden hamsters (Raether and Seidenath, 1984). Its effectiveness in man was also indicated by successful treatment of cutaneous and mucocutaneous lesions (Urcuyo and Zaias, 1982; Weinrauch *et al*., 1983a, 1983b). Investigations on the sterol biochemistry of *Leishmania* have shown the locus of ketoconazole action to be the sterol biosynthetic pathway. Detailed analysis of the sterol composition of promastigotes of several *Leishmania* species identified the major sterols as ergosterol and ergosta-5,7,24(28)-trien-3β-ol, with the latter compound usually predominating, particularly in *L. mexicana mexicana* (Goad *et al*., 1984, 1985a). A number of other minor sterols were also identified, including lanosterol, a precursor of sterols found in fungi. From the sterol composition and results of labelling studies with (2-^{14}C)mevalonic acid, the sterol biosynthetic pathway shown in Figure 1 has been deduced (Beach *et al*., 1988), which closely resembles that operative in fungi (Mercer, 1984). However, promastigotes grown in a medium with added serum, or amastigotes cultured in macrophage cells, also contain a significant amount of cholesterol which is of exogenous origin from the serum or macrophages (Goad *et al*., 1984, 1985a; Beach *et al*., 1988).

When *L. mexicana mexicana* promastigotes were exposed to ketoconazole, their growth was retarded and radioactivity from (2-^{14}C)mevalonic acid accumulated in a sterol fraction with the chromatographic properties of 4α-methylsterols which act as precursors to ergosta-5,7,24(28)-trien-3β-ol and ergosterol (Berman *et al*., 1984; Goad *et al*., 1985a; Beach *et al*., 1988). The main accumulating sterol was identified (Goad *et al*., 1985a) by ^{1}H and ^{13}C nuclear magnetic resonance and mass spectrometry as 4α,14α-dimethy lcholesta-8,24-dien-3β-ol. It was accompanied by smaller amounts of 4α,14α-dimethy lergosta-8,24-dien-3β-ol (obtusifoliol), 14α-methylcholesta-8,24-dien-3b-ol and 14α-methylergosta-8,24(28)-dien-3F"Symbol"b-ol (see Figure 1). Similar effects of ketoconazole on sterol synthesis have been demonstrated in several strains of Old and New World *Leishmania* species (Beach *et al*., 1988). The appearance of 14α-methylsterols was accompanied by a dramatic decrease in ergosterol and ergosta-5,7,24(28)-trien-3β-ol (Beach *et al*., 1988). Moreover, ketoconazole was also effective in blocking sterol production in *L. mexicana mexicana* amastigotes cultured in murine macrophage cells, again with accumulation of 4α,14α-dimethy lcholesta-8,24-dien-3β-ol (Berman *et al*., 1986). The ketoconazole-induced changes in sterol composition of *Leishmania* thus parallel those observed in treated fungi and reveal the susceptibility of the 14α-methylsterol 14α-demethylase to azole inhibition, presumably through the cytochrome P450 system. Similar effects on sterol biosynthesis and inhibition of growth have been demonstrated with the triazole antifungal, itraconazole, when tested against promastigotes of 36 strains of *Leishmania* (Beach *et al*., 1988), but fluconazole was much less effective. The anti-leishmanial activity of

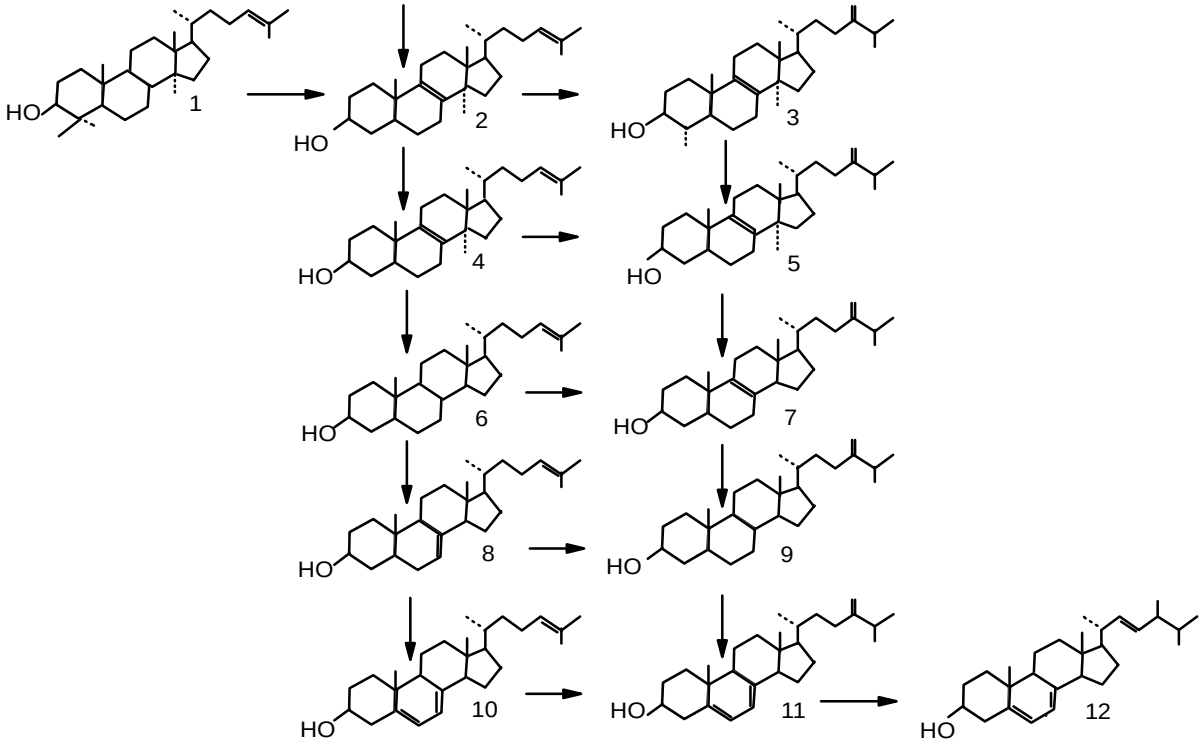

FIGURE 1. Sterols of *Leishmania* and postulated biosynthetic pathway. (1) Lanosta-8,24-dien-3β-ol (lanosterol); (2) 4α,14α-dimethy lcholesta-8,24-dien-3β-ol; (3) 4α,14α-dimethy lergosta-8,24(28)-dien-3β-ol; (4) 14α-methylcholesta-8,24-dien-3b-ol; (5) 14α-methylergosta-8,24(28)-dien-3F"Symbol"b-ol; (6) cholesta-8,24-dien-3β-ol (zymosterol); (7) ergosta-8,24(28)-dien-3β-ol (fecosterol); (8)

itraconazole, together with inhibition of the sterol pathway, has also been demonstrated against amastigotes of *L. mexicana mexicana* (Hart *et al.*, 1989).

The antifungal compounds that inhibit sterol biosynthesis and have been tested against *Leishmania* are the allylamines. Terbinafine (SF86-327) effectively retarded growth of *L. mexicana mexicana* promastigotes and, as in fungi, inhibited squalene epoxidase, causing an accumulation of squalene and a decrease in the sterol content of the cells (Goad *et al.*, 1985b; Beach *et al.*, 1989). Naftifine and terbinafine were also active against growth of *L. major* amastigotes in human monocyte-derived macrophages (Berman and Gallalee, 1987).

It is now becoming clear that *Leishmania* have a sterol biochemistry, and requirements for sterol to support growth, which are remarkably similar to fungi. The need for sterol during cell proliferation may be to fulfil two functions. The first is a `bulk' role for incorporation into newly formed membranes. In fungi it is suggested that the reduction in ergosterol and the appearance of abnormal amounts of 14α-methylsterols in response to azole treatment has a disruptive effect on membrane integrity and function. It is speculated that the 14α-methyl group of the accumulating sterols does not permit correct alignment of the sterol in the membrane for interaction with the fatty acyl chains of the phospholipid components (Bloch, 1983; Weete, 1987). The second need may be for small amounts of specific sterols with a C_{24} substituent, such as ergosterol, to act in a `metabolic' role to stimulate some essential process(es) required for cell division. This would be analogous to the `sparking' or `synergistic' role played by ergosterol in yeast proliferation (Pinto and Nes, 1983; Ramgopal and Bloch, 1983; Rodriguez *et al.*, 1985; Weete, 1987), where the sterol stimulates phospholipid synthesis (Kawasaki *et al.*, 1985), polyphosphoinositol metabolism (Dahl and Dahl, 1985), and phosphatidylinositol kinase and protein kinase activities (Dahl *et al.*, 1987).

The potential value of ketoconazole for the treatment of Old and New World cutaneous leishmaniasis has been examined by a number of groups. Somewhat mixed results are reported, but treatments with a positive response (Urcuyo and Zaias, 1982; Weinrauch *et al.*, 1983a, 1983b; Belazyoug *et al.*, 1985; Jolliffe, 1986; Viallet *et al.*, 1986; Dutz *et al.*, 1987; Weinrauch *et al.*, 1987; Saenz and Berman, 1988) outweigh the negative results (Weinrauch *et al.*, 1983a, 1983b; Dan *et al.*, 1986; Jolliffe, 1986; Kubba *et al.*, 1986) by about 2:l, although the fact that cutaneous leishmaniasis can often self-cure in a few months to a year indicates that caution is needed when interpreting data. One report has also indicated encouraging results using itraconazole (Dedet *et al.*, 1986), but clinical trials with the allylamine terbinafine are awaited with interest.

Some observations on the effects of ketoconazole on promastigote cultures and the sterol metabolism of amastigotes may be relevant to the apparent failure of some cases of cutaneous leishmaniasis to respond to ketoconazole treatment. First, the growth of some species (*L. braziliensis* and *L. donovani*) is somewhat more sensitive to inhibition by low doses of ketoconazole than is growth of others (*L. aethiopica, L. major, L. tropica* and *L. mexicana mexicana*). Strains of some of the less sensitive *Leishmania* species can be subcultured repeatedly in the presence of ketoconazole, in a medium containing serum, with growth

rates about 30–60% of the untreated controls (Beach *et al*., 1988). The sterol composition is changed dramatically with a massive build-up of 14α-methylsterols and a significantly increased cholesterol content derived by absorption of the serum cholesterol from the medium (Beach *et al*., 1988). The normal sterols, ergosterol and ergosta-5,7,24(28)-trien-3β-ol, are reduced to just a few percent of the total sterol. It appears that these cultures may have adapted to use the accumulated 14α-methylsterols, perhaps in conjunction with the exogenous cholesterol, to meet the bulk sterol function in the membranes. The traces of ergosterol and ergosta-5,7,24(28)-trien-3β-ol still produced, or possibly the 14α-methylergosta-8,24(28)-dien-3F"Symbol"b-ol that accumulates, may be adequate to satisfy the `metabolic' role to sustain cell proliferation (Beach *et al*., 1988). It is possible that development of tolerance to sublethal concentrations of ketoconazole in leishmaniasis patients may occur and this needs to be considered as a possible explanation for treatment failures. A resistance to terbinafine has also been noted, in this case with a build-up of squalene in the *Leishmania* cells but with sufficient sterol still being produced, and supplemented by cholesterol absorbed from the medium, to maintain growth (Beach *et al*., 1989).

The second point concerns the possible ability of the amastigotes to use sterols derived from the host macrophage. Analysis of the amastigote sterols of *L. mexicana mexicana* and *L. braziliensis guyanensis* showed that they had a rather different sterol composition from that of the promastigotes. Ergosterol and ergosta-5,7,24(28)-trien-3β-ol were much reduced compared to the promastigotes, while two C_{29}-sterols, stigmasta-5,7,24(28)-trien-3β-ol and stigmasta-7,24(28)-dien-3β-ol, recognized as only trace components in promastigotes, appeared in substantial amounts (Borelli, 1987). Cholesterol and desmosterol (cholesta-5,24-dien-3β-ol), products of macrophage sterol biosynthesis, were present in amastigotes together with ergosta-5,24(28)-dien-3β-ol in *L. mexicana mexicana* and in addition ergost-5-en-3β-ol and ergosta-5,22-dien-3β-ol in *L. braziliensis guyanensis*.

As indicated in Figure 2, it can be postulated that the latter three sterols can be produced by the amastigote using the desmosterol obtained from the macrophage. This transformation would not be susceptible to ketoconazole inhibition and could provide 24-alkylated sterols suitable to fulfil the `metabolic' role. Cholesterol obtained from the macrophage could then meet the `bulk' membrane sterol requirement and so permit the amastigote, in its host macrophage environment, to evade the toxic effects of ketoconazole.

The epimastigotes of *Trypanosoma cruzi*, the causative organism of Chagas' disease, have a sterol composition rather similar to *Leishmania*, with ergosterol and ergosta-5,7-dien-3β-ol as major sterols but with considerably enhanced amounts of the C_{29}-sterols stigmasta-5,7,22-trien-3β-ol and stigmasta-5,7-dien-3β-ol (Karn *et al*., 1969; Beach *et al*., 1986; Goad *et al*., 1989). Sterol biosynthesis in this organism is also very sensitive to azole compounds. Both ketoconazole and itraconazole inhibited the 14α-demethylation step, leading to a build-up of the precursor 24-methylenedihydrolanosterol and a decline in the

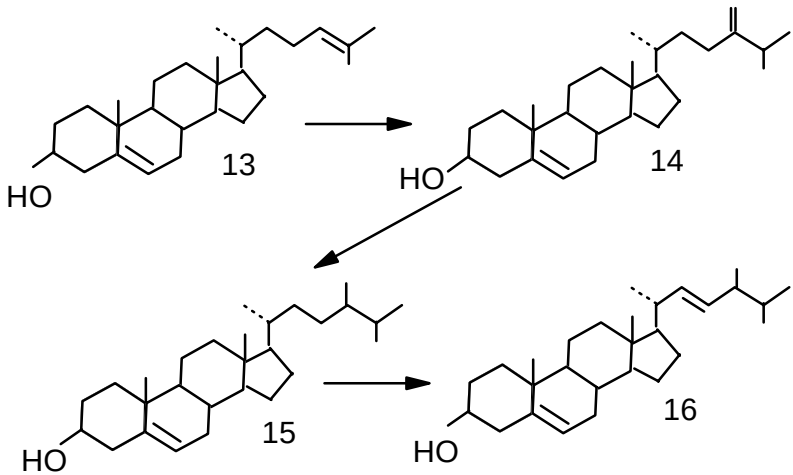

FIGURE 2. Suggested route for production of 24-methylsterols from desmosterol (13) in *Leishmania* amastigotes. (14) ergosta-5,24(28)-dien-3β-ol; (15) ergost-5-en-3β-ol; (16) ergosta-5,22-dien-3β-ol.

C_{28}- and C_{29}-sterols (Beach *et al*., 1986; Goad *et al*., 1989). There was a coincident reduction in growth rate of the epimastigotes (Beach *et al*., 1986), and amastigotes in VA-13 cells (human diploid lung cells) did not survive ketoconazole (5 ng/ml) treatment (Goad *et al*., 1989). Both sterol synthesis and growth of epimastigotes were less sensitive to fluconazole treatment (Goad *et al*., 1989).

These results with *T. cruzi* suggest that azoles may prove effective for chemotherapy, and indeed promising results are now appearing showing that ketoconazole and itraconazole are very active against *T. cruzi* infections of mice (Raether and Seidenath, 1984; McCabe *et al*., 1984, 1986, 1987). However, there are reports of failure of ketoconazole against chronic murine Chagas' disease (McCabe, 1988) and a murine infection of *T. brucei* (Jennings, 1988). Terbinafine has been shown to inhibit proliferation of the epimastigote and amastigote forms of *T. cruzi* and, moreover, to potentiate the anti-trypanosomal activity of ketoconazole, thus pointing to the possibility of effective combined therapy with these drugs (Urbina *et al*., 1988).

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Trypanosome alternative oxidase

A.B. Clarkson, Jr.*, E.J. Bienen†, G. Pollakis*, M. Saric*, L. McIntosh‡ and R.W. Grady§

*New York University School of Medicine
New York, New York 10016, USA

†International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

‡Michigan State University
East Lansing, Michigan 48824, USA

§Cornell University Medical Center
New York, New York 10021, USA

`We know a great deal and understand nothing.' This quote from a recent *Trends in Biochemical Sciences* book review is appropriate for an introduction to a discussion of mitochondrial function in African trypanosomes. The situation has improved, however, with the recent addition of a key finding: the role of coenzyme Q (CoQ) in the electron transport system of these mitochondria. Starting with this, we have reinterpreted a 25-year accumulation of data to draw a new picture of the metabolic processes in bloodstream-form trypanosome mitochondria.

One of the most widely recognized peculiarities of bloodstream trypanosome biochemistry is the so-called glycerophosphate oxidase (GPO) system. This is responsible for the cyanide-insensitive respiration of African trypanosomes and is supposed to be unique to these cells. Another interesting feature is the mitochondrial differentiation that occurs during transformation from bloodstream to procyclic forms. The generally accepted outline is that tricarboxylic (TCA) acid cycle enzymes are synthesized and begin to function quite early during differentiation, but oxidative phosphorylation does not begin until later because it is dependent on the subsequent development of a functional cytochrome chain. Exactly how a partial TCA acid cycle is to function prior to the development of cytochromes, and to what purpose, is a problem that has not been addressed.

A common error made in discussions of bloodstream-trypanosome mitochondria is the statement that they are physiologically inactive: the rate of respiration in bloodstream mitochondria is, in fact, fifty times greater than that in the cells of the mammalian host. The overall role of this respiration has been understood since Grant and Sargent (1960) first described what they called the α-glycerophosphate (glycerol-3-phosphate) oxidase system 30 years ago. A catalytic amount of dihydroxyacetone phosphate (DHAP) is reduced to

glycerol-3-phosphate (GP) by the NADH produced by glycolysis. This step is required for regenerating the NAD^+ needed to support the continued glycolysis upon which these cells depend. The DHAP used in the production of GP is catalytic because it is promptly reoxidized to DHAP. The reduction of DHAP by NADH occurs in the glycosome, a specialized microbody, and the reoxidation to DHAP occurs in the mitochondrion. This `GP shuttle' of electrons from the glycosome to the mitochondrion is shown in Figure 1. Although Grant and Sargent (1961) were not aware that the reoxidation of GP to DHAP occurs in the mitochondrion, they knew that at least two enzymes were involved: a dehydrogenase and an oxidase. Opperdoes *et al.* (1977) demonstrated that the reoxidation of GP occurs in the mitochondrion and Fairlamb and Bowman (1977) described the dehydrogenase as a typical FAD (flavin adenine dinucleotide)-containing mitochondrial enzyme. A suggestion was made that the dehydrogenase and oxidase components are linked by CoQ (Meshnick *et al.*, 1978). Results from our studies on inhibition of glycolysis suggested that inhibitors of respiration were CoQ analogues and thus acted as inhibitors by interfering with the transfer of electrons from the dehydrogenase to the oxidase (Grady *et al.*, 1986b). The concept of CoQ involvement was partially derived from results of workers studying a plant system that was sensitive to similar inhibitors, notably salicylhydroxamic acid and related N-aryl hydroxamates (Rich *et al.*, 1978). A conclusion by plant biochemists that the salicylhydroxamic-acid-sensitive system in plants was CoQ mediated was also speculative, but this role has recently been convincingly demonstrated (Bonner *et al.*, 1986).

We examined the role of CoQ in the trypanosome system because this was important for work aimed at interfering with its function as an approach to chemotherapy (Clarkson *et al.*, 1989a). We confirmed an earlier observation (Folkers *et al.*, 1983) that *Trypanosoma brucei brucei* contains CoQ_9 and determined the concentration to be 206 ng/mg protein. We showed that the redox state of this coenzyme is dependent on respiratory function in a manner consistent with it serving as an electron carrier between the dehydrogenase and oxidase components of the mitochondria. The action of salicylhydroxamic acid on the redox state of CoQ is consistent with interference in the reoxidation of this coenzyme after reduction by the dehydrogenase. Extraction of *T. b. brucei* with acetone to remove CoQ_9 inhibited respiration, which could be partially restored by the addition of CoQ_9 to the extracted cells. Dispersal of mitochondrial components by the detergent digitonin inhibited respiration and this could be partially reversed by liposomes, which provide a site for reassociation. Incorporation of CoQ into the liposomes enhanced this reversal. We synthesized an analogue of CoQ_9 that contains an unbranched alkyl chain in place of the 45-carbon isoprenoid chain; we refer to this compound as decyl-CoQ. This substitution provides a degree of water solubility such that decyl-CoQ can be used in aqueous buffer to measure the activity of a ubiquinol oxidase independent of a dehydrogenase. Chemically reduced decyl-CoQ supported salicylhydroxamic-acid-sensitive oxygen uptake in the absence of GP. Non-reduced decyl-CoQ relinked digitonin-dispersed components of the mitochondria, fully reversing digitonin inhibition of GP-supported respiration.

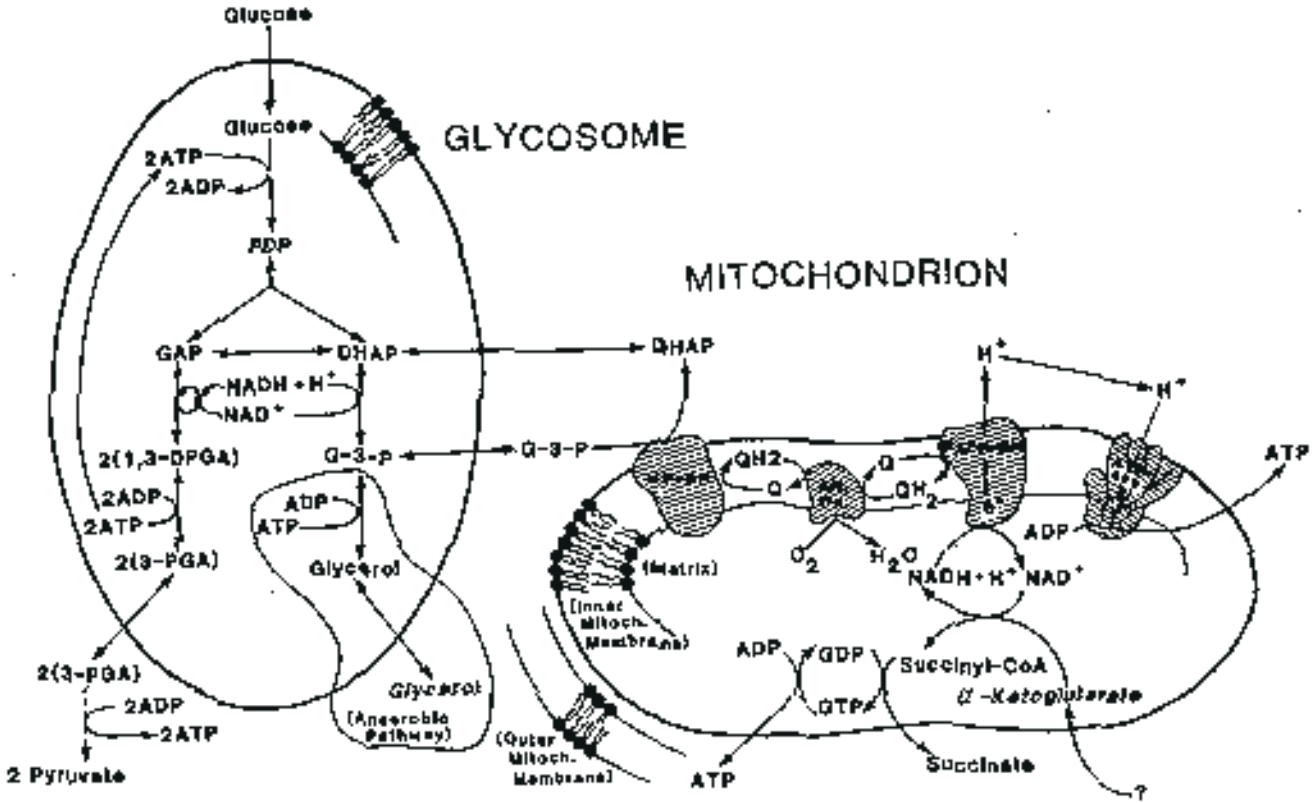

Figure 1. Bioenergetic pathways of *Trypanosoma brucei brucei* including the partial TCA cycle and NADH hydrogenase-associated oxidative phophorylation of transitional intermediate and short, stumpy forms.

From the findings reported above, we conclude that CoQ_9 links the dehydrogenase and oxidase components of the mitochondria. This defines the trypanosome terminal oxidase as an alternative oxidase. In contrast to what is generally assumed, the cyanide-insensitive respiratory system is therefore not unique to bloodstream African trypanosomes but is a conventional CoQ-mediated electron transport system, the only unusual feature being the terminal oxidase employed. We refer to this oxidase as the trypanosome alternative oxidase (TAO). We use the term TAO to replace GPO because GPO implies that the entire trypanosome respiratory system is unique, whereas TAO correctly identifies the only unusual feature of this respiratory chain.

To provide a further comparison of the plant and parasite systems, we examined the effect of a wide range of compounds we had previously found to be inhibitors of parasite respiration (Clarkson *et al*., 1981; Grady *et al*., 1986a, 1986b) for their effect on the plant system (Clarkson *et al*., 1989b). Every class of compound that inhibited parasite respiration also inhibited the plant system, thus supporting the similarity of these alternative oxidases. However, the relative degree of inhibition among the inhibitors varied between plant and parasite. We had found that increasing the length of the alkyl side chain greatly increased activity against the parasite (Grady *et al*., 1986a, 1986b) but this was not as important in the plant system. We concluded that the parasite and plant alternative oxidases are similar but the milieu, the inner mitochondrial membrane, of the two systems is different.

Monoclonal antibodies were recently prepared against the alternative oxidase of *Sauromatum guttatum* (voodoo lily) (Elthon *et al*., 1989). These monoclonals also revealed the alternative oxidase of *Neurospora crassa* on Western blot analysis (Lambowitz *et al*., 1989). We performed a Western blot analysis of *T. b. brucei* mitochondria with these monoclonals and it showed a band at 36.5 kilodaltons, the molecular weight of both the voodoo lily and *N. crassa* alternative oxidases. Not only are the plant alternative oxidase and TAO functionally equivalent, but the structure of these enzymes is apparently very highly conserved. Other evidence indicates that the plant enzyme is a single polypeptide and ultrafiltration data support this conclusion for the parasite system. These similarities are especially interesting because the presence of an alternative oxidase has never before been reported in any protozoan or metazoan.

As shown in Figure 1, the TAO provides a substitute for a cytochrome chain in transferring electrons from CoQ to molecular oxygen. In the general mitochondrial electron transport chain, the action of Complex I, that is, NADH dehydrogenase, is to transfer electrons to CoQ while concomitantly pumping protons from the matrix, thus creating an electromotive force across the inner mitochondrial membrane. This electromotive force is used by the oligomycin-sensitive ATPase to generate ATP. In trypanosomes, the presence of an oligomycin-sensitive ATPase had already been demonstrated, although it had been presumed to be non-functional in bloodstream cells (Opperdoes *et al*., 1976). The presence of a partial TCA cycle had also been demonstrated by the cytochemical detection of NADH-dependent diaphorase activity in the mitochondria of intermediate and short, stumpy bloodstream forms (Giffin and McCann, 1989). Furthermore, intermediate and short, stumpy cells had been

shown to be able to depend on a TCA cycle intermediate, α-ketoglutarate, as an energy source (Vickerman, 1965; Bowman *et al*., 1972; Flynn and Bowman, 1973), which implies the function of at least a partial TCA cycle. Through the use of the vital stain rhodamine 123, we were able to show that intermediate and short, stumpy cells generate an electromotive force and that this can be blocked by salicylhydroxamic acid or rotenone, thus indicating that the electromotive force is dependent on both Complex I and the TAO (Bienen *et al*., 1989). Furthermore, α-ketoglutarate-dependent motility is also blocked by rotenone and by salicylhydroxamic acid. Taken together, these results support the overall metabolic scheme shown in Figure 1.

In summary, the demonstration that bloodstream trypanosome respiration is dependent on a CoQ-mediated electron transport system has led to a better understanding of African trypanosome physiology. These data, old data showing the presence of F_1F_0 ATPase, other old data showing the ability of transitional cells to use TCA intermediates, and our rhodamine 123 uptake data have together produced a new picture of mitochondrial function. Based on this new picture, a better estimate can be made of the range of biochemical effects expected of chemotherapeutic agents that block the TAO. We also found a strong similarity among the TAO and the alternative oxidases of phylogenetically diverse cells. Perhaps the most interesting result is the potential of a better interpretation of both physiological and molecular biological data relating to the processes involved in parasite differentiation between mammalian and insect vector forms.

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Intermediary metabolism in *Trypanosoma cruzi*

J.F. Turrens* and F. Villalta†

*Department of Biochemistry
University of South Alabama
Mobile, Alabama, USA

†Division of Biomedical Sciences
Meharry Medical College
Nashville, Tennessee, USA

Trypanosoma cruzi is the causal agent of Chagas' disease, an endemic disease in Central and South America affecting more than 15 million people. *T. cruzi* can infect a variety of mammals, including dogs and cats. Mammals other than man may therefore serve as reservoirs, thereby increasing the chances of infecting vectors and spreading the disease at a faster rate than if man were the only host (Beaver *et al*., 1984). Chagas' disease in man initially presents as an acute form lasting 20 to 30 days, progressing into a chronic form after this period (Beaver *et al*., 1984). The acute phase can be treated with very toxic drugs (such as nifurtimox [Docampo and Moreno, 1984]) and may be fatal if untreated (Beaver *et al*., 1984). Once the disease becomes chronic, there is no effective treatment, and it is fatal after several years due to extensive damage to vital organs such as the heart, colon and brain (Beaver *et al*., 1984).

Like most eukaryotic cells, *T. cruzi* has many characteristics in common with mammalian cells. A rational way to develop a non-toxic treatment against the parasite is to identify major differences in metabolic pathways between the parasite and the host and to develop inhibitors against these pathways.

Energy metabolism is probably one of the best targets, since prolongation of a parasitic infection depends upon the parasite producing enough energy to propagate itself. Unlike other trypanosomes, *T. cruzi* depends upon cyanide-sensitive respiration (i.e., a mitochondrial cytochrome electron transport system) for survival throughout its life cycle. As a consequence, the parasite is more efficient in obtaining energy from substrate oxidation and better adapted for living intracellularly in the host cells.

Several differences in the mechanisms of energy conservation between *T. cruzi* and the mammalian host have been identified and may become useful targets for designing effective chemotherapeutic agents. This paper summarizes some of these differences between parasite and host that are shared among members of the genus *Trypanosoma*.

As with most trypanosomatids, *T. cruzi* has a glycosome (Cannata and Cazzulo, 1984a) which contains most of the glycolytic enzymes (Fairlamb and Opperdoes, 1986). In the glycosome, glucose is oxidized to phosphoenol-

pyruvate (PEP) through the same reactions described for glycolysis in mammals. After this point, there are several differences in energy metabolism between host and parasite. In mammals, subsequent to synthesis of PEP, the next reaction in the glycolytic pathway is catalysed by pyruvate kinase, an enzyme that is missing in several trypanosomatids (Fairlamb and Opperdoes, 1986). Even though this enzyme is present in *T. cruzi* epimastigotes, it has very low activity (Juan *et al*., 1976), and the relative contribution of this step to the process of aerobic glycolysis seems to be negligible (Cannata and Cazzulo, 1984a). Instead, most PEP is used by the enzyme PEP carboxykinase, producing oxaloacetate as the product (Cannata and Cazzulo, 1984a). Oxaloacetate is in turn the substrate of the glycosomal malate dehydrogenase, a reaction that requires NADH. This reaction is favoured by its low free energy (–8.1 kcal/mol) and the accumulation of NADH during the oxidative step of glycolysis. Thus, malate rather than pyruvate is the main product of glycolysis in these parasites, similar to what has been reported for other members of this genus (Fairlamb and Opperdoes, 1986).

In mammalian cells, glycolysis leads to the formation of pyruvate, which is further oxidized to acetyl-CoA in the mitochondria by pyruvate dehydrogenase, producing NADH. Acetyl-CoA reacts with oxaloacetate to produce citrate, which is oxidized back to oxaloacetate in the Krebs cycle. During this process, NAD^+ is reduced in three different reactions and reoxidized by the respiratory chain. In *T. cruzi* mitochondria, most of the steps where NADH would be produced seem to be missing. The enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase have not been detected in *T. cruzi* (Cannata and Cazzulo, 1984b; Adroher *et al*., 1988). The enzyme isocitrate dehydrogenase exists but is $NADP^+$, rather than NAD^+, dependent. However, the activity of several enzymes of the Krebs cycle are up to 25-fold higher in metacyclic trypomastigotes than in epimastigotes (Adroher *et al*., 1988), suggesting that parts of the Krebs cycle are operational and that their activity is required. In summary, the main product of glycolysis in *T. cruzi* (as well as in *T. brucei*) is malate rather than pyruvate, which upon entering the mitochondrial matrix is unlikely to be used by the Krebs cycle at the same rate as in the mammalian mitochondria.

When malate enters the mitochondrion of *T. cruzi*, it may be dehydrated to fumarate (a reaction with an equilibrium constant very close to 1) if oxaloacetate (the product of malate oxidation) is not removed efficiently. The Krebs cycle may continue in reverse through the reaction catalysed by the enzyme NADH-fumarate reductase. This enzyme is very active in both *T. brucei* procyclic trypomastigotes and *T. cruzi*, with activities comparable to the rate of oxygen consumption by intact cells (Table 1; Boveris *et al*., 1986; Turrens, 1987, 1989). The product of this enzyme is succinate, which can be oxidized efficiently in the respiratory chain back to fumarate through succinate dehydrogenase (Table 1). In addition, *T. cruzi* may secrete succinate, indicating that the product of the enzyme fumarate reductase has a dual role in the intermediary metabolism of these parasites. Through this enzyme, fumarate may be used directly as a substrate for energy production or as a sink of reducing equivalents (NAD[P]H),

TABLE 1. Mitochondrial activities in *Trypanosoma cruzi* epimastigotes. For these experiments, the cells were frozen and thawed and disrupted by passing the homogenate three times through a 24-gauge hypodermic needle. The `mitochondrial fraction' was isolated by centrifugation at 12,000 g for 10 minutes.

Enzyme	Specific activity (mU/mg protein)
Oxygen consumption by intact cells	12 ± 2
NADH-dehydrogenase*	180 ± 8
Succinate dehydrogenase	17 ± 2
Fumarate reductase	18 ± 3
NADH-dependent H_2O_2 production	2.3†
Cytochrome oxidase‡	0 ± 0

*Determined as NADH-ferricyanide reductase.
†Average of two experiments.
‡Determined using horse heart cytochrome *c* as substrate. The content of cytochrome a_{611} was 0.16 nmol/mg protein.

thus explaining how these parasites can either oxidize or accumulate and secrete succinate depending on their metabolic needs.

The mitochondrial cytochrome electron transport system also presents some peculiarities when compared with the mammalian system. In trypanosomes with a functional electron transport system (i.e., *T. cruzi* and *T. brucei* procyclic trypomastigotes), oxygen consumption is insensitive to rotenone, suggesting that either the segment between NADH-dehydrogenase and ubiquinone is somehow different from mammalian mitochondria or that most oxygen consumption is dependent upon succinate oxidation rather than NADH oxidation. The second possibility may occur in intact cells since most of the NADH-producing enzymes of the Krebs cycle appear to be missing (see above) and an active fumarate reductase may keep producing succinate. However, studies using isolated mitochondrial fragments from either *T. brucei* or *T. cruzi* indicate that they do not oxidize NADH at significant rates (Boveris *et al.*, 1986; Turrens, 1989). We proposed the scheme drawn in Figure 1 as a model respiratory chain for *T. cruzi* and *T. brucei* procyclic trypomastigotes, in which most of the reducing equivalents are channelled to fumarate, producing succinate, which in turn is oxidized by succinate dehydrogenase. We have recently reported additional data for *T. brucei* procyclic trypomastigotes using either intact or permeabilized cells, also suggesting that in intact cells succinate may be the major oxidizable substrate (Turrens, 1989).

There is evidence that the mitochondrial membrane of trypanosomes contains an active NADH-dehydrogenase. The transcription product of a gene that may code for a subunit of NADH-dehydrogenase from maxicircles of *T. brucei* kinetoplasts has been isolated from different forms of the parasite (Jasmer *et al.*, 1985; Feagin *et al.*, 1986). Moreover, a NADH-dehydrogenase activity

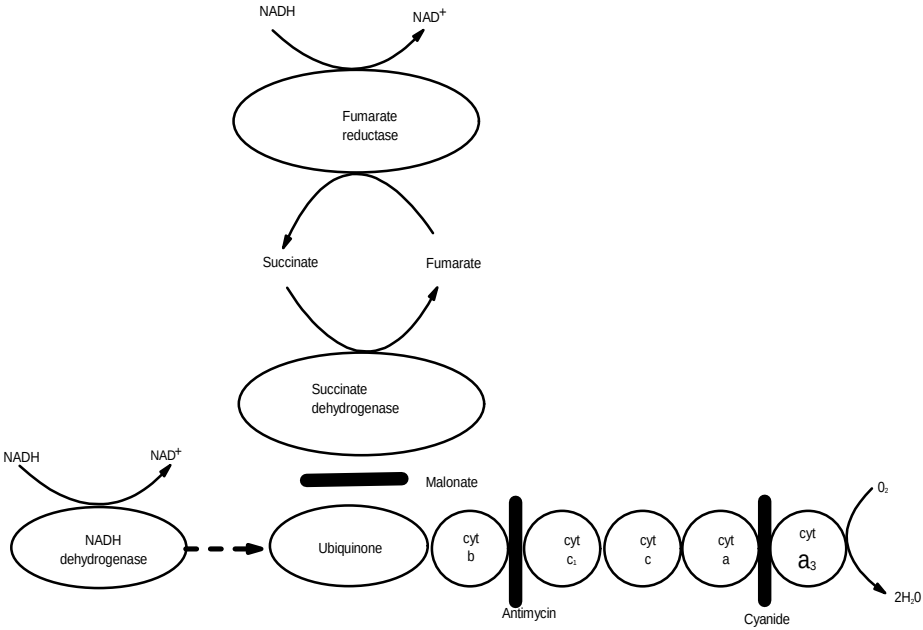

FIGURE 1. Suggested role for the enzyme NADH-fumarate reductase in the mitochondria of *Trypanosoma cruzi* epimastigotes and *T. brucei* procyclic trypomastigotes. According to this scheme, succinate appears as both a respiratory substrate and a final sink of reducing equivalents to be exported out of the cell.

(determined as NADH-ferricyanide reductase) is present in *T. cruzi* (Boveris *et al.*, 1986) (Table 1), but its specific function is not clear. The NADH-dehydrogenase may transfer electrons to the terminal electron donor of the NADH-fumarate reductase rather than to ubiquinone.

If the enzyme fumarate reductase is vital for the life cycle of these cells, the inhibition of this enzyme should be trypanocidal. We tested several inhibitors of the NADH-fumarate reductase of *Haemonchus contortus* developed at Merck, Sharp and Dohme Laboratories and found six compounds (Figure 2) that were effective inhibitors of the enzyme in *T. brucei* procyclic trypomastigotes. These compounds were added to cultures of either *T. cruzi* epimastigotes or *T. brucei* procyclic trypomastigotes. Four of them were effective against both trypanosomatids (Table 2).

The same inhibitors were tested on the electron transport chain of rat heart mitochondria, to see how specific they were against trypanosomes (Table 2). The experiments suggested that these inhibitors of NADH-fumarate reductase may be useful agents against trypanosomes, either *in vivo* or for blood banks. The mode of trypanocidal action of these drugs is under investigation. Some of the drugs may also inhibit the enzyme succinate dehydrogenase, thereby inhibiting cell growth by blocking succinate oxidation.

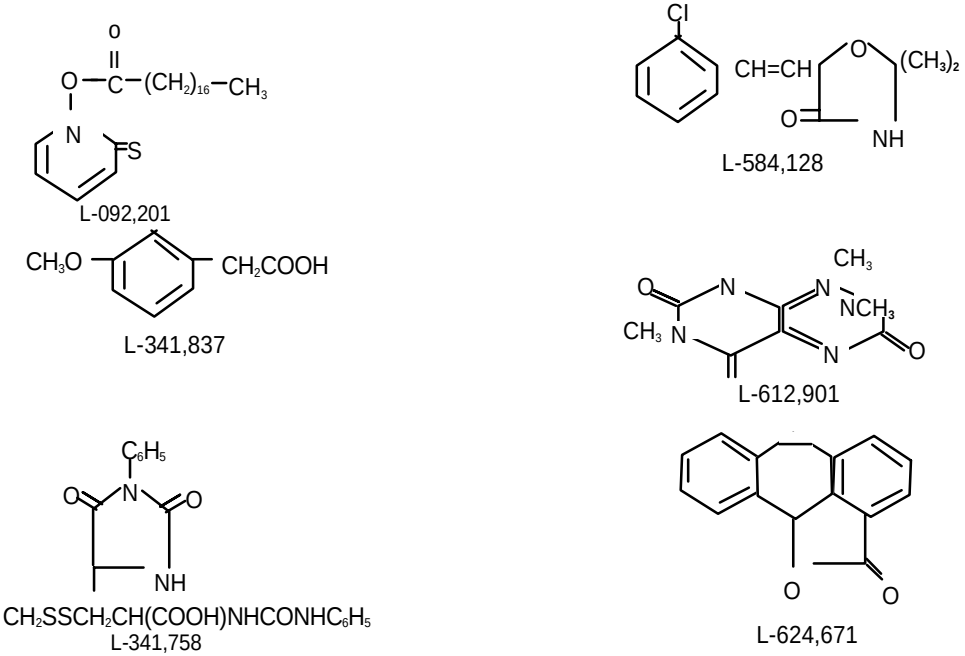

FIGURE 2. Molecular structures of six inhibitors of the enzyme fumarate reductase from *Trypanosoma brucei* procyclic trypomastigotes, four of which also inhibit *T. cruzi* epimastigote growth in culture.

TABLE 2. Effect of several inhibitors of the enzyme NADH-fumarate reductase on cultured *Trypanosoma cruzi* epimastigotes and *T. brucei* procyclics and on two enzymes of the electron transport chain of rat heart mitochondria. The values indicate the concentration of drug that inhibits either cell growth or electron transport by 50%.

Drug	*T. cruzi* growth in culture	*T. brucei* growth in culture	NADH oxidase in rat heart mitochondria	Succinate oxidase in rat heart mitochondria
L-092,201	10	2	4	> 30
L-341,758	24	2	> 30	No effect
L-341,837	60	15	> 30	No effect
L-584,128	No effect	4	No effect	No effect
L-612,901	60	3	No effect	No effect
L-624,671	32	7	> 30	No effect

Although more research is needed to fully understand the mechanism of aerobic glycolysis and energy conservation in *T. cruzi*, it is clear that there are significant differences with the well-characterized mammalian systems that are

worth studying not only for their potential pharmacological applications, but also for their unique biochemical characteristics.

ACKNOWLEDGEMENTS

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Characterization of trypanosomal glycolytic enzymes

P.A.M. Michels

Research Unit for Tropical Diseases
International Institute of Cellular and Molecular Pathology
ICP/TROP 74.39, Avenue Hippocrate 74
B–1200 Brussels, Belgium

THE GLYCOSOME: METABOLIC COMPARTMENTALIZATION IN TRYPANOSOMES

In trypanosomes, as in all other members of the protozoan order Kinetoplastida examined, a unique form of metabolic compartmentalization is found. The majority of the glycolytic enzymes, responsible for the conversion of glucose into phosphoglycerate, are contained within a microbody-like organelle, called the glycosome (Opperdoes and Borst, 1977).

It has been suggested that cells may use microbodies to sequester certain enzymatic systems when an obvious advantage can be gained (Borst, 1989). One example is enzymes that deal with toxic or reactive metabolites, such as peroxidase and catalase found in microbodies often called peroxisomes. Other examples are increasing the flux through a pathway by clustering its enzymes within an organelle or creating a special environment in a compartment of the cell to allow reactions that in the cytosol could normally not occur. These two latter advantages may have been the evolutionary force for the development of the glycosome. Glycolysis is a pathway of vital importance to trypanosomes, particularly of the genus *Trypanosoma*, comprising the well-studied parasites that are responsible for important diseases such as sleeping sickness and *nagana*. These parasites, when they live in the bloodstream of the mammalian host, are entirely dependent on the conversion of glucose into pyruvate for their energy supply. In this stage of the life cycle, they lack a functional Krebs cycle and the mitochondrial system for oxidative phosphorylation. Their dependence on an inefficient energy production is compensated for by an extremely high glucose consumption (Opperdoes, 1987). The sequestering of the glycolytic enzymes within an organelle most likely contributes to this high glycolytic flux by locally increasing the concentration of metabolites (Misset *et al*., 1986). Moreover, the capacity to maintain substrate concentrations, phosphate potential and redox state within the glycosome at levels different from those in the cytosol can significantly change the equilibrium state of some reactions. This is particularly the case under anaerobic conditions, when net ATP synthesis during the conversion of glucose into pyruvate and glycerol is made possible by the reversal of the glycerol kinase reaction as a result of the high concentra-

tion of glycerol-3-phosphate and the low ATP/ADP ratio in the glycosome (Visser *et al*., 1981; Hammond *et al*., 1985; Opperdoes, 1987).

The glycolytic pathway is a potential target for trypanocidal drugs for two reasons: first, because glycolysis is of vital importance for the energy supply of bloodstream trypanosomes, and second, because the glycolytic enzymes may be expected to have some structural features not present in the proteins of the mammalian host due to the large phylogenetic distance between trypanosomes and mammals (Sogin *et al*., 1986) and the unique cellular location of the enzymes in the parasite. The proteins are encoded in the nucleus, synthesized on free ribosomes and then transported into the glycosome (Clayton, 1987; Hart *et al*., 1987). This transport is not accompanied by any detectable processing of the polypeptides, indicating that a targeting signal has to be present in the structure of the mature protein.

CHARACTERIZATION OF THE GLYCOLYTIC ENZYMES OF *T. BRUCEI*

As part of our search for specific features in the glycosomal proteins of the parasite not shared by its host counterparts, we have purified all nine glycosomal enzymes involved in glucose and glycerol metabolism of *T. brucei* (Misset *et al.*, 1986). Cytosolic forms that were found for two glycolytic enzymes—glyceraldehydephosphate dehydrogenase (cGAPDH) and phosphoglycerate kinase (cPGK)—have been purified as well (Misset *et al*., 1977; Misset and Opperdoes, 1987). In addition, the genes for several glycolytic enzymes have been cloned and sequenced. These include the genes for the glycosomal enzymes glucosephosphate isomerase (Marchand *et al*., 1989), fructose-biphosphate aldolase (Clayton, 1985; Marchand *et al*., 1988), triosephosphate isomerase (Swinkels *et al*., 1986), glyceraldehydephosphate dehydrogenase (Michels *et al*., 1986) and phosphoglycerate kinase (Osinga *et al*., 1985), as well as genes for two enzymes located in the cytosol, cGAPDH (M. Marchand and P. Michels, unpublished) and cPGK (Osinga *et al*., 1985), and also the gene for pyruvate kinase (S. Allert and P. Michels, unpublished), a glycolytic enzyme that is present only in the cytosol. The structural and functional properties of the purified enzymes, including their primary structures predicted from the gene sequences, were compared with each other and with those of the glycolytic enzymes of other organisms. The overall sequence similarity of the trypanosomal enzymes to those of other organisms is rather limited: the positional identity of the amino acid sequences varies between 38% and 57% for the different enzymes, regardless of whether the comparison is made with other eukaryotic organisms or with prokaryotes. As for the subunit composition, with the exception of hexokinase, most glycosomal enzymes have an organization comparable with the mammalian ones (Table 1). However, significant differences are found in the subunit masses: all *Trypanosoma* glycosomal enzymes, except triosephosphate isomerase, have subunits that are 1–5 kilodaltons larger than their cytosolic counterparts from other organisms (Table 1). These larger subunits are due to the presence of unique insertions or

TABLE 1. The subunit and native molecular weights of the glycosomal enzymes from *Trypanosoma brucei*[*] and those of their cytosolic homologues from mammalian cells

Enzyme[†]	Native M_r (kilodalton) *T. brucei*		Subunit M_r (kilodalton) *T. brucei*		Subunit M_r (kilodalton) Mammalian	Subunit composition *T. brucei*	Subunit composition Mammalian
Hexokinase	295 ± 12	(4)	50.3 ± 0.8	(10)	97–110	6	1
Glucose-P isomerase	105 ± 6	(5)	62.4 ± 1.6	(8)	59–61	2	2
Phosphofructokinase	196 ± 15	(3)	50.3 ± 0.8	(10)	79–84	4	4
Aldolase	157 ± 3	(3)	40.5 ± 0.5	(9)	39–40	4	4
Triose-P isomerase	55 ± 0	(3)	27.0 ± 0.5	(8)	26–28	2	2
Glyceraldehyde-P dehydrogenase	139	(1)	38.5 ± 0.5	(10)	36–37	4	4
Phosphoglycerate kinase	48 ± 4	(2)	47.0 ± 0.7	(11)	43± 46	1	1
Glycerol-3-P-dehydrogenase	66 ± 6	(3)	37.2 ± 0.7	(9)	33–35	2	2
Glycerol kinase	82	(1)	52.5 ± 0.4	(8)	No data	2	No data

[*]Data from Misset *et al*., 1986.
[†]P: phosphate.
(1) Opperdoes and Borst, 1977.
(2) Borst, 1989.
(3) Opperdoes, 1987.
(4) Misset *et al*., 1986.
(5) Visser *et al*., 1981.
(8) Hart *et al*., 1987.
(9) Clayton, 1987.
(10) Misset and Opperdoes, 1987.
(11) Misset *et al*., 1977.

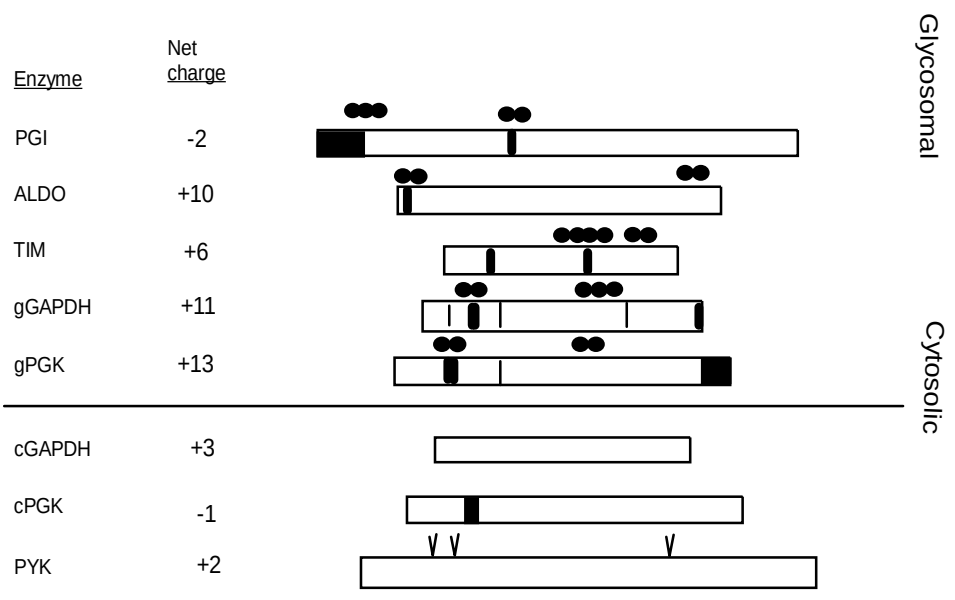

FIGURE 1. Schematic representation of unique features associated with the glycolytic enzymes of *Trypanosoma brucei*. The bars represent the amino acid sequence of the respective enzymes and their size has been drawn relative to the length of the polypeptide chain. Black blocks indicate the position and size of trypanosome-specific amino acid insertions and extensions. Dots represent residues with positive charge. Arrow heads indicate the position of deletions.

extensions of the glycosomal proteins (Figure 1). Furthermore, all glycosomal proteins except glucosephosphate isomerase are characterized by an excess of basic residues, which confer a very high isoelectric point (pI 8.7–10.0) to the proteins. These pIs are significantly higher than those of the cytosolic isoenzymes cGAPDH and cPGK (pI 7.9 and 6.2, respectively) and those reported for the corresponding enzymes of any other organism. Modelling of the amino acid sequences of three glycosomal proteins (triosephosphate isomerase, glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase) into a three-dimensional structure, using the structural information of the homologous enzyme from other organisms, indicated that many of the additional charged residues form a particular spatial configuration in the proteins: two clusters of charges on the protein's surface separated from each other by a distance of 40 Å (Wierenga *et al.*, 1987). This configuration may be common to all glycosomal proteins, because clusters of positively charged residues, specific for the *T. brucei* enzymes, could also be recognized in the primary structures of fructose-biphosphate aldolase and glucosephosphate isomerase, despite the overall neutral charge of the latter enzyme. In contrast, they were not detected in *T. brucei's* cytosolic enzymes cGAPDH, cPGK and pyruvate kinase (Figure 1). It has been proposed that these unique charged patches constitute the signal that directs the proteins to the glycosome after their synthesis in the cytosol (Wierenga *et al.*, 1987). However, a serious objection arguing against this hypothesis is the observation that no charge differences exist between the glycosomal and cyto-

solic phosphoglycerate kinase (PGK) isoenzymes of *Crithidia fasciculata* (Swinkels *et al*., 1988), an organism related to *T. brucei*. Because the only major difference between these two proteins is a C-terminal extension, Swinkels *et al*. (1988) drew the conclusion that the topogenic signal should be located at the carboxy-terminus of that protein. Interestingly, a stretch of amino acids was found in the C-terminal sequence of the *T. brucei* glycosomal phosphoglycerate kinase with considerable similarity to a peptide in the N-terminal extension of glycosomal glucosephosphate isomerase. However, a search for similar peptides in other glycosomal proteins has not yet led to the unambiguous identification of a common, putative topogenic signal.

The functional properties of most of the purified glycosomal enzymes have been analysed in detail (Misset *et al*., 1977; Cronin and Tipton, 1985, 1987; Lambeir *et al*., 1987; Misset and Opperdoes, 1987; Marchand *et al*., 1989; M. Callens, A.-M. Lambeir, D. Kuntz and F. Opperdoes, unpublished). These analyses have shown that the kinetic parameters of some of the enzymes (glucosephosphate isomerase and triosephosphate isomerase) are very similar to those of their mammalian counterparts, whereas other enzymes (hexokinase, fructose-biphosphate aldolase, glyceraldehydephosphate dehydrogenase and PGK) display more unusual characteristics. This dissimilar kinetic behaviour may be attributed to the participation of residues in the catalytic process different from their mammalian counterpart, such as differences in residues either at the active-site, or in residues that may play a role in binding of the co-factors. Detailed knowledge of the three-dimensional structures of the glycosomal proteins will be required to interpret the kinetic differences between these enzymes and their homologues from other organisms.

SUMMARY

Analysis of the cell biology of trypanosomes has shown that glycolysis is of vital importance for the energy supply of these organisms. Moreover, most of the enzymes of the glycolytic pathway appear to be organized in a unique manner, that is, in a microbody called the glycosome. Further unravelling of the glycolytic process, using the approaches of enzymology and molecular biology, demonstrated that the glycosomal enzymes in *T. brucei* have a number of unusual structural and functional features. These features may be involved in the entry of the proteins into the organelle, in their functioning within the specific environment, or they may simply be the result of the large phylogenetic distance between these protozoans and all other organisms studied so far. Whatever their function and origin, these unusual features are potential targets for compounds that should specifically block the parasite's glycolysis without interfering with the pathway of the mammalian host's cell. A more detailed analysis of the special aspects of some glycosomal enzymes of *T. brucei*, by crystallographic methods, and examples of the design of selective inhibitors that should specifically interact with those aspects, are described by F.M.D. Vellieux (this volume).

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Protein crystallography and the design of new drugs against sleeping sickness

F.M.D. Vellieux*, R.K. Wierenga†, H. Groendijk*, R.J. Read‡, M.E.M. Noble†, C.L.M.J. Verlinde* and W.G.J. Hol*

*BIOSON Research Institut
Department of Chemistry
University of Groningen
Nijenborgh 16
9747 AG Groningen, The Netherlands

†European Molecular Biology Laboratory
Postfach 10.2209
6900 Heidelberg, West Germany

‡Department of Medical Microbiology and Infectious Diseases
University of Alberta
Edmonton, Alberta T6G 2H7, Canada

INTRODUCTION: RATIONAL DRUG DESIGN AND TRYPANOSOMIASIS

Because X-ray crystallography can be used to determine three-dimensional structures of biomacromolecules at close to atomic resolution, this technique can be used for the rational design of drugs (Hol, 1986). To understand how this is possible, it is useful to consider a `rational drug design cycle' (Figure 1). Analysis of the three-dimensional structures of a well-chosen target protein complexed with several inhibitors will reveal their modes of binding. Computer graphics and theoretical calculations can be used to propose modifications of these inhibitors. Promising compounds are then synthesized and tested for inhibitory activity, and the three-dimensional structures of the target protein complexed to these new inhibitors are examined. In this manner, our understanding of the mechanisms by which inhibition takes place can be increased until we are able to obtain very selective inhibitors. We are using this approach for the design of new drugs against sleeping sickness.

Sleeping sickness is considered by the World Health Organization to be one of the major tropical infectious diseases, and has been included in the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. The causative agent of sleeping sickness is a unicellular protozoan, *Trypanosoma brucei*, which is transmitted by the tsetse fly. When present in the bloodstream of the human host, the single mitochondrion of trypanosomes is inactive, and these

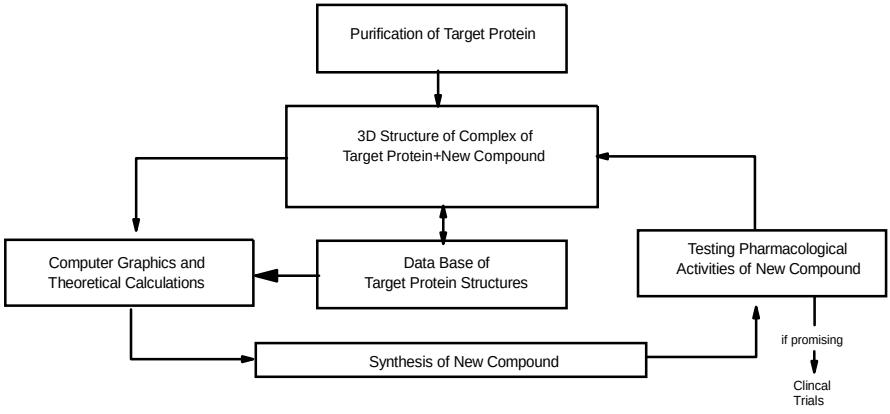

FIGURE 1. The protein-structure-based drug design cycle.

TABLE 1. The nine glycosomal enzymes from *Trypanosoma brucei* involved in the glycolytic pathway

Name	Molecular abbreviation	Subunit Number of weight	Subunits	pI
Hexokinase	HK	49.500	6	10.0
Glucosephosphate isomerase	PGI	52.500	2	7.5
Fructosephosphate kinase	PFK	49.000	4	8.7
Fructose-biphosphate aldolase	ALDO	39.000	4	9.1
Triosephosphate isomerase	TIM	27.000	2	9.8
Glyceraldehydephosphate dehydrogenase	GAPDH	37.000	4	9.3
Phosphoglycerate kinase	PGK	48.000	1	9.4
Glycerol phosphate kinase	GK	41.000	2	9.0
Glycerol phosphate dehydrogenase	GDH	33.000	2	10.0

protozoa depend entirely on glycolysis for survival. In trypanosomes, unlike other organisms, an essential part of glycolysis takes place in a peroxisome-like organelle, the glycosome (Opperdoes and Borst, 1977), in which nine of the glycolytic enzymes are sequestered (Table 1).

The glycolytic enzymes can be purified by chromatographic techniques (Misset *et al*., 1986). Since interference with glycolysis will rapidly kill the parasites (Clarkson and Brohn, 1976; Fairlamb *et al*., 1977), the nine glycolytic enzymes have been selected as a target for protein-structure-based drug design.

GLYCOSOMAL ENZYMES AS TARGETS FOR DRUG DESIGN

There are three general areas of possible interference with the proper functioning of the glycosomal enzymes in the trypanosome:

- prevention of entry into the glycosome, since these enzymes are synthesized in the cytosol and subsequently imported into the glycosomes (Hart *et al*., 1987)
- prevention of assembly into multimers, since eight of the nine glycolytic enzymes function as multimers (Table 1)
- inhibition of catalytic activity by blocking the active site

Due to space limitations, we will refrain from discussing interference with the assembly of glycolytic enzymes in this short communication. To interfere with import of glycolytic enzymes into the glycosome, the topogenic signal for import must be known. The following are two hypotheses about this.

(1) Glycosomal enzymes from *T. brucei* contain clusters of positive charges at their surface, or `hot spots', which are missing in the mammalian enzymes. These hot spots have been proposed to be the import signal into the glycosome (Wierenga *et al*., 1987b). Compounds designed as potential hot spot ligands were found to specifically inhibit the trypanosomal enzymes (Opperdoes *et al*., 1989), and crystallographic studies of glycosomal triosephosphate isomerase complexed with these compounds have been initiated to shed light on the mechanism of inhibition.

(2) Recent sequence comparison studies have revealed the existence of a short fragment with significant sequence similarity in both the C-terminal extension of glycosomal phosphoglycerate kinase (gPGK) (Osinga *et al*., 1985) and in the N-terminal extension of glycosomal glucosephosphate isomerase (gPGI) (Marchand *et al*., 1989) (Table 2). Such a peptide sequence could be the signal that directs these two proteins to their biological compartment, the glycosome. This attractive hypothesis is supported by the presence of a C-terminal extension in phosphoglycerate kinase from the trypanosome-related *Crithidia fasciculata*, which contains a similar peptide sequence (Swinkels *et al*., 1988).

The testing of these hypotheses will require an ingenious experimental set-up to overcome the problems created by the fragility of purified glycosomes.

CRYSTALS OF GLYCOSOMAL ENZYMES FROM *T. BRUCEI*

The third area of interference with trypanosomal glycolytic enzymes is selective inhibition of the catalytic mechanism. Crystallization of trypanosomal enzymes is quite difficult owing to the small amounts of material available. So far, our crystallization experiments have yielded useful crystals for three of the nine

TABLE 2. Alignment of the C-terminal segment of glycosomal phosphoglycerate kinase with the N-terminal segment of glycosomal glucosephosphate isomerase

Protein	Residue numbers	Putative import signal sequence
Glycosomal phosphoglycerate kinase	421–433	A V V S Y A S A G T G T L
Glycosomal glucosephosphate isomerase	12–24	L A AS P A S G G S A S I

glycosomal enzymes, namely, triosephosphate isomerase, glyceraldehydephosphate dehydrogenase and hexokinase. Crystals of hexokinase were obtained very recently and have only been partially characterized.

X-RAY CRYSTALLOGRAPHIC STUDIES OF GLYCOSOMAL GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *T. BRUCEI*

Two crystal forms were grown from glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) samples (Read *et al*., 1987). For crystal form II, synchrotron radiation was essential to obtain data to 2.82 Å resolution. With these data, Read *et al*. (unpublished results) were able to solve the gGAPDH structure using the molecular replacement method (Rossman, 1972), with the structure of the similar enzyme from *Bacillus stearothermophilus* (Skarzynski *et al*., 1987) as a search model. The asymmetric unit of this crystal form contains six gGAPDH monomers, or 1.5 GAPDH tetramers having a total molecular weight of approximately 220 kilodaltons.

While refinement of this crystal form is in progress, we are exploring possibilities of obtaining data for gGAPDH inhibitor complexes. In particular, we are using the very rapid Laue data collection method (Campbell *et al*., 1987) (in collaboration with Dr. J. Hadju, University of Oxford) to collect data for the form I GAPDH crystals. We were able to collect a partial 3.2 Å resolution data set using four film packs, and we are currently attempting to solve this gGAPDH structure using the molecular replacement method.

Even in the absence of a fully refined structure, the amino acid sequence obtained by Michels *et al*. (1986) allowed us to make general statements concerning the type of molecules that may selectively inhibit the glycosomal enzyme. Comparison of the NAD binding site in GAPDH, using both human and *T. brucei* sequences (Tso *et al*., 1985; Michels *et al*., 1986), revealed that most residues in the neighbourhood of NAD are identical, except near the adenine binding pocket (Table 3). Thus, interesting candidates for the development of drugs targeted against gGAPDH would be NAD analogues suitably modified at the level of the adenine moiety.

TABLE 3. Residues in the vicinity of the adenine moiety of NAD which differ between glycosomal and human glyceraldehyde-3-phosphate dehydrogenase

Trypanosoma brucei	Human
ASN 7	ASP 7
VAL 36	ASN 33
ASN 39	PHE 36
GLN 90	GLU 78
ASN 92	ASP 80
LEU 112	VAL 100

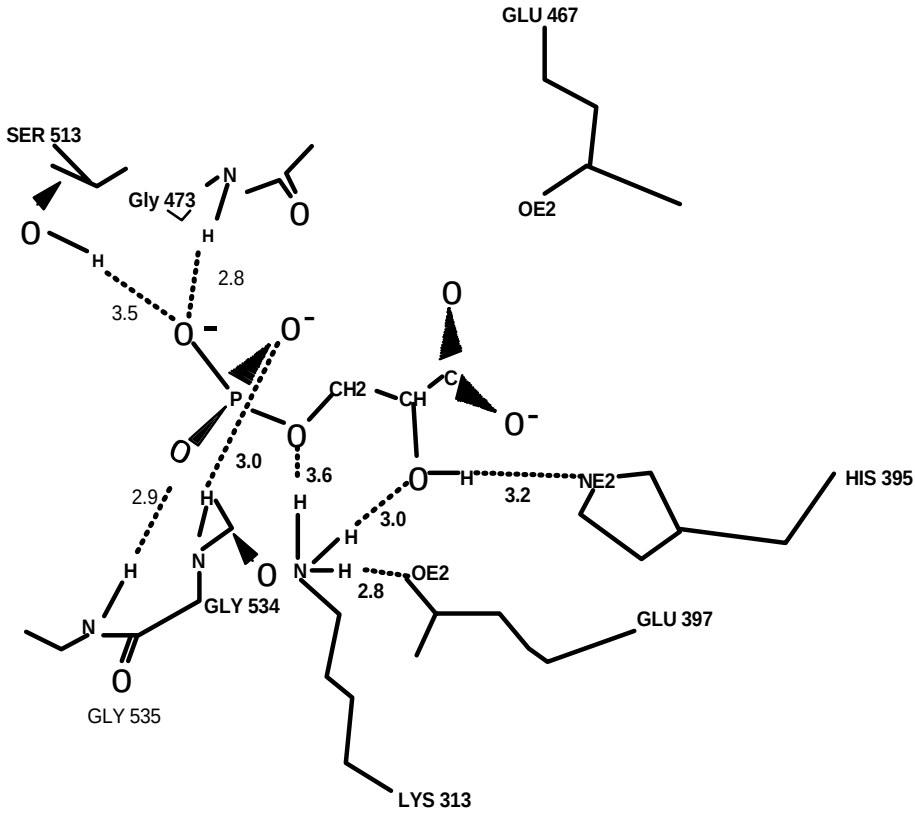

FIGURE 2. The observed interactions between the bound inhibitor D-3-phosphoglycerate and the active site residues of glycosomal triosephosphate isomerase.

CRYSTALLOGRAPHIC STUDIES OF GLYCOSOMAL TRIOSEPHOSPHATE ISOMERASE FROM *T. BRUCEI*

Glycosomal triosephosphate isomerase (gTIM) crystals were grown from 2.4 M ammonium sulphate solutions (Wierenga *et al*., 1984). A total of 600 μg of protein were required to establish crystallization conditions and to solve the structure to 2.4 Å resolution (Wierenga *et al*., 1987a). Subsequently, 1.83 Å resolution data were collected using synchrotron radiation. The native gTIM structure is nearing the completion of its refinement, and has an R-factor of 19.3% for data between 6.0 and 1.83 Å resolution. The model comprises 286 water molecules, plus a sulphate ion bound in the active site of subunit 2.

Following the development of a procedure to transfer crystals from the ammonium sulphate mother liquor to solutions of polyethylene glycol (Schreuder *et al*., 1988), inhibitor binding studies could be initiated. X-ray data

for three inhibitors (D-glycerol-3-phosphate, D-3-phosphoglycerate and 3-phosphopropionate) complexed with gTIM are now available. From these studies, a detailed picture of the binding mode of the inhibitors is emerging: each of these inhibitors contains a phosphate group that binds at the sulphate ion binding site, and recognition of the inhibitor by gTIM involves several hydrogen bonds with the enzyme (Figure 2). Also, the gTIM loop comprising residues 170 to 180 partially blocks the entrance of the active site cavity.

With our current knowledge we are now able to use the gTIM structure to design inhibitors that extend out of the active site region. These inhibitors may allow us to bridge the active site with those surface residues that differ between mammalian and trypanosomal TIM (Table 4).

This new generation of TIM inhibitors will be one step further in the path leading to new anti-trypanosomal drugs, since they should be more specific for the parasite enzyme.

TABLE 4. Triosephosphate isomerase (TIM) surface residues that differ in the human and the trypanosome enzyme

Residue number	Human TIM	*T. brucei* TIM	D (Å)*
100	HIS	ALA	14.2
101	VAL	TYR	11.6
102	PHE	TYR	15.9

*D represents the distance from the phosphorus atom of the inhibitor D-3-phosphoglycerate to the terminal atom of the glycosomal triosephosphate isomerase side chain.

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DRUG RESISTANCE

In vitro techniques for identifying and quantifying drug resistance in *Trypanosoma brucei brucei*

R. Kaminsky

International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi, Kenya

Drug resistance in African trypanosomes is only one of many possible explanations for the failure of treatment with trypanocides in the field. Even if drugs are accurately administered, it has yet to be shown whether the parasites have developed a degree of resistance to the drugs or whether the apparent `resistance' is due to other factors, such as the inaccessibility of trypanosomes in host tissues to trypanocidal drug concentrations. Detailed laboratory investigations on drug resistance have been largely restricted to experiments using mice. To maintain drug-resistant trypanosomes *in vitro* would facilitate detailed studies on the mechanisms of such resistance in the parasites. However, a number of difficulties and questions such as the isolation and initiation of *in vitro* cultures from mammalian hosts, the effect of *in vitro* cultivation on the stability of drug resistance, and the expression of drug resistance *in vitro* have to be solved before *in vitro* techniques can be used to study drug resistance in African trypanosomes.

INITIATION OF *IN VITRO* CULTURES

The low parasitaemias that usually occur in the natural host have been found insufficient to initiate *Trypanosoma brucei brucei* bloodstream-form cultures. Successful initiation of *in vitro* cultures required a minimum inoculum of 2×10^3-1×10^5 trypanosomes/ml, separating parasites from mouse blood during a rising parasitaemia (Hirumi *et al*., 1977; Baltz *et al*., 1985). A novel technique for the successful initiation of bloodstream-form cultures has been applied by direct inoculation of a drop of infected blood into feeder-layer-containing culture wells (Zweygarth *et al*., 1989). Briefly, blood samples were aspirated with a 5 or 10 µl Eppendorf pipette and each was transferred into a well of a culture plate containing feeder-layer cells. The blood was deposited in a corner at the bottom of the wells. This technique has been improved so that it was possible to isolate *T. b. brucei* from pigs and cattle with low parasitaemias, undetectable in wet blood films, using feeder-layer cell cultures and axenic conditions (Zweygarth and Kaminsky, in press). After successful initiation of cultures, trypanosome populations were continuously propagated *in vitro* in a cell-free system (Kaminsky and Zweygarth, 1989b).

STABILITY OF DRUG RESISTANCE

Adaptation of any parasite to *in vitro* culture conditions is associated with a selection for a variety of parameters such as fast growth. Therefore, we investigated whether *in vitro* cultivation of trypanosomes changed their sensitivity to drugs. The susceptibility of a multidrug-resistant *T. b. brucei* stock was examined before and after *in vitro* propagation as bloodstream forms in the absence of any drug, and after full cyclical development *in vitro*. There was no significant change in susceptibility to diminazene aceturate, quinapyramine sulphate, suramin, Mel B or isometamidium chloride following *in vitro* maintenance (Kaminsky and Zweygarth 1989a; illustrated in Figure 1). These observations confirm earlier results showing that transmission of trypanosomes

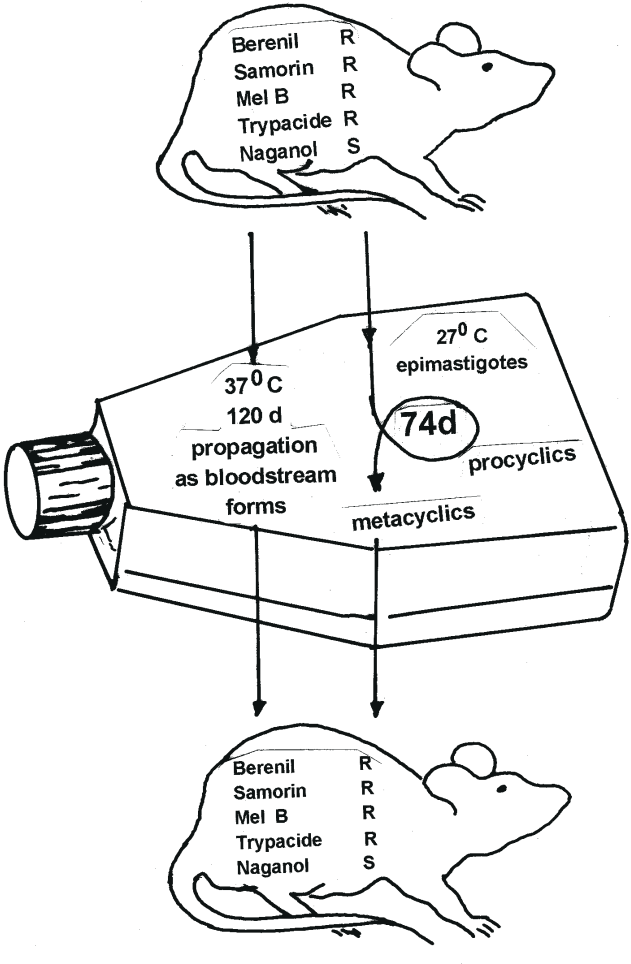

FIGURE 1. Schematic diagram demonstrating the stability of drug resistance after *in vitro* propagation. R: resistant to drug in mice, S: susceptible to drug in mice.

through tsetse flies did not change their drug sensitivity pattern (Gray and Roberts, 1971; Schonefeld *et al.*, 1987).

EXPRESSION OF DRUG RESISTANCE *IN VITRO*

The next step towards the development of *in vitro* assays for the identification and quantification of drug resistance in trypanosomes was to clarify whether drug resistance can be demonstrated *in vitro*. *T. brucei* stocks with different drug susceptibilities to diminazene aceturate and isometamidium chloride were adapted to *in vitro* culture conditions. The effect of the drugs on the trypanosomes was investigated *in vitro* and compared with the drug susceptibilities in mice.

Procyclic forms are not usually exposed to trypanocides; for *in vitro* drug testing, they nevertheless have several advantages over bloodstream forms: easy maintenance (no incubator and no CO_2-enriched atmosphere required) and the possibility of direct isolation from tsetse flies. When procyclic forms were incubated for 48 hours at 27 °C with various concentrations of isometamidium chloride, the growth of trypanosomes was inhibited. The growth of the drug-resistant *T. b. brucei* stock CP 547 was less inhibited by isometamidium than that of susceptible stocks. However, growth of the resistant stock CP 2469 and clone 2469/cl 1 was inhibited in the same manner as that of susceptible stocks. Thus, procyclic forms do not seem to be suitable for the differentiation of drug-susceptible from drug-resistant trypanosomes.

Growth inhibition, however, is a suitable parameter to distinguish between susceptible and resistant trypanosomes when the assay is applied on bloodstream trypomastigotes. Differences in diminazene aceturate-induced growth inhibition after incubation for 24 hours with various concentrations of drug can be demonstrated graphically (Figure 2). In the 24-hour growth inhibition, there were only minor differences in EC_{50} values for isometamidium chloride. However, concentrations of 0.001–0.1 μg/ml inhibited susceptible and, to a lesser degree, drug-resistant stocks (CP 547 and CP 2469). Thus, differences in growth inhibition due to isometamidium occurred when trypanosomes were incubated with drug concentrations (0.001–0.05 μg/ml) below the EC_{50} values (Kaminsky *et al.*, 1989).

T. b. brucei stocks that were propagated *in vitro* as bloodstream forms in the presence of drugs for a period of 10 days differed greatly in their viability. In contrast to susceptible trypanosomes, the drug-resistant stocks CP 547 and CP 2469 were propagated without loss of viability in the presence of 0.01 μg/ml diminazene aceturate or 0.0001–0.001 μg/ml isometamidium chloride. This long-term viability assay offers an alternative to the 24-hour growth inhibition assay, for it does not necessarily require feeder-layer-free culture conditions. However, if trypanosome isolates have been adapted to cell-free cultures, the 24-hour growth inhibition assay using bloodstream forms appears to be a rapid and reliable test to distinguish between drug-resistant and drug-susceptible *T. b. brucei* stocks (Kaminsky *et al.*, 1989).

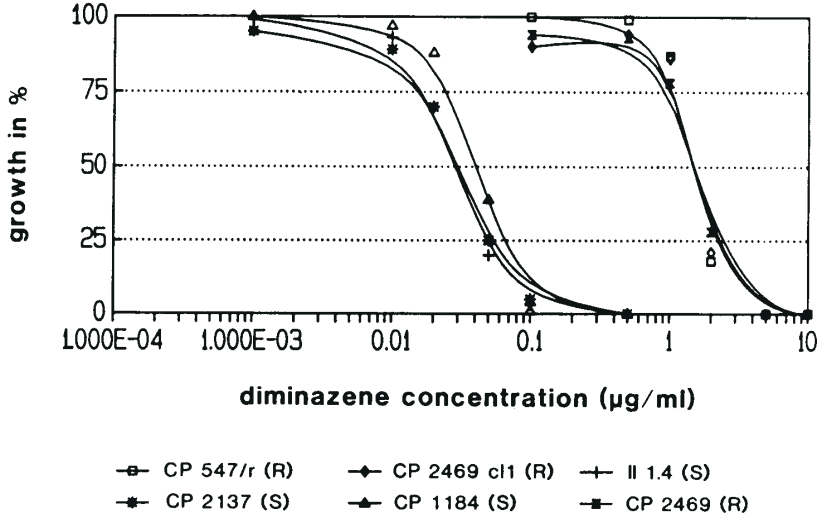

FIGURE 2. Growth inhibition of *Trypanosoma brucei brucei* stocks produced by diminazene aceturate after incubation in culture medium containing various concentrations of drug for 24 hours at 37 °C in 4% CO_2 in air. The number of trypanosomes was determined with a Coulter counter and compared with control cultures. R: resistant to diminazene aceturate in mice, S: susceptible to diminazene aceturate in mice.

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Inhibition of type II topoisomerase activity in the mitochondria of trypanosomes

T.A. Shapiro*† and P.T. Englund*

*Department of Biological Chemistry and
†Division of Clinical Pharmacology
Department of Medicine
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205, USA

The family Trypanosomatidae includes a number of parasitic haemoflagellates pathogenic to man and animals. Each of these protozoa has a single mitochondrion, which contains a unique type of DNA called kinetoplast DNA (kDNA; see Simpson [1987] and Ryan *et al*. [1988] for reviews on kDNA). This DNA is in the form of a single network of thousands of topologically interlocked DNA circles, a structure unique in nature.

The complex nature of kDNA structure and replication creates an unusually high demand for topoisomerase activity. The topoisomerases are enzymes that can supercoil, relax, catenate, decatenate, knot and unknot circular DNA molecules (Maxwell and Gellert, 1986). Such enzymes undoubtedly function at several steps in the minicircle replication process, probably play an important role in maxicircle replication, and are likely to be involved in the division of double-size networks. In addition to these enzymatic functions, type II topoisomerases in the mitochondria of trypanosomes might have a structural role (analogous to the topoisomerase in the scaffold of mammalian nuclei [Earnshaw *et al*., 1985]), perhaps tethering replicating free circles to the network or constraining the network to one region of the mitochondrion. A type II topoisomerase has been purified from *Crithidia fasciculata*, a related kinetoplastid organism. On the basis of immunofluorescence microscopy, this enzyme localizes to two distinct sites at the periphery of the kDNA network (Melendy *et al*., 1988).

In bacteria and higher eukaryotes, the function and the mechanisms by which topoisomerases act have been greatly clarified by inhibitor studies (Drlica and Franco, 1988). Certain antibacterial agents (Gellert *et al*., 1977) and a variety of intercalative and non-intercalative antitumour agents (Chen *et al*., 1984; Tewey *et al*., 1984a) stabilize a `cleavable complex' between the type II topoisomerase and its DNA substrate; a covalent protein-DNA adduct is trapped upon denaturation of the complex with sodium dodecylsulphate.

VP16-213 TRAPS A MINICIRCLE DNA-PROTEIN COMPLEX

To explore for a drug-sensitive mitochondrial type II topoisomerase in *Trypanosoma equiperdum*, freshly harvested trypanosomes in tissue culture medium

were exposed to VP16-213 (for experimental details see Shapiro *et al*., 1989), a potent and highly specific inhibitor of eukaryotic nuclear type II topoisomerases (Chen *et al*., 1984). The DNA was purified and resolved by agarose-gel electrophoresis. To examine minicircle DNA, a Southern blot was probed with ^{32}P-labelled *T. equiperdum* minicircle DNA. Figure 1a, lane 1, depicts the normal distribution of DNA among the major components of the free minicircle pool (networks do not enter the gel); the predominant free forms are nicked or uniquely gapped circles (II), linearized circles (III) and covalently closed circles (I). VP16-213 perturbed this distribution, causing an increase in the mass of the free minicircle DNA population, most strikingly in the linearized form (Figure 1a, lane 7). These drug-induced linearized minicircles are protein-linked. If proteinase K treatment was omitted, the aqueous phase after phenol extraction contained essentially no linear forms (data not shown). Exonuclease treatment demonstrated that the protein trapped from the mitochondria of VP16-213-treated cells is linked to both 5' ends of the linearized minicircles (data not shown).

SUSCEPTIBILITY TO OTHER TYPE II TOPOISOMERASE INHIBITORS

Live trypanosomes were exposed to various compounds known to affect type II topoisomerases in other systems. These compounds included inhibitors of gyrase (oxolinic and nalidixic acids) (Gellert *et al*., 1977), agents active against eukaryotic type II topoisomerases (the epipodophyllotoxins, 2-methyl-9-hydroxyellipticine, 4'-[9-acridinylamino]-methanesulphon-*m*-anisidine and acriflavine) (Chen *et al*., 1984; Riou *et al*., 1986) and novobiocin (which competes with ATP for binding to both prokaryotic and eukaryotic type II topoisomerases, but does not stabilize cleavable complexes [Hsieh and Brutlag, 1980]). The compounds were tested at concentrations reported to be effective against intact cells in culture or in animals. Like VP16-213 (Figure 1a, lane 7), the other inhibitors of eukaryotic type II topoisomerases promoted minicircle DNA-protein complex formation (Figure 1a, lanes 3 to 6). However, novobiocin or compounds known to generate DNA-protein complexes with gyrase (nalidixic and oxolinic acids) (Figure 1a, lane 2, and data not shown) did not cause significant cleavage of *T. equiperdum* minicircles. Recent studies have shown that some anti-trypanosomal drugs (including Berenil®, Samorin®, ethidium bromide and pentamidine), at concentrations of 10 μM or less, promote minicircle linearization. This suggests that the mitochondrial topoisomerases may be a target for these drugs.

MINICIRCLE CLEAVAGE PATTERNS

The sites of minicircle cleavage were mapped by digesting DNA samples from control or drug-treated cells with *Bgl* II (an enzyme that cuts minicircles once at nucleotide 345; see map, Figure 2). The linearized minicircles naturally

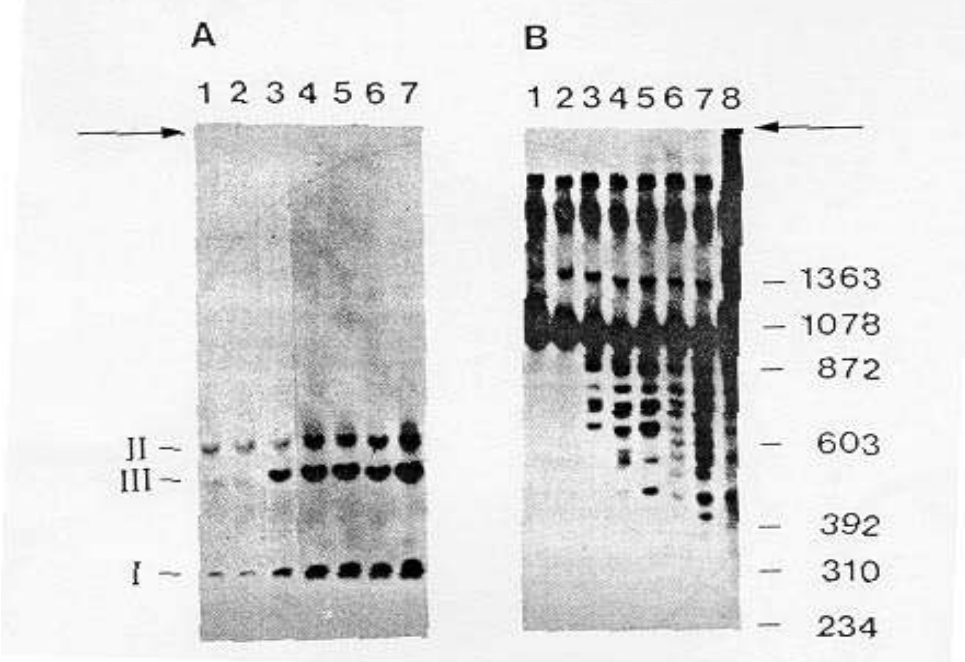

FIGURE 1. Type II topoisomerase inhibitors generate protein-bound linearized minicircles. *Trypanosoma equiperdum* in medium were treated for 30 minutes at 37 °C with drug or with solvent, as indicated. *(Panel a)* The sodium dodecylsulphate lysate was digested with proteinase K, then with RNase A and RNase T_1. After phenol extraction and ethanol precipitation, the DNA from 3×10^7 cells was electrophoresed in a 1.5% agarose gel with buffer containing 1 μg/ml ethidium bromide, transferred to a nylon membrane, and probed with ^{32}P-labelled minicircle sequence. *(Panel b)* An equal quantity of each sample was digested with *Bgl* II before electrophoresis in a 2% agarose gel with buffer containing 1 μg/ml ethidium bromide. I: Covalently closed minicircles, II: minicircles containing nicks or small gaps, III: linearized minicircles. Arrows indicate origin.

Panel a. Lane 1: DNA from control cells, *lane 2:* from cells treated with nalidixic acid (400 μM), *lane 3:* from cells treated with acriflavine (50 μM), *lane 4:* from cells treated with 2-methyl-9-hydroxyellipticine (5 μM), *lane 5:* from cells treated with 4'-(9-acridinylamino)-methanesulphon-*m*-anisidine (6 μM), *lane 6:* from cells treated with VM-26 (100 μM), *lane 7:* from cells treated with VP16-213 (100 μM). Samples from cells treated with dimethyl sulphoxide or with drug solvent were identical to control DNA *(lane 1)*.

Panel b. Lanes 1 to 7: Bgl II digests of DNA as described in *Panel a* (the autoradiograph was exposed for 3 hours). *Lane 8:* same as *lane 1* except autoradiograph was exposed for 16 hours. Size markers are indicated.

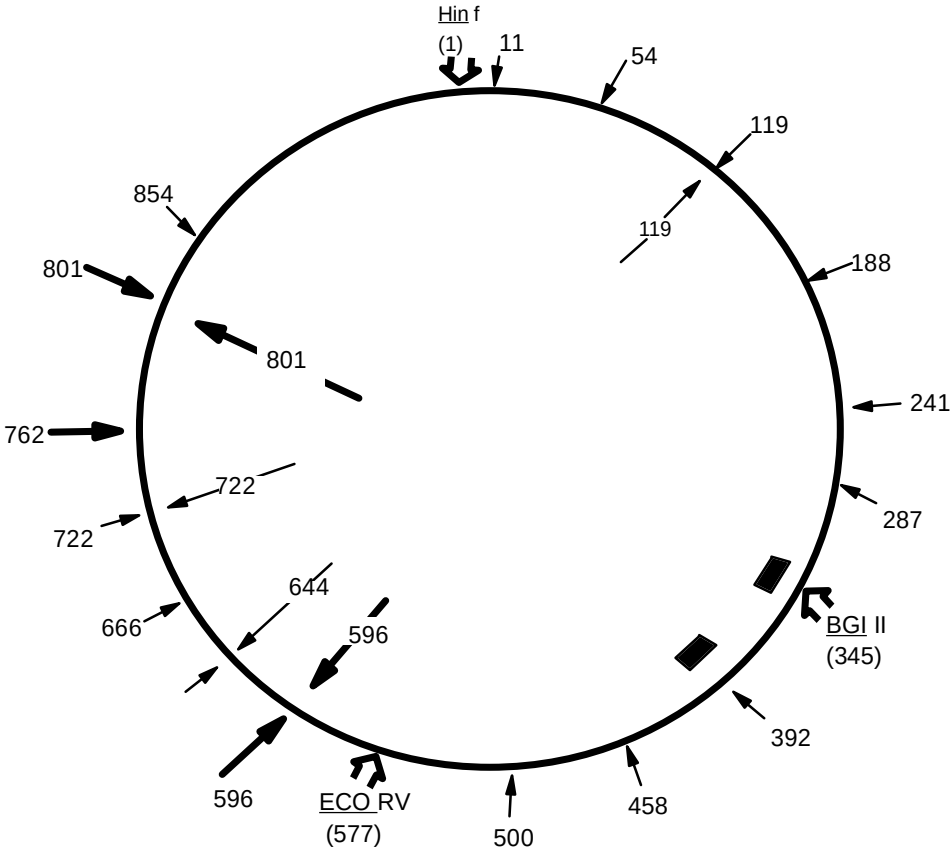

FIGURE 2. Map of cleavage sites induced in *Trypanosoma equiperdum* kinetoplast DNA minicircles by VP16-213. This map is based on the published nucleotide sequence (1012 base pairs); numbering begins at the *Hinf* I site (Barrois *et al.*, 1981). Natural' sites (produced by sodium dodecylsulphate lysis in the absence of drug) are indicated by outward pointing arrows. VP16-213-induced breaks are depicted by inward pointing arrows; heavy arrows represent major cleavage points.

present in samples not treated with drug create a distinct pattern of bands (Figure 1b, lane 8; these bands are not detectable in the shorter exposure shown in lane 1). More complex patterns are generated by drug treatment (Figure 1b, lanes 3 to 7). As anticipated, digests of DNA from cells treated with compounds that do not generate linearized minicircles (nalidixic acid, Figure 1b, lane 2, and longer exposures) display only the natural' cleavage pattern like that in lane 8. As has been reported for other cell types, and with purified nuclear type II topoisomerases *in vitro*, drugs of different chemical classes generate strikingly different cleavage patterns (Tewey *et al.*, 1984b). Interestingly, within the

resolution of agarose-gel electrophoresis, drugs appear to enhance the `natural' cut sites, perhaps by stabilizing type II topoisomerase at these preferred locations. Figure 2 is a map of naturally occurring and VP16-213-induced cleavage sites in *T. equiperdum* minicircles.

CONCLUSIONS

Treatment of intact *T. equiperdum* with compounds known to be active against mammalian type II topoisomerases results in DNA cleavage after sodium dodecylsulphate lysis. Because minicircle DNA is present in the cleaved complex, the type II topoisomerase that has been trapped must be mitochondrial in origin. As is characteristic of the type II topoisomerase-DNA complexes that have been evaluated, the enzyme from *T. equiperdum* mitochondria binds to both 5⌈ ends of the substrate DNA. Gyrase inhibitors, even at concentrations 25-times greater than those effective against *Escherichia coli* (Wolfson and Hooper, 1985), do not promote minicircle linearization or give evidence of type II topoisomerase inhibition. In terms of inhibitor susceptibility, the mitochondrial type II topoisomerase of *T. equiperdum* appears to resemble most closely the type II topoisomerase in the nuclei of higher eukaryotes, rather than gyrase, the type II topoisomerase of prokaryotes.

Interference with mitochondrial type II topoisomerase activity can explain the well-documented effects of some of the anti-trypanosomal drugs on kDNA. For instance, a high proportion of trypanosomes that survive acriflavine treatment become `dyskinetoplastic'. Dyskinetoplastic cells retain mitochondrial membranes (Trager and Rudzinska, 1964), but the characteristic densely staining kDNA disc is absent on light or electron microscopy, and no DNA homologous to minicircle or maxicircle sequences is detectable (Riou and Saucier, 1979). Acriflavine promotes minicircle DNA/type II topoisomerase complexes (Figure 1a, lane 3), and is an inhibitor of isolated topoisomerase catalytic activity (Riou *et al.*, 1986). It therefore seems likely that the genesis of dyskinetoplastic trypanosomes by acriflavine is mediated, at least in part, by inhibition of mitochondrial type II topoisomerase activity. As another example, ethidium bromide and the diamidines interfere at a very early stage, and preferentially, with the structure and replication of kinetoplast, rather than nuclear, DNA (Newton, 1974). The basis for this selectivity may reside in selective stabilization of minicircle DNA/mitochondrial type II topoisomerase complexes.

The type II topoisomerase inhibitors will undoubtedly prove important in understanding the role of type II topoisomerases in trypanosomes and may also provide a much needed new approach to anti-trypanosomal chemotherapy.

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Characterization of chloroquine resistance in *Plasmodium falciparum*

D.J. Krogstad*, I.Y. Gluzman*, T.E. Wellems†, B.L. Herwaldt* and P.H. Schlesinger*

*Washington University Schools of Medicine and Dental Medicine
St. Louis, Missouri 63110, USA

†Laboratory of Parasitic Diseases
National Institutes of Health
Bethesda, Maryland 20892, USA

Chloroquine is an exceedingly useful antimalarial because it is relatively safe for children and pregnant women (Schlesinger *et al.*, 1988). It is effective against all strains of *Plasmodium vivax, P. ovale* and *P. malariae,* and against many strains of *P. falciparum* (Krogstad and Schlesinger, 1987; Schlesinger *et al.*, 1988). However, the value of chloroquine as an antimalarial has been compromised by the increasing prevalence of chloroquine-resistant *P. falciparum.* These strains are now prevalent in South America, Southeast Asia and sub-Saharan Africa (Krogstad *et al.*, 1988a). In this review, we examine the action of chloroquine against susceptible *P. falciparum,* chloroquine resistance in *P. falciparum* and the genetic basis of chloroquine resistance.

CHLOROQUINE ACTION AGAINST SUSCEPTIBLE *P. FALCIPARUM*

Chloroquine is a diprotic weak base that raises the pH of acid intracellular vesicles in mammalian cells, as indicated by its two pK values (8.3 and 10.2) (Roos and Boron, 1981; Krogstad and Schlesinger, 1986a). Although Homewood *et al.* (1972) first postulated that chloroquine might act against plasmodia by raising vesicle pH, it has not been possible to test this hypothesis until recently.

Using hypotonic shock, we labelled unparasitized red cells with fluorescein isothiocyanate linked to dextran, resealed these cells by restoring the osmolality to normal, and infected them with synchronous cultures of *P. falciparum* (Krogstad *et al.*, 1985). The parasites grew at the same rate as synchronous cultures in normal red cells and had the same susceptibility (or resistance) to chloroquine *in vitro.* After incubation with these red cells, parasites containing vesicles labelled with fluorescein isothiocyanate/dextran were examined by spectrofluorimetry. This revealed that the parasite vesicle has a MgATP-dependent proton pump similar to that of mammalian acid vesicles. Spectrofluorimetry has also shown that the concentrations of chloroquine that inhibit parasite growth raise parasite vesicle pH within 3–5 minutes (Krogstad *et al.*, 1985). These results

suggest that chloroquine acts against the parasite by raising intravesicular pH. However, they do not establish whether the ability of chloroquine to raise parasite vesicle pH can be explained entirely by its properties as a diprotic weak base. To address this question, we have performed two types of experiments: measurements of parasite vesicle buffering capacity and measurements of ^{3}H-chloroquine uptake.

The measurements of vesicle buffering capacity indicate that parasite and mammalian acid vesicles buffer a base equally well in the form of NH_4Cl (a typical weak base). However, with chloroquine, the parasite vesicle buffers the base load 600- to 800-fold less well than the mammalian vesicle (Krogstad and Schlesinger, 1986b). Potential explanations for this apparent discrepancy include a chloroquine-concentrating mechanism that permits the parasite vesicle to concentrate more chloroquine than predicted by its properties as a diprotic weak base. We have tested this hypothesis by comparing the uptake of ^{3}H-chloroquine by parasite and mammalian vesicles with that predicted (by the pKs of chloroquine and the pH of the medium and the vesicle interior) (Roos and Boron, 1981; Krogstad and Schlesinger, 1986a). These experiments revealed a 600- to 800-fold greater concentration of chloroquine by the parasite than predicted (Table 1). Taken together, these observations suggest that the parasite vesicle has a chloroquine-concentrating mechanism that is absent from the mammalian vesicle, which accounts for its relative inability to buffer a base load in the form of chloroquine, and thus for the selective action of chloroquine against the parasite (including its low toxicity for the mammalian host). Recent studies performed in our laboratory indicate that subcellular preparations of membrane vesicles obtained from parasitized red cells concentrate chloroquine with an apparent K_m of 21 μM for ATP and with an apparent V_{max} of 2.3 pmols/mg protein/hour. These results indicate that it is possible to study the process of chloroquine accumulation by *P. falciparum* in a cell-free system.

TABLE 1. Intravesicular accumulation of chloroquine (mM)*

Cells	Observed	Predicted
Chloroquine-susceptible *P. falciparum*	68.7	0.014
Chloroquine-resistant *P. falciparum*	3.6	0.014
J774.2 macrophages	0.11	0.14

*Data are based on the moles of ^{3}H-chloroquine accumulated divided by the volume of the intravesicular compartment (Krogstad *et al*., 1988b).

CHLOROQUINE RESISTANCE IN *P. FALCIPARUM*

Studies by Fitch (1970) and others have shown that chloroquine-resistant *P. falciparum* accumulate less chloroquine than susceptible *P. falciparum*. However, the basis for this difference in chloroquine accumulation has been unclear. Our recent studies have shown that the resistant parasite has an efflux mecha-

nism by which it releases chloroquine 40- to 50-fold more rapidly than the susceptible parasite (initial chloroquine efflux half-times of 2 versus 75–150 minutes) (Krogstad *et al.*, 1987, 1988a).

We believe that the process of chloroquine efflux is energy-dependent because it is inhibited by the removal of glucose or by the addition of vanadate (a broad-spectrum inhibitor of ATPases). In the parasite, the process of chloroquine accumulation is also energy-dependent, consistent with a need for ATP to run the MgATP-dependent proton pump that acidifies the parasite vesicle (Krogstad *et al.*, 1985).

The phenomena of chloroquine efflux and resistance in *P. falciparum* are remarkably similar to multidrug resistance (MDR) in mammalian cells (Fojo *et al.*, 1985). Indeed, MDR mammalian cells (Chinese hamster ovary cells) handle chloroquine similarly to the resistant parasite, although the magnitude of the difference in the efflux rate is smaller (3- to 4-fold, rather than 40- to 50-fold). Chloroquine efflux is energy-dependent in both systems and is inhibited by several drugs with calcium channel-blocking activity (verapamil, diltiazem and TMB-8) (Krogstad *et al.*, 1987). Indeed these agents and anti-cancer drugs (vinblastine and daunomycin) can reverse chloroquine resistance in both *P. falciparum* (Martin *et al.*, 1987) and mammalian cells *in vitro*. However, none have yet been shown to be curative for resistant *P. falciparum in vivo* (Bitonti *et al.*, 1989).

THE GENETIC BASIS OF CHLOROQUINE RESISTANCE

The genetic basis of chloroquine resistance in *P. falciparum* is unclear. To address this question, a cross was performed recently between chloroquine-susceptible and chloroquine-resistant clones of *P. falciparum*. Linkage analysis and chromosome mapping were used to analyse the unique progeny derived from this cross, as in previous studies of pyrimethamine resistance (Peterson *et al.*, 1988; Wellems, 1988). These studies revealed no evidence of linkage between *mdr*-like genes in the parasite and chloroquine resistance. Although amplification of *mdr*-like genes in resistant *P. falciparum* has been reported by other investigators (Wilson *et al.*, 1989; Foote *et al.*, 1989), it occurred in both susceptible and resistant clones derived from the aforementioned cross and was absent from four of the eight resistant clones. For this reason, we believe that a rapid diagnostic test to detect chloroquine resistance in *P. falciparum* cannot be based on a *mdr* or *mdr*-like probe at this time. Our preliminary studies with a test based on ^{3}H-chloroquine accumulation suggest that such a test can be performed within 2–3 hours and has a sensitivity to 0.1% parasitaemia (4×10^{3} parasites per μl of blood).

SUMMARY

The action of chloroquine on *P. falciparum* is consistent with its ability to raise parasite vesicle pH. Several other antimalarials that inhibit parasite growth *in*

vitro (quinine and mefloquine) also raise vesicle pH at the same concentrations. Chloroquine resistance results from an efflux mechanism, which is energy-dependent and remarkably similar to multidrug resistance in mammalian cells. Both resistances are inhibited or reversed by several calcium-channel blockers and by anti-cancer agents such as vinblastine.

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The antimalarial mechanisms of chloroquine and qinghaosu: relevance for trypanosomiasis

S.R. Meshnick

City College of New York
138th Street and Convent Avenue
New York, New York 10031, USA

Attempts to develop synthetic antimalarials began with Paul Ehrlich in the late 19th century. Chloroquine was developed during the Second World War and soon became the standard prophylactic and therapeutic agent. Chloroquine-resistant strains of *Plasmodium falciparum*, however, appeared and have now become quite common. This spurred attempts to develop other antimalarial agents, including qinghaosu (artemisinin), isolated from *Artemisia annua*, an ancient Chinese herbal remedy (for review, see Peters, 1987).

We have been studying the mechanisms of action of both qinghaosu and chloroquine. We believe that qinghaosu acts via the generation of activated oxygen, and that chloroquine acts as a DNA intercalating agent. Since African trypanosomes are known to be sensitive to activated oxygen generators and to intercalators, studies on these antimalarials may serve as models for similar studies on anti-trypanosomal drugs.

QINGHAOSU

Activated oxygen species, including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$), are highly toxic substances that may mediate the toxic effects of a variety of pharmacological agents and of leukocytes. Since most aerobic cells normally encounter low levels of activated oxygen, they are equipped with a variety of defensive systems (Fridovich, 1986). These oxidant defences include superoxide dismutase, glutathione peroxidase, catalase (Fridovich, 1986), and in trypanosomes, trypanothione peroxidase (Henderson *et al.*, 1987).

African trypanosomes and plasmodia are both exquisitely sensitive to activated oxygen. A variety of activated oxygen generators such as haemin (Meshnick *et al.*, 1977), haematoporphyrin D (Meshnick *et al.*, 1978a), menadione (Meshnick *et al.*, 1978b) and quinone imines (Grady *et al.*, 1984) have been shown to have anti-trypanosomal activity *in vitro* and/or *in vivo*. Other anti-trypanosomal agents, such as the arsenicals (Fairlamb *et al.*, 1989) and certain *bis*-catechols (Meshnick *et al.*, 1985), may act by inhibiting the oxidant defences of trypanosomes, namely, trypanothione and superoxide dismutase, respectively.

Qinghaosu contains a dioxygen bridge that has been shown to be essential for its antimalarial activity. (The analogue deoxyqinghaosu, which has only a single oxygen bridge, has been shown to be inactive [Brossi *et al.,* 1988]). Earlier work by Krunkai and Yuthavong (1987), showing that free radical scavengers antagonize qinghaosu and that oxidants potentiate it, suggested the participation of activated oxygen in the mechanism of its antimalarial activity.

Our laboratory, in collaboration with laboratories at Oakland Children's Hospital and at Peking Union Medical Center, has accumulated several lines of evidence that qinghaosu is an oxidant antimalarial drug.

First, qinghaosu acts as a classical oxidant in uninfected erythrocytes. Treatment of red cells with qinghaosu, but not deoxyqinghaosu, led to reductions in cell deformability that are characteristic of oxidant effects, and to an increased susceptibility to cumene hydroperoxide. Furthermore, qinghaosu but not deoxyqinghaosu caused reductions in reduced glutathione levels and increases in methaemoglobin levels, which are also characteristic of oxidant effects. These effects were greater in certain oxidant-sensitive red cells such as those containing haemoglobin AE or EE (Scott *et al.,* 1989).

Second, treatment of infected and uninfected erythrocytes with artesunate, a water-soluble derivative of qinghaosu, led to the production of thiobarbituric-acid-reacting substances, which are a characteristic end-product of lipid peroxidation caused by the reaction of activated oxygen and unsaturated fatty acids. More thiobarbituric-acid-reacting substances were produced in red cells infected with plasmodia than in uninfected red cells (Meshnick *et al.,* 1989).

Third, ascorbic acid and reduced glutathione, two well-known antioxidants, antagonized the antimalarial effects of qinghaosu *in vitro* (Meshnick *et al.,* 1989).

CHLOROQUINE

The mechanism of action of chloroquine remains controversial. During the 1970s and 1980s, most studies suggested that chloroquine exerted its antimalarial effect by binding to haemin or by alkalinizing lysosomes (for reviews, see Fitch *et al.,* 1984; Krogstad and Schlesinger, 1987). However, a great deal of data had accumulated in the 1950s and 1960s indicating that chloroquine acts via intercalation into DNA (for review, see Yielding *et al.,* 1971).

The sensitivity of trypanosomes to intercalators is well known. Indeed, the classic intercalator, ethidium bromide, was first developed as an anti-trypanosomal agent. Acriflavine is also an intercalator with well-known anti-trypanosomal activity (for review, see Meshnick, 1989).

Our laboratory has shown that chloroquine binds to DNA in a salt-dependent manner. The K_d varies from 27 μM to 2.6 mM, depending on the salt concentration. Unfortunately, the intranuclear concentration of chloroquine in treated malarial parasites is not known, but assuming it is 10- to 100-times more concentrated than the serum concentration, 0.03–1.0% of intranuclear binding sites may be occupied by chloroquine when a parasite is exposed to physiological concentrations (Kwakye-Berko and Meshnick, 1989).

We have also now shown that binding of chloroquine to DNA may potentially affect DNA function. At concentrations as low as 20 μM, chloroquine prevents the formation of left-handed DNA helices (Z-DNA), a form of DNA believed to be important in gene activation.

CONCLUSIONS

Both plasmodia and trypanosomes are sensitive to the toxic effects of intercalators and activated oxygen generators. Many of the techniques used to study the pharmacology of these drugs in malaria have not yet been applied to the study of anti-trypanosomal drugs. Such studies might aid in the development of new anti-trypanosomal agents.

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Multidrug resistance genes in eukaryotic cells

I.B. Roninson

Department of Genetics
University of Illinois at Chicago
Chicago, Illinois 60612, USA

PROPERTIES OF MULTIDRUG-RESISTANT MAMMALIAN CELL LINES

Selection of mammalian cells for resistance to lipophilic cytotoxic drugs usually results in cross-resistance to many other drugs, which share little structural or functional similarity with the primary selective agent. The resulting cell lines are characterized by resistance to *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, colchicine, taxol, puromycin, actinomycin D, trimetrexate, gramicidin D and some other compounds (Riordan and Ling, 1985). This phenomenon of multidrug resistance (MDR) is of particular significance in cancer chemotherapy, since it affects the response of tumour cells to some of the most commonly used anti-cancer agents. Multidrug resistance is associated with decreased intracellular accumulation of the drugs, due to increased energy-dependent drug efflux from MDR cells (Dano, 1973). In 1976 Juliano and Ling found that several MDR cell lines expressed an increased amount of a 170-kilodalton membrane glycoprotein, termed P-glycoprotein. The presence of P-glycoprotein(s) proved to be the most consistent marker of MDR cells, but the role of this protein in the phenotype of these cells remained unknown before molecular genetic analysis.

IDENTIFICATION OF GENES RESPONSIBLE FOR MULTIDRUG RESISTANCE

In 1984 we found that two MDR Chinese hamster cell lines, independently selected with different drugs, contained commonly amplified DNA sequences (Roninson *et al.*, 1984). These sequences included a transcribed gene, designated *mdr*, whose increased expression is specific to MDR cell lines (Gros *et al.*, 1986c). Two genes related to the hamster *mdr* were detected in human DNA, but only one of these genes, called *mdr*1, was consistently expressed in different MDR human cell lines (Roninson *et al.*, 1986). Gene transfer studies demonstrated that expression of the *mdr*1 gene was sufficient to produce the complete MDR phenotype in drug-sensitive cells (Gros *et al.*, 1986a; Ueda *et al.*, 1987). Furthermore, the phenomenon of preferential resistance to the selective agent,

observed in some MDR cell lines, was shown to result from point mutations in the *mdr*1 gene, at least in some cases (Choi *et al.*, 1988). The second human gene, *mdr*2, does not appear to provide resistance to lipophilic drugs upon transfer into drug-sensitive cells (Van der Bliek *et al.*, 1988). In rodent cells, three *mdr* genes have been detected. Two of these genes appear to correspond to the human gene in their structure and ability to confer multidrug resistance; the third rodent gene corresponds to the human *mdr*2 (Ng *et al.*, 1989).

STRUCTURE AND FUNCTION OF P-GLYCOPROTEINS

In an independent line of investigation, Riordan *et al.* (1985) have isolated cDNA (complementary DNA) clones of hamster P-glycoprotein. P-glycoprotein and *mdr* cDNA clones were found to cross-hybridize, indicating that P-glycoprotein was the product of the *mdr* genes (Ueda *et al.*, 1986). Amino acid sequences of the human and rodent P-glycoproteins were determined by sequence analysis of *mdr* cDNA clones (Chen *et al.*, 1986; Gerlach *et al.*, 1986; Gros *et al.*, 1986b). P-glycoprotein, encoded by the human *mdr*1 gene, is 1280 amino acids long and consists of two homologous halves (Chen *et al.*, 1986). Each half of the protein includes a hydrophobic region with six predicted transmembrane segments and a relatively hydrophilic region, which contains consensus sequences for a nucleotide-binding site. The hydrophilic regions of P-glycoprotein share homology with a group of ATP-binding bacterial proteins, which include energy-coupling subunits of multicomponent periplasmic transport systems for the uptake of various metabolites. The highest levels of homology to P-glycoprotein are found in efflux-associated bacterial proteins *hlyB* and *ndv*A (Higgins *et al.*, 1986; Stanfield *et al.*, 1988). Homology with bacterial transport proteins and the presence of potential channel-forming membrane-spanning segments suggest that P-glycoprotein functions as an active efflux pump, responsible for the removal of drugs from MDR cells. The hypothesized efflux pump function of P-glycoprotein has received indirect confirmation from biochemical studies, in which P-glycoprotein was shown to bind drugs and to hydrolyze ATP *in vitro* (Cornwell *et al.*, 1986; Hamada and Tsuruo, 1988). The role of nucleotide-binding sites in P-glycoprotein function was demonstrated by site-directed mutagenesis of human *mdr*1 cDNA. Both nucleotide-binding sites were found to be required for normal function of P-glycoprotein (B. Morse and I.B. Roninson, unpublished data).

MDR GENE EXPRESSION IN NORMAL AND TUMOUR TISSUES

Analysis of *mdr*/P-glycoprotein expression in normal human tissues revealed relatively high levels of expression in the adrenal cortex, on the lumenal surfaces of the kidney, liver, colon, jejunum and pancreas, in capillary endothelial cells of the brain and testes, and in placental trophoblasts (Thiebaut *et al.*, 1987; Sugawara *et al.*, 1988; Cordon-Cardo *et al.*, 1989). These expression

patterns suggest that P-glycoprotein-mediated membrane transport may be involved in a variety of physiological processes. Such processes might include removal of potentially cytotoxic natural compounds present in the diet, maintenance of blood-tissue barriers, regulation of metabolite exchange between mother and foetus and transport of steroid hormones in the adrenal gland. In clinical samples of human tumours, high P-glycoprotein expression has been observed in many untreated tumours, which are known to be non-responsive to chemotherapy, as well as in treated tumours, where P-glycoprotein expression was often found to increase after treatment (Goldstein *et al.*, 1989).

MDR GENES IN MALARIAL *PLASMODIUM*

Resistance of *Plasmodium falciparum* to chloroquine and other aminoquinolines was found to be associated with increased drug efflux (Krogstad *et al.*, 1987). This efflux was reversible by verapamil, a drug known to reverse multidrug resistance in mammalian cells, suggesting that a similar mechanism may be responsible for chloroquine resistance (Martin *et al.*, 1987). Recently, two *mdr*-like genes were isolated from the genome of *P. falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989). The complete sequence determined for one of these genes showed identical structural organization and strong homology with mammalian P-glycoproteins (Foote *et al.*, 1989). This gene was amplified in two out of five chloroquine-resistant field isolates of *P. falciparum* (Foote *et al.*, 1989) and in one mefloquine-selected strain (Wilson *et al.*, 1989). Gene amplification in these cases was accompanied by increased transcription of *mdr*. It remains to be seen if chloroquine resistance in the non-amplified isolates might be associated with increased transcription or structural changes in the *mdr* genes. Thus, P-glycoprotein-mediated active efflux appears to be a general mechanism for drug resistance in both protozoa and metazoa.

PERSPECTIVES FOR CLINICAL RESEARCH

Development of a molecular diagnostic test for multidrug resistance in cancer, based on detection of *mdr*1 messenger RNA (mRNA) or protein expression, represents the most immediate clinical application of the studies described above. More ambitious and long-term directions of clinical research involve the development of novel therapeutic strategies for prevention or reversal of multidrug resistance in cancer cells, and possibly also of drug resistance in parasites. One actively pursued strategy involves the use of chemical agents that inhibit P-glycoprotein function. Most of the known multidrug-resistance reversing agents appear to function as competitive inhibitors of drug binding and/or transport by P-glycoprotein (Akiyama *et al.*, 1988). Initial clinical trials using P-glycoprotein inhibitors have been reported (Dalton *et al.*, 1989), and the development of improved P-glycoprotein inhibitors is under way. Other approaches involve inhibition of P-glycoprotein function or selective destruction of MDR cells using monoclonal antibodies against P-glycoprotein (Hamada and

Tsuruo, 1986; FitzGerald *et al.,* 1987), and using modified antisense oligonucleotides complementary to *mdr*1 mRNA in an attempt to selectively inhibit *mdr*1 gene expression. Another potential therapeutic approach is based on introducing a constitutively expressed *mdr*1 gene into normal bone marrow cells of a patient prior to chemotherapy, with the aim of reducing bone marrow toxicity of lipophilic chemotherapeutic drugs.

ACKNOWLEDGEMENTS

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Multidrug resistance and gene amplification in cultured *Leishmania:* relevance to current and prospective chemotherapy

S.M. Beverley

Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
250 Longwood Avenue
Boston, Massachusetts 02115, USA

At the Department of Biological Chemistry and Molecular Pharmacology at the Harvard Medical School, we have been investigating the occurrence, biochemical mechanisms, and genetic basis of drug resistance in laboratory stocks of the human parasite *Leishmania major.* We have focused primarily on drugs and their gene targets as model systems for these processes, including resistance to the antifolate methotrexate (MTX). The cellular target of MTX is the enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS), an essential enzyme for nucleotide biosynthesis. MTX constitutes a model drug in the sense that MTX is far more toxic to humans than to *Leishmania* (Peixoto and Beverley, 1987). However, the success in designing anti-DHFR agents in other organisms such as malaria and bacteria and the extensive amino acid sequence divergence between *L. major* and human enzymes (Beverley *et al.,* 1986) suggest that drugs directed against the leishmanial DHFR will be feasible (Sirawaraporn *et al.,* 1988). Our studies of MTX resistance lead to the identification of the amplified DHFR-TS structural gene, showing that, somewhat paradoxically, studies of resistance can lead towards improved chemotherapy (even when clinical resistance is not a problem).

There are at least three mechanisms by which resistance to MTX occurs in *Leishmania* (reviewed by Beverley *et al.,* 1988):

- amplification of the DHFR-TS gene, encoded on a segment of DNA termed the R region
- decreased cellular MTX accumulation
- amplification of a region of DNA termed the H region, which is completely distinct from the R region

We have shown that these three mutations can occur independently or in combination in the same cell, indicating that the parasite has diverse mechanisms it can marshall to survive chemotherapeutic attack (Ellenberger and Beverley, 1987, 1989). Amplified DNA such as R or H occurs generally as extrachromosomal circular DNA, which can be readily detected due to a high copy number or unusual mobility properties in conventional or pulsed-field electrophoresis gels (reviewed by Beverley *et al.,* 1988). Gene amplification has provided a convenient method for directly identifying and then working

with a resistance locus as the absence of methods for genetic manipulations of trypanosomatid parasites has proven a formidable problem until quite recently.

MULTIDRUG RESISTANCE AND THE H-REGION AMPLIFICATION

The H region was first observed in cells that also exhibited DHFR-TS gene amplification, and we were uncertain whether this DNA could independently mediate MTX resistance or was a non-functional segment of DNA that had merely been non-specifically co-amplified (Beverley *et al.*, 1984). However, recent data from several other laboratories as well as our own indicate that this DNA indeed mediates drug resistance.

The first clue emerged when it was shown that certain unselected laboratory stocks of the lizard parasite *L. tarentolae* contained amplified DNA (Petrillo-Peixoto and Beverley, 1986, 1988; White *et al.*, 1988), which proved to be the H-region homologue of this species similarly amplified as an extrachromosomal circular DNA. Not all lines of *L. tarentolae* carried this amplification, but all that did had a resistance to MTX that was twentyfold greater than that of the other lines. This indicated that H-region amplification could confer MTX resistance. However, we were puzzled why a lizard parasite had become resistant to MTX. Because MTX is not often used other than in cancer chemotherapy, it seemed unlikely that *L. tarentolae* would ever have been exposed to this agent.

Concurrent with these studies our laboratory had been selecting *L. major* for resistance to numerous drugs, and two of these had developed gene amplification that turned out to be amplification of the H region (Ellenberger and Beverley, 1989). The three drugs that resulted in H amplification—MTX, primaquine (PQ, an 8-aminoquinoline whose target is unknown in *Leishmania)* and terbinafine (TB, an allylamine inhibitor of squalene epoxidase)—shared no obvious structural or pharmacological similarity. When a variety of lines were tested, it was found that all lines containing H-region amplification were highly cross-resistant to MTX and weakly cross-resistant to PQ and TB.

This observation of multidrug resistance suggested to us that the *L. tarentolae* had actually developed H-region amplification in response to an agent other than MTX. This situation seemed similar to the emerging story of multidrug resistance in tumour cell lines, in that overexpression of the mammalian multidrug resistance gene (often due to amplification) had been shown to lead to resistance to a spectrum of drugs, with no obvious structural or functional similarity, by causing decreased drug accumulation (reviewed by Gottesman and Pastan, 1988).

Accordingly, we characterized MTX and folate transport in *L. major* promastigotes and then asked whether H-region amplification mediated decreased MTX uptake. The answer was clearly negative because the H-region amplified PQ- and TB-resistant lines were completely normal in MTX influx, accumulation and efflux properties (Ellenberger and Beverley, 1989). Verapamil, an agent that reverses multidrug resistance associated with gene amplification, had

no effect on the MTX resistance of the H-amplified lines. We went on to show that the PQ- and TB-resistant lines did not exhibit increased levels of MTX metabolism, due to increases in the activity of a MTX hydrolase activity present in wild-type lines or other metabolic alterations (Ellenberger *et al.*, 1989). Current data suggest that the form of multidrug resistance mediated by the H region is novel and distinct from that seen in other systems.

The story at this point took an unexpected twist. Lines of *L. mexicana* selected for resistance to arsenite also contained an amplification of the H region; correspondingly, this line was cross-resistant to MTX (Katakura and Chang, 1989; Detke *et al.*, 1989). We then showed that the H-amplified *L. major* lines described above were cross-resistant to arsenite as well (Ellenberger and Beverley, 1989), thereby implicating the H-region amplification in resistance to an astonishingly diverse group of compounds. Since arsenite has certain chemical similarities with trivalent antimonials, we investigated whether the H-region amplification could be involved in mediating resistance to these compounds. Figure 1 shows the results of preliminary studies of the primaquine-resistant PQ-R30 line, which bears high levels of H-region amplification; this line is significantly resistant to potassium antimony tartrate. Berman (1982) showed that clinical isolates resistant to trivalent antimonials were also resistant to pentavalent antimonials. It will be interesting to test whether H-region amplification mediates resistance to pentavalent antimonials, especially in the amastigote stage, and whether isolates clinically unresponsive to antimonials show H-region amplification.

IMPLICATIONS FOR CHEMOTHERAPY

The potential association of H-region amplification and antimonial resistance is simultaneously a disturbing and exciting finding. Since natural isolates of *L. tarentolae* can exhibit H-region amplification, it is possible that natural isolates of human leishmaniasis also possess this amplification. Moreover, our studies show that *Leishmania* possesses a remarkable ability to amplify the H region locus *de novo* in response to drug pressure. At a broader level, the overall ability of the parasites to develop resistance by a plethora of mechanisms to every compound we have tested suggests that concerns over drug resistance will be as much a part of the development of a practical anti-leishmanial chemotherapy as they are of malaria control today.

Our findings are exciting in that very little is known about either the true cellular target(s) of antimonials or the mechanisms of antimonial resistance. Analysis of the large collection of H-region amplifications in different species has allowed us to genetically identify this DNA as a resistance locus. Characterization of the molecules and mechanism of resistance encoded by the H region should prove informative to questions concerning both resistance and drug targets. As genetic methods emerge and become more powerful in *Leishmania*, we should be able to extend these studies to the far larger class of drug-resistance mutations not mediated by gene amplification.

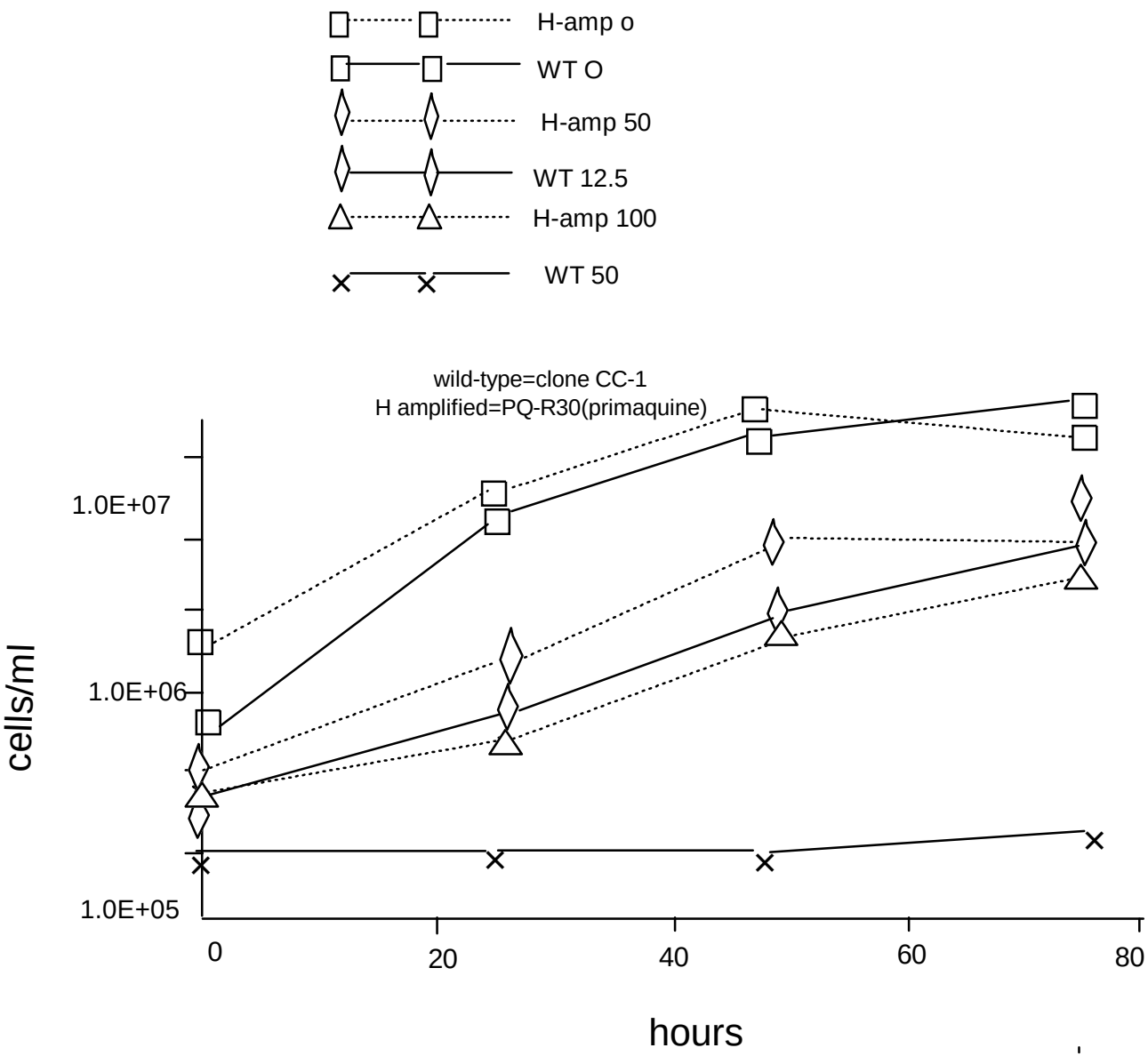

FIGURE 1. Effect of potassium antimony tartrate on wild-type *Leishmania major* and on lines whose H region has been amplified.

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Pyrimethamine resistance in *Plasmodium falciparum*

J. Inselburg, M. Tanaka, H. Gu, D. Bzik and W.B. Li

Department of Microbiology
Dartmouth Medical School
Hanover, New Hampshire 03756, USA

Pyrimethamine binds to and inhibits the dihydrofolate reductase activity of the essential, bifunctional *Plasmodium falciparum* enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Ferone *et al.,* 1969; Garrett *et al.,* 1984). We have been studying pyrimethamine-resistant (Pyr^R) parasite mutants with the intention of understanding the mechanism(s) by which resistance is achieved and to find ways to design a more effective antimalarial. To do this, we have isolated and examined Pyr^R mutants (Banyal and Inselburg, 1986; Inselburg *et al.,* 1987), and have also determined the nucleotide sequence of the *P. falciparum* DHFR-TS gene (Bzik *et al.,* 1987). We and others have further shown that specific amino acid changes, which arise from DHFR gene mutations, are found in *P. falciparum* Pyr^R mutants (Cowman *et al.,* 1988; Inselburg *et al.,* 1988; Peterson *et al.,* 1988). In the study reported here, we further examined the nucleotide changes associated with pyrimethamine resistance and found that parasites with such alterations often contained demonstrable changes in the electrophoretic migration of the chromosome that carries the DHFR-TS gene.

MATERIALS AND METHODS

P. falciparum parasites Honduras 1 (Pyr^R), FCR3 (pyrimethamine sensitive [Pyr^S]) and FCR3 Pyr^R mutants D4, D5, D6, D7 and D8 were studied (Inselburg, 1983; Banyal and Inselburg, 1986).

Polymerase chain reaction. Oligonucleotides for primers were derived from the DHFR-TS sequence of Bzik *et al.* (1987). The location of primers are indicated in Figure 1. The specific primer sequences are presented elsewhere (Gu *et al.,* submitted for publication). The genomic DNA was amplified in a thermocycler (Ericomp, USA) using *Thermus aquaticus* polymerase (Perkin-Elmer Cetus, USA) according to the manufacturers instructions.

DNA sequencing. Sequencing of amplified DNA was by the dideoxy-termination method using the modified T7 DNA polymerase, Sequenase (U.S. Biochemicals), as described by Higuchi *et al.* (1988).

Pulsed-field gel electrophoresis. P. falciparum chromosomes were prepared in agarose blocks containing approximately 3×10^8 parasites per ml, as described elsewhere (Gu *et al.,* submitted for publication), and separated using

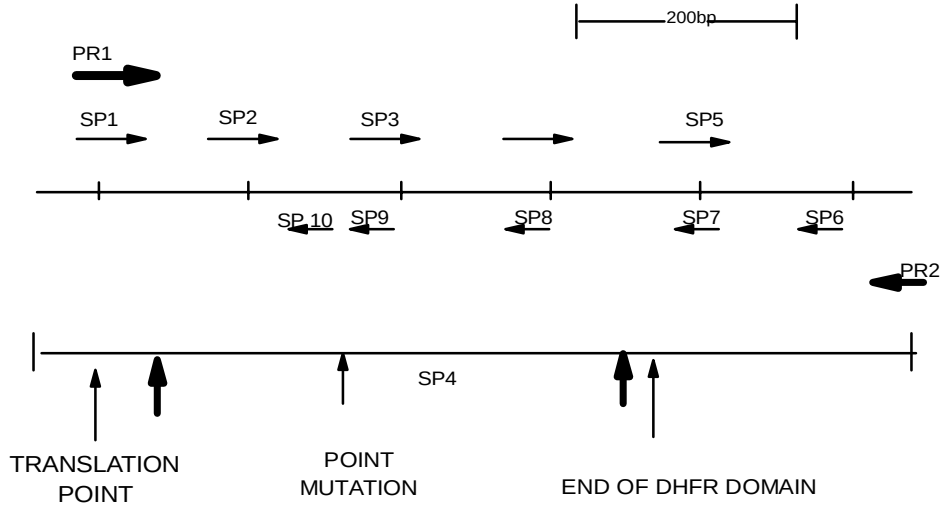

FIGURE 1. The location of polymerase chain reaction primers used to amplify and sequence the *Plasmodium falciparum* DHFR-TS gene. The location and direction of primers are shown. The base pair locations of point mutations are shown (by heavy vertical arrows) that encode amino acids 16, 54 and 223. PR are primers used to amplify the total region. SP are primers used to amplify and sequence the DNA.

contour-clamped homogeneous electric-field electrophoresis (Chu *et al.*, 1986; Gu *et al.*, submitted for publication). A pulse time of 11 minutes, an electric field strength of 4 V/cm and a total running time of 149 hours were used for chromosome separations in this report. The gels were stained with ethidium bromide (0.5 μg/ml).

Southern blotting and probing of chromosomes. Separated chromosomes were transferred to Zeta probe membrane (BioRad, USA) using the Southern blot transfer method after sequentially soaking the gel in 0.25 M HCl at room temperature for 30 minutes and in 0.4 M NaOH at 4°C overnight. The transfer was done using 0.4 M NaOH. The blotted DNA was probed with appropriate ^{32}P-labelled DHFR-TS DNA. The ^{32}P-labelled probes were labelled by the random priming method described by Feinburg and Vogelstein (1983).

RESULTS

We have examined the DHFR sequences in the Pyr^{R} Honduras 1 strain, in several isolates of the original FCR3 strain and in five Pyr^{R} mutants of FCR3: FCR3 clones D4, D5, D6, D7 and D8 (Banyal and Inselburg, 1986). FCR3 clones D4, D5, D6 and D7 were cloned, at intervals, from a culture to which progressively higher concentrations of pyrimethamine were added. FCR3-D8 was isolated as a clone after treating the culture from which FCR3-D7 was isolated with the mutagen N-methyl-N-nitro-N⌈-nitrosoguanidine and then

TABLE 1. The locations of amino acid and nucleotide changes in the mutant dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene

	Amino acid number of DHFR					
Parasite	16	51	54	59	108	223
FCR3	GTA*(Val)	AAT(Asn)	GAT(Asp)	TGT(Cys)	ACC(Thr)	TTT(Phe)
Honduras 1	GCA(Ala)	AAT(Asn)	GAT(Asp)	TGT(Cys)	AAC(Asn)	TTT(Phe)
FCR3-D4-D8	GTA(Val)	AAT(Asn)	GAT(Asp)	TGT(Cys)	ACC(Thr)	TCT(Ser)
FCR3-D8†	GTA(Val)	AAT(Asn)	AAT(Asn)	TGT(Cys)	ACC(Thr)	TCT(Ser)

*Nucleotides encoding amino acid (Bzik *et al*., 1987).

†These sequence data are the same as those reported previously (Inselburg *et al*., 1988). However, FCR3-D8 cells grown in the absence of pyrimethamine have been found eventually to lose the mutation affecting amino acid #54.

growing the survivors in a culture to which had been added a higher level of drug. The results of the sequence analyses of the parasites are shown in Table 1. Only those nucleotides and amino acids that are different from the FCR3 sequence are shown.

The original isolate of FCR3-D8, which was obtained from two DNA clones, is also shown (Inselburg *et al.*, 1988). It differed from FCR3 at amino acids #54 and #223. The recent DNA sequencing of FCR3-D4, D5, D6, D7 and D8, using DNA amplified by the polymerase chain reaction method, indicated that each mutant differed from FCR3 at only amino acid #223. The originally sequenced FCR3-D8 clones were resequenced using the polymerase chain reaction amplification method and again showed the original two amino acid differences. We concluded that the extended *in vitro* cultivation of FCR3-D8 cells without pyrimethamine led to the selection of the mutant without change at amino acid #54. The mutation at amino acid #54 had been reported to reduce DHFR activity in mouse cells by several hundredfold (Howell *et al.*, 1986). We have found that the new FCR3-D8 culture, unlike the original isolate (Banyal and Inselburg, 1986), had a detectable level of DHFR activity consistent with there having been a reversion at amino acid #54.

We previously showed that FCR3-D7 contained a gene duplication and overproduced a DHFR-TS enzyme that had a lower specific activity (Inselburg *et al.*, 1987). Our sequence analysis indicated that the amino acid sequence change in the FCR3-D7 DHFR sequence cannot explain its higher level of resistance than that of FCR3-D4, D5 or D6 and therefore must be related to some other change(s).

The Honduras 1 DHFR differed from that of FCR3 at two amino acids: #16 and #108.

We have examined the chromosomes of FCR3, FCR3-D4, D5, D6, D7 and D8 and Honduras 1. The wild type DHFR-TS gene is on chromosome #4. We found detectable chromosome changes in only the smaller chromosomes of some of the Pyr^R FCR3 and therefore have shown only those chromosome separations. It was noted that there was no longer a structure the size of the FCR3 chromosome

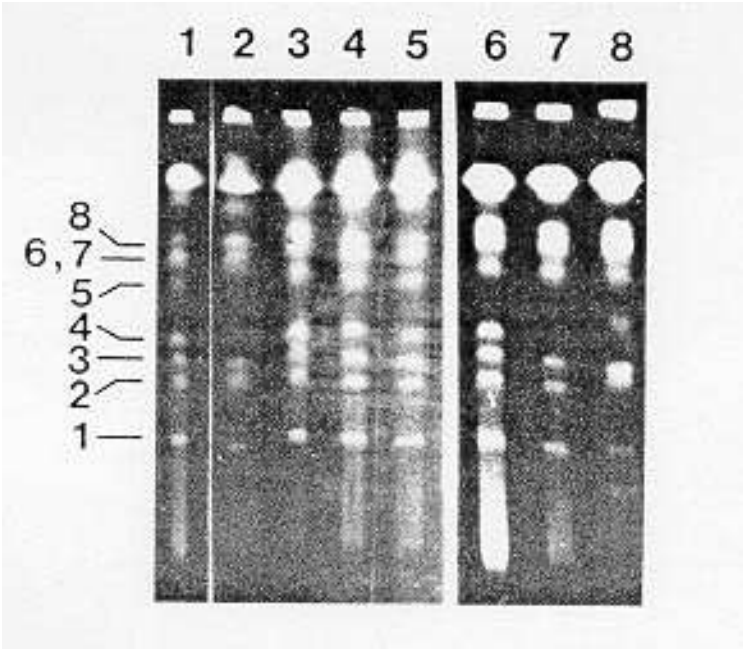

FIGURE 2. Contour-clamped homogeneous electric-field electrophoresis separation of the small- and intermediate-size chromosomes of *Plasmodium falciparum*. The chromosomes were from the following cultures. Lane 1: FCR3, lane 2: Honduras 1, lane 3: FCR3-D4, lane 4: FCR3-D5, lane 5: FCR3-D6, lane 6: FCR3, lane 7: FCR3-D7, lane 8: FCR3-D8. Chromosome numbering of some FCR3 chromosomes are on the left of the figure. The smallest FCR3 chromosome is #1. Chromosomes separated in lanes 1–5 were from one gel and in lanes 6–8 from a second gel.

#4 in FCR3-D7 and FCR3-D8 (Figure 2). In FCR3-D8, a second small chromosome was also seen. We then used a ^{32}P-labelled, cloned DHFR DNA (Bzik *et al.*, 1987) to probe the chromosomes of FCR3, FCR3-D7 and FCR3-D8 (Figure 3). The DHFR-TS gene in FCR3-D7 was in a chromosome that was larger than chromosome #4, while in FCR3-D8 the gene was in two chromosomes, one much smaller and one slightly larger than chromosome #4. Honduras 1 was missing structures the size of chromosome #4, #5 and #10. The higher levels of drug resistance shown by FCR3-D7 and D8, which have the same primary DHFR structure as FCR3-D4, D5 and D6, and the resistance of Honduras 1, therefore appears to be associated with changes in chromosome #4.

DISCUSSION

We and others have now shown that a number of *P. falciparum* DHFR mutants exhibit the Pyr^R phenotype. It has been suggested that a change of amino acid #108 was essential for the expression of pyrimethamine resistance (Peterson *et*

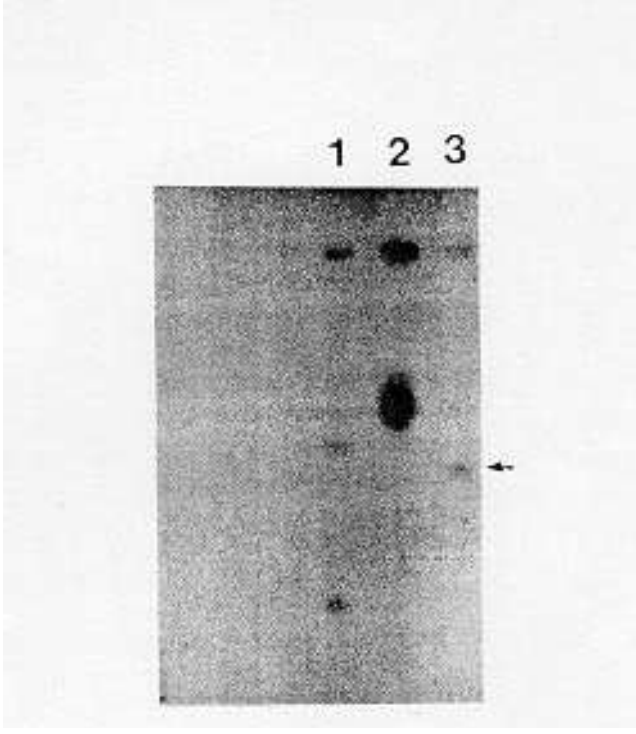

FIGURE 3. Hybridization of ^{32}P-DHFR DNA to *Plasmodium falciparum* chromosomes in Southern blots. The blotted chromosomes were derived from the following cultures. Lane 1: FCR3-D8, lane 2: FCR3-D7, lane 3: FCR3. FCR3 chromosome #4 is marked on the right.

al., 1988). The Pyr^R mutants we have described do not contain such a change. We also found that in three Pyr^R mutants containing identical DHFR point mutations, an electrophoretically detectable change had occurred in chromosome #4, the chromosome on which the wild type DHFR-TS gene resides. We suggest that chromosome-size changes that contribute to either gene duplications or possible gene regulatory processes probably play an important role in the development of higher levels of pyrimethamine resistance in the parasite. In FCR3-D7 the duplicated genetic material resides on one electrophoretically separable chromosome. In FCR3-D8, DHFR coding sequences reside on two chromosomes. However, we must confirm that the FCR3-D8 culture was a pure cloned population since we previously found that the DNA content of FCR3 and FCR3-D8 were about the same, while the enzyme level of FCR3-D8 was measurably higher. The study of Pyr^R mutants may provide an important approach to studying the formation of chromosome-size polymorphisms in malaria.

We also noted that maintaining a pyrimethamine-resistant culture in the absence of drug (FCR3-D8) led to the selection of a parasite population with a diminished level of resistance. Similarly, some drug-resistance genotypes in bacteria are unstably maintained. Thus, control of pyrimethamine usage may eliminate a selective drug pressure that is required to maintain populations of

some highly resistant organisms and may therefore restore its effectiveness in some areas where it is no longer effective in the treatment of malaria.

Finally, the demonstrated affinity of pyrimethamine for DHFR has been the basis for explaining the mechanism of resistance. In preliminary studies that examined *in vivo* ^{3}H-pyrimethamine binding to the DHFR-TS protein, we found that the drug also becomes associated with other parasite and/or red blood cell proteins. It may prove worthwhile to consider the ability of other parasite proteins to titrate the drug as another mechanism of pyrimethamine resistance.

ACKNOWLEDGEMENT

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NEW DRUG-DELIVERY SYSTEM

Protein conjugates as trypanocide delivery systems

J.B. Mitchell

School of Biology and Tropical Diseases
Chemotherapy Research Unit
Sunderland Polytechnic
Sunderland SR1 3SD, UK

Attachment of a drug to a macromolecular carrier system results in substantial changes in the mechanism of its interactions at the cellular level. Although a drug with a low molecular weight may enter cells by simple diffusion, the same drug attached to a macromolecular carrier, such as a protein, enters the cell only by endocytosis. Because endocytosis can be a cell-specific mechanism, this provides possibilities for targeting drugs to cells that will endocytose the carrier. In the design of such drug-carrier systems, it is important that the linkage is stable in plasma but is susceptible to hydrolysis in lysosomes of the target cells or organisms.

The work discussed in this paper relates to the antitumour antibiotic, daunorubicin. This diffuses into mammalian cells (Peterson *et al.*, 1980), whereas its macromolecular conjugates are endocytosed by tumour cells and lysed by lysosomal enzymes to release the drug within the cell (Trouet *et al.*, 1982).

The trypanocidal activity of daunorubicin is greater *in vitro* than that of any other drug (Williamson and Scott-Finnigan, 1975), yet *in vivo* it is completely inactive (Williamson and Scott-Finnigan, 1978). To investigate this unexpected difference, Brown *et al.* (1982) examined the distribution of daunorubicin and its metabolites in infected mice. They found that drug accumulates in trypanosomes and in the cellular components of the blood. However, as its concentration in the plasma declines, so also does its concentration in the trypanosomes. They also showed, by fluorescence microscopy, that daunorubicin reaches the nucleus of the trypanosomes *in vivo* but its concentration in this site declines rapidly, indicating that adequate concentrations of drug may not be maintained long enough in the organisms for the trypanocidal effect to be achieved (Brown *et al.*, 1982).

In attempting to prolong the exposure of trypanosomes to daunorubicin *in vivo*, Williamson *et al.* (1981) investigated the effects of coupling it to macromolecules, including the proteins bovine serum albumin (BSA) and ferritin. It seemed likely that these would act as true carriers because they would be expected to be endocytosed by trypanosomes (Brown *et al.*, 1965; Langreth and Balber, 1975; Fairlamb and Bowman, 1977, 1980a, 1980b). Indeed, Fairlamb and Bowman (1980a) have suggested that suramin, though administered as the free drug, forms a complex with plasma albumin before being taken up by

endocytosis from the flagellar pocket and subsequently released in secondary lysosomes.

Williamson *et al.* (1981) prepared two different daunorubicin-BSA complexes. One, containing stable secondary amine bonds, was inactive; the other, with a labile glutaraldehyde-produced covalent linkage (D-BSAG), was active *in vitro* and retained its activity *in vivo*. It was, indeed, the first daunorubicin preparation to clear trypanosomes from the blood of infected mice for prolonged periods. (Daunorubicin linked by the same method [Hurwitz *et al.*, 1975] to ferritin was also trypanocidal both *in vitro* and *in vivo* [Williamson *et al.*, 1981]). Daunorubicin has also been covalently linked to BSA by hydrazide and oxime linkages, which are far more labile than the glutaraldehyde-produced linkages, and both these conjugates are inactive against trypanosomes *in vivo* (Hardman *et al.*, 1983). This supports the hypothesis that the drug is not active while it remains coupled to its carrier. It must first be released from the conjugate before its trypanocidal activity is exhibited. However, if it is too readily released, presumably in the plasma, it will equally fail to exert its effects.

Our investigations into the distribution of D-BSAG in *Trypanosoma brucei rhodesiense* from infected mice provide information on the mode of action of the conjugate *in vivo*.

After infected mice were given an intraperitoneal dose equivalent to 25 mg/kg daunorubicin, the subcellular distribution of free and bound daunorubicin in trypanosomes was determined (Brown *et al.*, 1981; Golightly *et al.*, 1983). The nucleus contained 85% of the unconjugated drug whereas the small particulate (lysosome) fraction contained 75% of the drug-protein conjugate. (Daunorubicin administered as free drug localizes, and is active, principally in the nucleus [Brown *et al.*, 1982; Williamson *et al.*, 1983]). We have also demonstrated that D-BSAG can be cleaved by enzymes contained in lysosome-rich fractions of trypanosomes obtained from Percoll density gradients. So it appears that daunorubicin can be released from the conjugate in lysosomes and, once released, can reach its intracellular targets where it may exert its effects.

In a series of tests we have compared the action of two covalently bound conjugates of daunorubicin and BSA, D-BSAG (dialysed for several weeks to ensure the removal of any free daunorubicin) and D-BSAS (a stable succinyl-linked conjugate) (Arnold *et al.*, 1983). When we looked at the effects on the ultrastructure of trypanosomes of exposure to various concentrations of free daunorubicin and the two conjugates for 4 hours *in vitro* (Golightly *et al.*, 1986), we found that: (1) segregation of the nucleolus caused by 1×10^{-7} M daunorubicin requires 1×10^{-6} M D-BSAG; (2) complete nucleolar fragmentation results from 1×10^{-6} M free drug but requires 5×10^{-6} M D-BSAG; and (3) D-BSAS produces no detectable changes.

When uptake of these same compounds was studied *in vitro* using fluorescence microscopy (Golightly *et al.*, 1985), D-BSAG was observed to be taken up by the trypanosomes in a similar way to free drug but to a lesser extent and more slowly. Fluorescence in the nucleus, kinetoplast and lysosomes was observed in D-BSAG-treated trypanosomes after 3.5 hours, while free drug produced the same effect in only 15 minutes. (However, the conjugate was marginally more effective at inducing loss of infectivity *in vitro* [Golightly *et*

al., 1988]). No fluorescence at all occurred in trypanosomes exposed to D-BSAS, indicating that this stable linked conjugate does not enter the organisms. It is not surprising, therefore, that this compound failed to affect the ultrastructure of the trypanosomes. *In vivo* doses of 10–30 mg/kg D-BSAG cleared trypanosomes from the bloodstream in 24–48 hours, but after a further 36 hours parasites reappeared in the bloodstream and multiplied normally (Golightly *et al.*, 1988). Repeated dosing following initial clearance failed to clear parasites in the relapsed infection. The high toxicity of daunorubicin is a major problem of the use of this drug, and its toxicity is not diminished by binding to BSA or by using multiple dosing regimes.

Multiple dosing of protein-bound drugs could lead to the development of hypersensitivity and this may influence the clinical applicability of proteins as carriers in drug-delivery systems. A `single shot' cure would be the ideal, but if multiple doses are required, it may be necessary to have a series of complexes involving the same drug bound to a number of different proteins.

We are now attempting to exploit the fact that cells of the immune system have the ability to endocytose foreign proteins and are using this to target protein-bound drugs to the amastigotes of *Leishmania*, which develop in macrophages. We have tested several drugs against *L. donovani* in infected mice and obtained the following ED_{50} figures: pentostam 65 mg/kg, daunorubicin 23.7 mg/kg and D-BSA 11.25 mg/kg. These results give a strong indication that there is a future for the use of protein conjugates as drug-delivery systems.

SUMMARY

Attachment of the drug daunorubicin to the protein bovine serum albumin by a labile (glutaraldehyde) linkage (D-BSAG) confers *in vivo* trypanocidal activity to a drug otherwise trypanocidal only *in vitro*. Experimental evidence supports a lysosomotropic mode of action: free drug can be cleaved from D-BSAG by isolated lysosomal enzymes of trypanosomes; and in trypanosomes from infected mice treated with D-BSAG, the drug-carrier complex is recovered largely from lysosomes while freed drug is in the nucleus. If the bonds between daunorubicin and the protein are either stable or too labile, the complex is not trypanocidal. *In vitro*, trypanosomes are damaged less by D-BSAG than by free drug. Against *L. donovani in vivo* the protein-bound form is the more effective, indicating that better targeting has been achieved. Protein binding does not diminish the toxicity of the drug *in vivo*.

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Drug delivery by immunoliposomes

B.T. Rouse*, S.J. Kennell† and L. Huang‡

*Department of Microbiology
University of Tennessee
Knoxville, Tennessee 73996, USA

†Biology Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37830, USA

‡Department of Biochemistry
University of Tennessee
Knoxville, Tennessee 37996, USA

Contemporary chemotherapy requires efficient means of delivering drugs to target tissues so as to minimize toxicity. Drug targeting can either be achieved by coupling a tissue-specific ligand to the drug, or by using microreservoirs that are themselves targeted. Intelligent homing devices represent the ideal targeting strategy, but at present one must arrange for close encounters to occur so that binding may result. Currently, targeting drug-containing microreservoirs with antibody represents the most promising strategy, and our approach to this issue is to use liposomes for the purpose. Such antibody-targeted liposomes are referred to as immunoliposomes. Numerous methods can be used to prepare immunoliposomes (Wright and Huang, in press), but the method we developed and favour uses the *N*-hydroxysuccinimide ester of palmitic acid to acylate immunoglobulin (Ig) (Huang *et al.*, 1980). The resulting hydrophobic antibody intercalates into the bilayers of liposomes. The achievement of optimal coupling stoichiometry must be ascertained for each antibody and type of liposome, but typically antibodies retain 20–30% of their binding activity and can be incorporated with high efficiency and stability into liposomes (Huang *et al.*, 1982).

Immunoliposomes for drug delivery can be prepared that exhibit a range of biological properties. To be effective in drug delivery, immunoliposomes must not only bind to target structures, but they or their contents must also be internalized and may need to be delivered to certain locations within the cell (Wright and Huang, in press). The latter activity is best achieved by controlled-release immunoliposomes, particularly those designed to disrupt in the presence of particular microenvironments. The value of acid-sensitive controlled-release liposomes for drug delivery as well as their potential for gene therapy was recently reviewed (Collins and Huang, 1988).

Drug delivery *in vivo* with immunoliposomes has many practical problems to overcome, of which avoiding uptake by the reticuloendothelial system (RES) and the constraints of anatomical compartmentalization are the most significant (Collins and Huang, 1988; Wright and Huang, in press). Methods to overcome

both problems are rapidly being developed (Allen and Chonn, 1987). Certain locations in the body are less constraining, such as the surface of the eye (Norley *et al.*, 1986) and the lung (Hughes *et al.*, 1989). The latter site represents a potential target for immunoliposomes placed in the venous circulation since such vehicles contact the lung before they are subject to entrapment by the RES. We have developed a monoclonal antibody to an antigen gp112 (Kennel *et al.*, 1988) found on murine lung capillary endothelial cells that can be acylated to make immunoliposomes that bind very effectively to the lung (Table 1). This

TABLE 1. Biodistribution of ^{125}I-labelled anti-gp112 labelled liposomes*

Organ†	Time		
	15 minutes	24 hours	1 week
Lung % per g	670	640	583
ratio‡	27.9	27.4	24.9
Liver % per g	9.4	4.3	4.5
ratio	0.4	0.2	0.3
Spleen % per g	14	13	10
ratio	0.5	0.5	0.7

*Data modified from Hughes *et al*. (1989).

†Values are the fraction of the percent of the recovered radioactivity in an organ per gram of wet weight of tissue. Values are averages of 3 animals per time point. Mice received tail vein injections of ^{125}I-labelled anti-gp112 or ^{125}I-labelled rat IgG_{2b} of no known specificity immunoliposomes.

‡Ratio of organ binding by anti-gp112 immunoliposomes to those not specifically targeted.

TABLE 2. Time course of liposome biodistribution*

Tissue	Target	% injected dose (minutes)					
		1	3	5	10	15	30
Lung	anti-gp112	37	34	34	32	31	28
	nonspecific	6	3	2	1	1	1
Liver	anti-gp112	21	24	24	24	20	17
	nonspecific	26	35	37	32	28	22
Blood	anti-gp112	12	8	7	5	4	4
	nonspecific	6	4	3	2	2	1

*Groups of mice were injected intravenously with a 200 µl inoculum of N-[3-(3-[^{125}I] iodo-4-hydroxy-benzyl)-propionyl] dipalmitoylphosphatidylethanolamine-labelled liposomes.

binding occurs extremely rapidly (Table 2) and liposome components can be found in the lung seven days after their administration. Specificity of the reaction was shown in a number of ways. Accordingly, immunoliposomes targeted with Ig with no anti-lung activity failed to bind to the lung (Table 1) and the effect could also be blocked by pretreating animals with anti-gp112 ascites fluid but not by pretreating them with other antibodies (Hughes *et al.*, 1989). Rather surprisingly, lung binding occurred within one minute after administration (Table 2). We also suspect that specific binding occurs only during the first few passes of immunoliposomes through the lung. Liposomes that do not achieve rapid binding are assumed to be entrapped by RES cells in various organs.

As mentioned above, binding to target structures by immunoliposomes is a necessary, but not in itself sufficient, requirement for effective drug delivery. Conditions must be such that the entrapped drug will be released to effect therapeutic activity. To assess this possibility, we entrapped the anti-herpes virus drug acycloguanosine in immunoliposomes and determined if the formulation was any more effective at controlling a respiratory infection with herpes simplex virus than control drug alone or drug administered within untargeted liposomes. The results of two experiments attest to the superior efficiency of the drug-containing immunoliposome formulation (Table 3). Currently, we are optimizing conditions to achieve maximum therapeutic efficiency as well as attempting to endow liposomes with stealth characteristics so that their uptake by the RES is minimized and their circulatory time prolonged.

TABLE 3. Virus recovery from lungs after intranasal infection with herpes simplex virus*

Vehicle	Drug	Mean virus titre ($\times 10^3$) Experiment 1	Experiment 2
Anti-gp112 liposome	No drug	3600	340
Anti-gp112 liposome	ACV†	2	5
Liposome	ACV	570	19
No vehicle	ACV	160	93
No vehicle	No drug	18,000	140

*Groups of five three-week-old mice each were used. Vehicles were given at times 0, 12, 24, 36 and 48 hours. Infections were administered at 14 hours and titrations were made at 60 hours.

†ACV: Acycloguanosine concentrations were equal for each mouse.

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The use of viral membrane fusion proteins in drug delivery

S.J. Doxsey

Department of Biochemistry and Biophysics
University of California
San Francisco, California 94143, USA

A major obstacle in delivering molecules into cells is the barrier imposed by the hydrophobic plasma membrane. The membrane is not freely permeable to ions, solutes, proteins or nucleic acid molecules. In spite of this, enveloped viruses have evolved an elegant strategy for transferring their membrane-encapsulated genome into the cytosol of host cells. They accomplish this by inducing fusion between viral and cellular membranes. The virus thus serves as a `transport vesicle' for delivery of its contents into its host cell.

DELIVERY SYSTEMS IN NATURE: ENTRY OF ENVELOPED VIRUSES INTO CELLS

After binding to the surface of their host cells, many viruses are taken up by the cell. The process of internalization is a constitutive cellular function: receptor-mediated endocytosis via coated pits and coated vesicles (Brown *et al.,* 1983). The internalization pathway of Semliki Forest virus, which serves as a general paradigm for viruses with endocytic entry pathways (Helenius *et al.,* 1980; Marsh and Helenius, 1989), is shown in Figure 1. Once inside the cell, the virus is delivered to an acid compartment, the endosome (Helenius *et al.,* 1983; Marsh and Helenius, 1989). Exposure to the acidic environment triggers the viral-mediated fusion of endosomal and viral membranes. The fusion event results in the release of the viral genome into the cytoplasm and begins the infection process.

In contrast to viruses that require low pH to fuse with target cells are those that display pH-independent fusion activity. These viruses can penetrate cells by fusing with the plasma membrane (White *et al.,* 1983; White, in press). Regardless of pH dependence, the fusion of enveloped viruses with target cells represents an effective mechanism for introducing the viral contents into the cytosol.

VIRAL MEMBRANE FUSION PROTEINS

The membrane fusion activity of the enveloped viruses is a property of `fusion proteins' on their surfaces (Marsh and Helenius, 1989; White, in press). Some

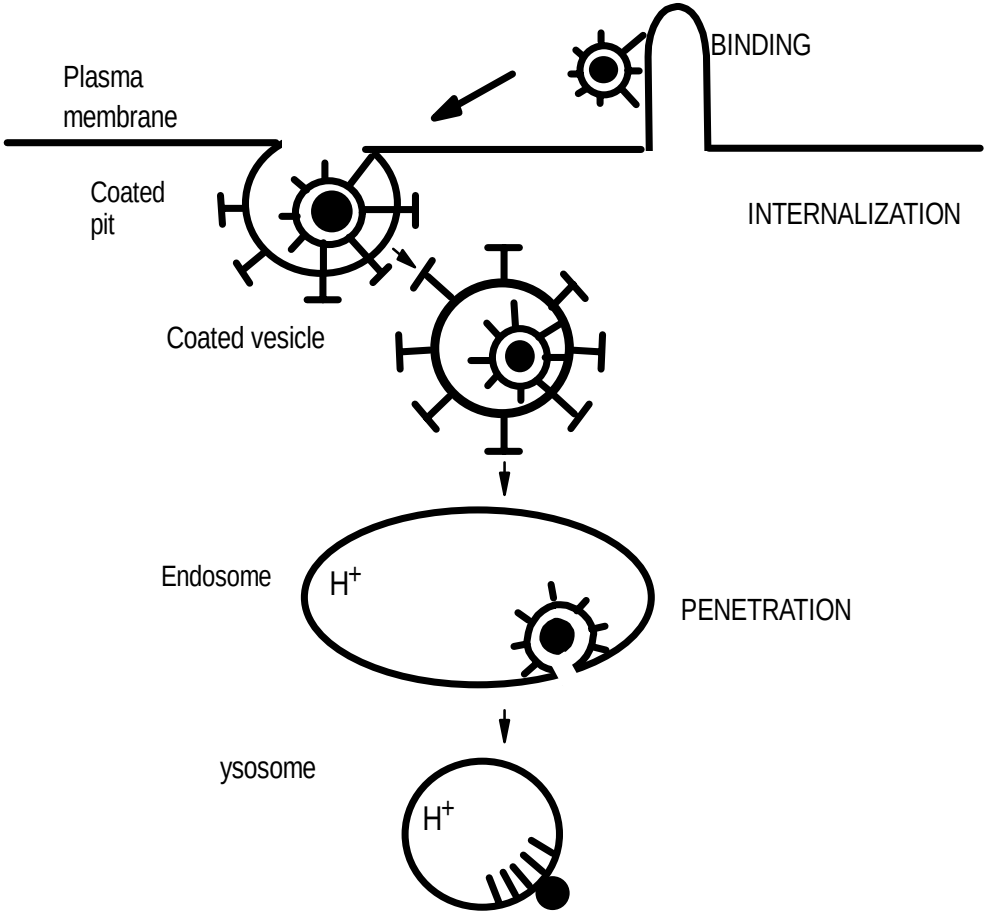

FIGURE 1. Uptake of Semliki Forest virus by cultured cells. The virus binds to the cell surface and moves laterally to coated pits. The virus is internalized by coated vesicles and transported to endosomes. The low pH of the endosome triggers the viral-mediated fusion of membranes, resulting in the delivery of the viral genome into the cytoplasm.

fusion proteins and their properties are listed in Table 1. They are generally integral membrane glycoproteins present as oligomers in the viral membrane. Multiple copies of these spike-like glycoproteins project from the membrane of the virion, the external spike comprising more than 85% of the protein mass. In many cases, proteolytic processing is required to produce the fusion-competent form of the molecule (Marsh and Helenius, 1989; White, in press). The best characterized spike glycoprotein to date, the haemagglutinin (HA) molecule of influenza virus, is schematically depicted in Figure 2.

The mechanism by which the viral fusion proteins promote membrane fusion is currently a topic of great interest. Recent evidence suggests that more than one molecule is necessary for fusion in the case of the influenza HA (Doms *et al.*, in press; Stegmann *et al.*, 1989; Ellens *et al.*, submitted for publication). In

TABLE 1. Properties of some viral fusion proteins

pH Dependence	Virus	Fusion Protein	Hydrophobic fusion sequence	Reconstituted into vesicles*
Low pH:				
5.1–5.8	Influenza	HA (HA1-HA2)	N-terminus of HA2	Yes
< 6.2	SFV	ENV (E1-E2-E3)	Internal E1	No
< 6.2	VSV	G	Not Apparent	Yes
Neutral pH:				
	Sendai	F (F1-F2)	Not Apparent	Yes
	HIV	ENV (gp120-gp41)	N-terminus gp41	No

SFV: Semliki Forest virus, VSV: Vesicular Stomatits virus, HIV: Human Immunodeficiency virus.
*Reconstitution of protein with fusion activity under physiological conditions.

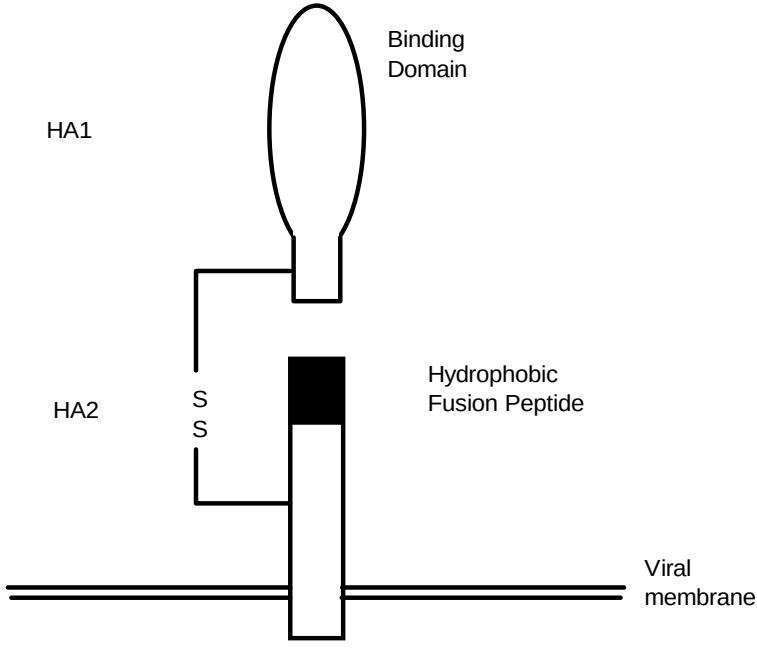

FIGURE 2. Schematic representation of the influenza haemagglutinin molecule. The molecule is present in the viral envelope as a trimer; a monomer is depicted here for simplification. Haemagglutinin is composed of two disulphide-linked polypeptide chains, HA1 and HA2. The binding site is at the tip of the globular head domain. The hydrophobic N-terminus of HA2 is thought to be responsible for mediating fusion with target membranes.

the ectodomains of many fusion proteins, there is a stretch of several apolar amino acids that seem to be involved in membrane fusion and have thus been termed `fusion peptides' (White, in press). A mechanism has been proposed for haemagglutinin-mediated membrane fusion whereby the `fusion peptide' is inserted into the target membrane, perturbing the lipid bilayer and leading to fusion of the two membranes (Harter *et al.*, 1988; Marsh and Helenius, 1989; Stegmann *et al.*, 1989). This and other hypotheses are being tested (White, in press).

ARTIFICIAL DELIVERY SYSTEMS BASED ON VIRAL FUSION PROTEINS

Soon after the discovery of the fusogenic proteins of viruses, attempts were made to harness the fusion activity for artificial delivery of drugs and other molecules into cells (Doxsey *et al.*, 1985; Celis, 1986; Schlegel and Rechsteiner, 1986; Helenius *et al.*, 1987). Three general delivery strategies developed to exploit the viral fusion proteins are shown in Figure 3 (Helenius *et al.*, 1987). The fusion protein can be expressed on the target cell (Figure 3a) or reconstituted into artificial lipid vesicles (virosomes, Figure 3c). Alternatively, intact virions can be used as fusogens to `bridge' the delivery vesicles and target cell (Figure 3b).

To achieve efficient binding and increase the probability of fusion, the appropriate receptors must be present in high concentrations on the target membrane. Once bound, the delivery vesicle can be fused with the cell either by brief acid treatment (low pH-dependent fusion proteins) or by prolonged incubation at neutral pH (pH-independent fusion proteins). One advantage of using acid pH-triggered fusion proteins is that molecules are delivered rapidly and simultaneously into the cells. Each of the three strategies has limitations and none has emerged as an accepted routine method for efficient bulk delivery. However, they all have potential for specific applications.

Virus particles as fusogens

Some success has been achieved using intact viruses (usually pH-independent) as `bridges' to fuse delivery vesicles with cells (Celis, 1986; Schlegel and Rechsteiner, 1986). Since a separate fusogen is used, potentially any number of target cells and delivery vesicles could be employed *in vitro*. However, our experience with acid-dependent viruses (Semliki Forest virus, influenza) has been discouraging owing to the low efficiency of delivery when compared to the HA-expressing target cell system (see below).

Target cells expressing the fusion protein

We have developed a method for the cytoplasmic delivery of molecules that uses target cells expressing the influenza HA molecule and erythrocytes as vesicular carriers (Doxsey *et al.*, 1985; Ellens *et al.*, 1989). Efficient surface

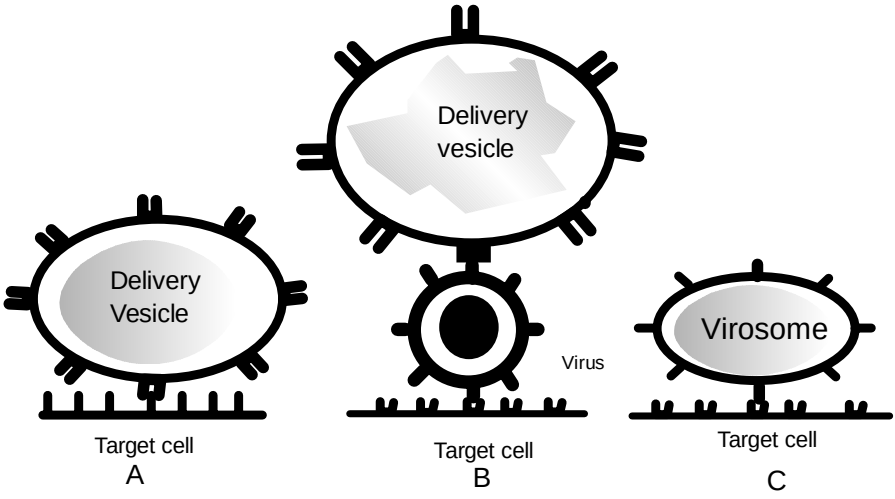

FIGURE 3. Three strategies that exploit viral fusion proteins for delivery of molecules into cells. *(a)* Target cells expressing the viral fusion protein bind delivery vesicles containing receptors. *(b)* Intact viruses act as bridges' to fuse delivery vesicles to target cells. The viral fusion protein binds receptors on both target cell and delivery vesicle. *(c)* Fusion proteins reconstituted into vesicles (virosomes) containing the molecules to be delivered bind to target cells containing receptors.

expression of the cloned HA molecule has been achieved by viral infection or by establishing permanent cell lines (Sambrook *et al.*, 1985; Whiley and Skehel, 1987) that have been optimized for efficient fusion (Ellens *et al.*, 1989).

Perhaps the single most important aspect of this technique is its high efficiency (Doxsey *et al.*, 1985). We routinely deliver molecules to 80–95% of the cells in a monolayer. Up to 200 million copies of some proteins have been introduced into each cell. In addition, delivery is easily controlled, highly reproducible and not deleterious to the recipient cells. Using this approach, a variety of macromolecules have been delivered using both erythrocytes (Doxsey *et al.*, 1985, 1987) and liposomes (Ellens *et al.*, 1989; Glenn *et al.*, in preparation) as vesicle carriers.

The utility of the technique for investigating biological problems has been demonstrated in several studies. We showed that delivery of antibodies to the clathrin heavy chain inhibited receptor-mediated endocytosis, thus providing evidence for a direct role for clathrin in endocytosis (Doxsey *et al.*, 1987). Delivery of erythrocyte-encapsulated oligonucleotides complementary to HA RNA (antisense RNA [see J.J. Toulmé, this volume]) were shown to inhibit HA biosynthesis by 60% (S. Froshauer, personal communication).

Liposomes have recently been used to introduce biologically active RNA into cells (Ellens *et al.*, 1989; Glenn *et al.*, in preparation). Following delivery of messenger RNA encoding an enzyme, target cells expressed 10^4 molecules per

cell. If an RNA amplification vector was used (Xiong *et al.*, 1989), expression levels were increased by two orders of magnitude. Efficient delivery of nucleic acids into cells should prove useful for expressing foreign proteins, for delivering antisense RNAs, and for examining the fate and effect of the delivered oligonucleotides. Finally, a similar approach has been used to implant lipids into defined domains of the plasma membrane to study the properties of the tight junction (Vanmeer *et al.*, 1985, 1986).

The results using HA-expressing cells demonstrate the effective use of viral fusion proteins in the efficient delivery of macromolecules into cells and the utility of this strategy in studying a wide range of biological problems. The technique, however, is limited to the use of cells expressing high levels of fusogenic proteins.

Lipid vesicles containing viral fusion proteins

Viral fusion proteins reconstituted into lipid vesicles (virosomes) have the potential to serve as universal carriers for delivery of molecules into any cell containing the appropriate receptor. Recent advances in solubilization and reconstitution methodologies have facilitated the development of virosomes that have fusion properties comparable to those of the intact virions (Metsikko *et al.*, 1986; Sechoy *et al.*, 1986; Stegmann *et al.*, 1987) (see Table 1).

A potential limiting factor in this strategy is that virosomes made by these reconstitution procedures are small (70–130 nm in diameter), so the number of molecules that can be entrapped and subsequently delivered is few. This may not be a problem if the delivered molecule is effective at low copy number (Eiklid *et al.*, 1980) or if the effective concentration of a protein can be increased by delivering RNAs capable of self replication (Xiong *et al.*, 1989). One advantage of the small virosomes is that they can be internalized by endocytosis and are capable of acid-dependent fusion with internal membranes (Stegmann *et al.*, 1987). Targeting to specific cell types using antibody-bearing liposomes has met with some success (Loyter *et al.*, 1986; B.T. Rouse, this volume) and could be applied to virosome technology. These and other results (Celis, 1986; Loyter *et al.*, 1986) suggest that this approach is feasible and has potential for the future.

POTENTIAL USE OF VIRUSES IN THE CHEMOTHERAPY OF TRYPANOSOMES

Although the use of viruses to deliver toxins or drugs to trypanosomes *in vivo* is far from a reality, there is potential for such a strategy starting perhaps with cells in culture. Trypanosomes have been shown to internalize ligands by receptor-mediated endocytosis (Coppens *et al.*, 1987; Webster and Grab, 1988) and thus possess the pathway for virus entry (see above). Virosomes could be targeted to molecules on the parasite cell surface that are invariant among different strains of trypanosomes and that are internalized along the endocytic pathway. Molecules that seem to fulfil these criteria have been found in the

flagellar pocket of trypanosomes, such as the receptors for transferrin and low-density lipoprotein (Coppens *et al.*, 1987, 1988; Webster and Grab, 1988). If access through the narrow mouth of the flagellar pocket is a problem (Coppens *et al.*, 1988), it may be possible to use pH-independent virosomes or to fuse acid-triggered virosomes at the cell surface by brief acidification of the extracellular medium *in vitro*. This approach could prove useful for studying cell biological problems of trypanosomes and for drug testing.

Since viruses are natural shuttle vectors for transferring their genome into host cells, they have been exploited as vectors for introducing foreign genes into cells (Nichols, 1988; Varmus, 1988). This technology could be applied to the targeting of toxic molecules to trypanosomes by engineering vectors that contain genes encoding such molecules (Nichols, 1988; Varmus, 1988; Xiong *et al.*, 1989). Furthermore, vectors could be engineered to contain nucleic acids that possess trypanosome-specific replication or splicing sites so they would be replication-competent only in trypanosomes. Vectors have recently been designed that do not produce infective particles, thus confining molecules to the targeted cells (Varmus, 1988). Although the success of such an approach is faced with the same difficulties mentioned above and others (Nichols, 1988; Varmus, 1988), it has potential.

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Antisense oligonucleotide analogues as therapeutic agents

J. Goodchild*, S. Agrawal*, P. Zamecnik* and P. Sarin†

*Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts 01545, USA

†Laboratory for Tumor Virology
National Cancer Institute
Bethesda, Maryland 20892, USA

The site of action for most drugs is a macromolecule, usually a protein but sometimes a nucleic acid. Our ability to design drugs from first principles is limited by a poor ability to predict the interaction of macromolecules with other compounds. A striking exception is the interaction between nucleic acids by Watson-Crick base pairing which is the best understood and most predictable mechanism for molecular recognition and binding in biology.

The first demonstration that this property might be a basis to predict therapeutically active compounds used an oligonucleotide complementary to Rous sarcoma virus RNA to inhibit its replication (Zamecnik and Stephenson, 1978). Messenger RNA (mRNA) can be inactivated similarly to limit gene expression. Although subsequent work on the therapeutic uses of complementary or `antisense' oligonucleotides has concentrated on viruses, the approach should be applicable to other types of infectious agents and to other clinical conditions where inhibiting the expression of a particular protein might be beneficial. As the sequence of the antisense oligonucleotides follows from that of the target RNA, this approach goes a long way towards rational drug design and, providing the target sequence is unique, should be highly specific.

There are now sufficient examples in the literature to be sure that `hybridization arrest' does work in many model situations, but so far there are no published examples of its successful use in animals and it is not clear whether the present compounds have sufficient potency for therapeutic application. The object of the present paper is to consider the factors that determine the activity of antisense oligonucleotides and how they may be modified to increase their therapeutic potential. Reference to the original literature may be obtained in two recent reviews (Stein and Cohen, 1988; Goodchild, in press).

Oligonucleotides may be grouped into three categories according to their mechanism of action.

Passive oligonucleotides are conceptually the simplest but mechanistically may be the most diverse and the least understood. When hybridized to strategic regions of a messenger or viral RNA, they are presumed to block the action of ribosomes, spliceosomes and various enzymes or other factors that read or interact with the RNA. In mRNA, the capped end and AUG initiator codon are

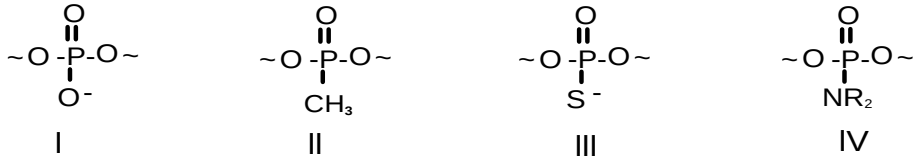

FIGURE 1. Internucleoside phosphate modifications used in antisense oligonucleotides. I: unmodified phosphodiester, II: methylphosphonate, III: phosphorothioate, IV: phosphoramidates.

generally the most effective sites. For reasons that will be discussed later, the phosphate groups are often modified as shown in Figure 1.

Reactive oligonucleotides carry a group such as an alkylating agent or free radical generator to cross-link or cleave the RNA irreversibly (Knorre and Vlassov, 1985). Little work has been reported on hybridization arrest with these derivatives. They may have advantage if activity is limited by dissociation of the RNA/oligonucleotide hybrid but are likely to be more toxic. The binding site on RNA is less critical with this category and the next than with the first.

Activating oligonucleotides also lead to irreversible damage to the target RNA but do so by activating endogenous host cell factors such as ribonuclease H that cleaves the RNA strand of RNA/oligodeoxynucleotide complexes. This approach is limited in applicability by the requirement for the appropriate ribonuclease but it has been claimed that this is widespread. An advantage of this mechanism is that the oligomer is not degraded by ribonuclease H and so catalyses' the cleavage of more than one equivalent of RNA whereas the chemically reactive oligonucleotides behave stoichiometrically. Ribonuclease H will accept phosphorothioate groups in the deoxy strand but not methylphosphonates and so the possibilities for modification are more limited in this case.

The activity of an oligonucleotide should be governed largely by its ability to form the desired hybrid in the target cell. This will depend on chemical and biological properties of the oligomer as well as the nature and accessibility of the target sequence.

Factors known to promote hybridization also increase antisense activity. These include increasing the length of the oligonucleotide, increasing the G–C content, attaching intercalating groups to shorter oligonucleotides or performing the assay at lower temperatures. *In vitro,* preannealing to the target RNA is generally not necessary for oligomers of chain length 15–20, which is encouraging for their use *in vivo.* Secondary structure within the target RNA can sometimes inhibit binding but usually only in the case of very stable structures or poorly binding oligomers.

The oligonucleotide must be long enough to differentiate between RNA molecules but the chance of forming undesired complexes will also increase with length. A reasonable compromise is 12–20 nucleotides. Activity generally increases with length over this range, but to minimize the mass and cost of material for a therapeutic dose, the chain length should be as short as possible.

Oligonucleotides probably enter cells by both pinocytosis and a cell surface protein-mediated mechanism. In culture, the intracellular concentration typically rises to about 10% of that in the medium. Degradation appears to be mainly by exonucleases and can take from less than 10 minutes to over 24 hours, depending on the cell. In attempts to increase cellular uptake, molecules are made more lipophilic with substituents that are either added to the ends or positioned so as to remove the negative charge from the internucleoside phosphates, such as the methylphosphonates introduced by Miller and Ts'o. These and other modifications to the internucleoside phosphates (shown in Figure 1) also increase resistance to degradation by nucleases. All of these phosphate modifications considerably increased activity against human immunodeficiency virus to give complete inhibition of replication at concentrations around 3 μM (Agrawal *et al.*, 1988; Sarin *et al.*, 1988). These derivatives are about as active in molar terms as AZT but have much larger molecular weights and so may require larger masses for therapeutic doses.

Of importance for any therapeutic potential are the questions of toxicity and bioavailability. Even unmodified oligomers were found to be fairly stable at 37⌈C in whole blood from humans or rabbits. There was no sign of degradation in 2 hours and substantial amounts of starting material were left after 24 hours. After intravenous injection of labelled material to rabbits, clearance from the blood was rapid and label was found distributed fairly evenly in the organs where it was apparently degraded quite rapidly. After 2 hours, about 20% of the oligomer was found in the urine still largely intact.

In mice, unmodified oligonucleotides as well as all those modified as shown in Figure 1 were found to have an LD_{50} of about 160 mg/kg body weight. Rats were more resistant.

There are probably three major problems that must be overcome for these compounds to be used clinically.

- Activity must be sufficient that the required mass of these high molecular weight compounds does not become excessive. It remains to be seen whether any of the compounds under investigation are sufficiently active or whether it will be necessary to develop further generations.
- Present solid support synthetic methods are rapid and highly efficient but the cost of these compounds is high due to the cost of starting materials. Currently, the cost for in-house syntheses on the gram scale is estimated at about US$4 per mg but this has been forecast to fall to US$0.04 over the next 5 years by a company involved in their production.
- Renal excretion is very fast. It was recently reported by J. Walder at a meeting that this could be decreased considerably by covalent attachment of cholesterol.

It is encouraging for therapeutic considerations that several diverse chemical modifications have resulted in increased activity that may make it possible to tailor these agents to the pharmaceutical requirements. For the goal of rational drug design, a mechanism of molecular recognition as predictable as Watson-Crick base pairing is certainly an appealing starting point.

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Antisense oligodeoxynucleotides as anti-parasitic agents

J.J. Toulmé*, P. Verspieren*, N.T. Thuong†, N. Loreau*, C. Cazenave* and C. Boiziau*

*Laboratoire de Biophysique
INSERM U201
Muséum National d'Histoire Naturelle
43 rue Cuvier
F-75005, Paris, France

†Centre de Biophysique Moléculaire
CNRS
F-45071 Orléans cédex, France

INTRODUCTION TO HYBRID-ARRESTED TRANSLATION

The formation of nucleic acid/nucleic acid complexes, based on the formation of double-stranded structures through hydrogen bonds between complementary nucleic acid bases, is highly specific. The association of a messenger RNA (mRNA) with a complementary DNA strand can block its translation. This was first used for gene identification (Paterson *et al.,* 1977). It was also demonstrated that some prokaryotic genes were regulated by a similar mechanism: transcripts complementary to part of a mRNA (so-called antisense RNA) formed duplexes that prevented the synthesis of the encoded protein (Simons, 1988). The expression of numerous genes was then specifically turned off by artificial antisense RNAs equally well in prokaryotic and eukaryotic cells (Green *et al.,* 1986).

Initially, it was proposed that antisense RNA (DNA) could act as a block to ribosome scanning or to ribosomal subunit binding. Subsequently, it was reported that only antisense molecules targeted to the 5' region of the mRNA (from the cap to the AUG initiation codon) were able to inhibit translation, owing to an unwinding activity associated with elongating ribosomes (Melton, 1985; Shakin and Liebhaber, 1986). However, in a few cases, antisenses complementary to the region downstream of the AUG induced specific inhibition of protein synthesis concomitant with a decrease of the target mRNA concentration. It was demonstrated that the regulation of the CII gene of bacteriophage λ by the antisense OOP RNA was due to the specific degradation of the RNA/RNA hybrid by *Escherichia coli* RNase-III (Krinke and Wulff, 1987). In wheat germ extracts and in injected *Xenopus* oocytes, RNase-H, an RNase that cleaves the RNA part of RNA/DNA hybrids, was shown to be

TABLE 1. Criteria and chemical modifications for the design of synthetic antisense oligonucleotides.

Problems	Possible solutions
Specificity (Target recognition)	Length
Affinity (Competition with ribosomes, . . .)	Modified bases Modified backbone Intercalating agents Active groups
Stability (Degradation by nucleases)	Phosphotriesters Alkylphosphonates a-oligome rs Phosphorothioates Phosphorodithioates
RNase-H induction (mRNA cleavage)	Phosphodie ster Phosphorothioate
Uptake (Membrane penetration, endocytosis)	Length/Charges Hydrophobic groups Polycations (poly-Lysine) Lipoproteins Liposomes
Toxicity (Non-specific binding, mutagenicity, carcinogenicity)	

responsible for the degradation of the target mRNA when antisense DNA was used (Cazenave *et al.*, 1987).

ANTISENSE OLIGODEOXYNUCLEOTIDES

Synthetic oligonucleotides (oligos) are now easily available. Antisense oligos were successfully used in cell-free extracts, in micro-injected cells and in cultured cells (Toulmé and Hélène, 1988). However, their sensitivity to DNases and their limited uptake by intact cells led to the development of chemically modified analogues. A number of criteria should be fulfilled to tailor an antisense oligo (see Table 1).

The specificity of the recognition of the mRNA target is due mainly to the length of the oligo: in a human cell, a single site will be hit by an oligomer 11–15 nucleotides long, depending on the sequence. The higher the affinity of the antisense oligomer for the complementary mRNA, the greater the efficiency of competition with the translational machinery. Lengthening the oligo results in increased affinity; however, this also reduces the uptake and can affect the specificity. Alternatively, removal of negative charges in the phosphate backbone or linkage to groups that will provide additional energy of interaction

results in derivatives of higher affinity. An example of such molecules is provided by oligomers linked to intercalating agents (see below). A number of chemical modifications have been proposed to overcome the sensitivity of the natural oligomers to nuclease attack (see Table 1). Unfortunately, some of these modifications led to inactive antisense oligomers: α-oligomers did not prevent polypeptide chain elongation, although they did bind to the target sequence with good affinity, because α-DNA/RNA hybrids are not recognized as substrates by RNase-H.

One way to increase the efficiency of the antisense molecules is to promote their uptake by intact cells in order to increase their intracellular concentration. Neutral derivatives are taken up more efficiently than negatively charged molecules (see J. Goodchild *et al.*, this volume). In contrast, phosphorothioate oligomers are trapped in the plasma membrane and penetrate cells very slowly (Boiziau *et al.*, unpublished results). Linking oligos to a polycation led to compounds that exhibited anti-viral properties in the nanomolar range (Lemaitre *et al.*, 1987). Liposome encapsulation also led to improved uptake (Loke *et al.*, 1988). Toxicity should be considered: most of the oligos exhibit sequence-independent inhibition at high concentrations. This is well documented for phosphorothioate (Cazenave *et al.*, 1989), acridine-linked oligos (Cazenave *et al.*, 1987) and poly-(L)-lysine conjugates (Lemaitre *et al.*, 1987). However, toxic doses are at least 100-fold higher than that required for specific inhibition. Cytotoxic concentrations depend on the oligomer length and sequence. With a view to therapeutic use, unmodified and phosphorothioate 20-mers were injected into mice: no effect was observed for up to 14 days at a dose of 40 mg/kg (Agrawal *et al.*, 1988; Sarin *et al.*, 1988).

THE MINI-EXON SEQUENCE: A TARGET FOR ANTISENSE OLIGONUCLEOTIDES

In trypanosomes and related parasites, gene expression involves discontinuous transcription followed by *trans*-splicing of a so-called mini-exon-derived RNA onto the major exon, giving rise to a sequence, thirty-five nucleotides long in *Trypanosoma brucei*, common to all mRNAs (Borst, 1986). Targeting this region with antisense oligos resulted in decreased, or even abolished, *in vitro* translation of *T. brucei* mRNA (Cornelissen *et al.*, 1986; Walder *et al.*, 1986).

Oligonucleotides linked to intercalating agents

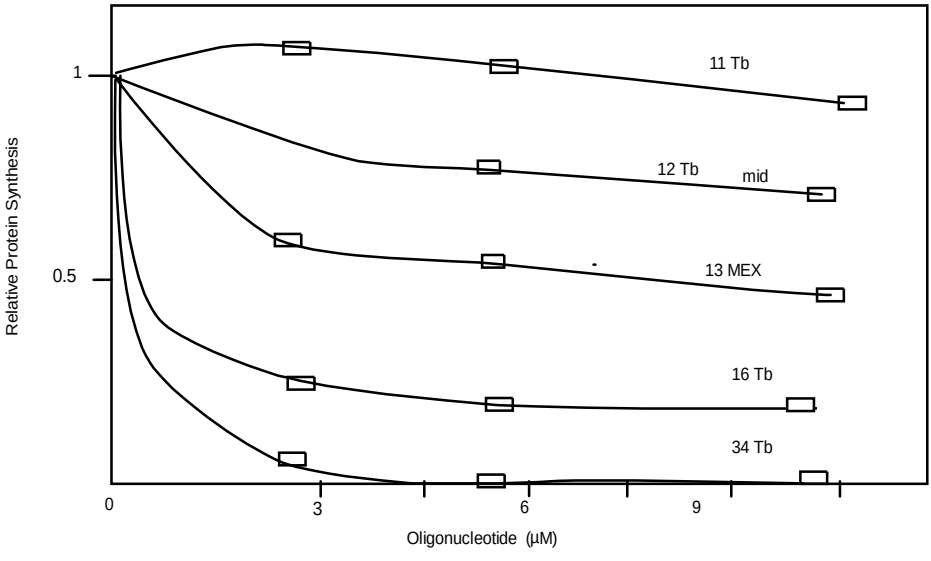
Oligos linked to an acridine derivative (2-methoxy, 6-chloro, 9-amino acridine) through a pentamethylene linker were shown to bind complementary RNA with a higher affinity than homologous unmodified ones (Toulmé *et al.*, 1986). This was ascribed to stacking interactions between the dye and base pairs of the oligo/RNA duplex. Such modified oligomers, complementary to the *T. brucei* mini-exon sequence, were used both in cell-free systems and with procyclic trypanosomes in culture.

Effect on in vitro *translation.* A complementary 9-mer linked at its 3⌈ end to the acridine derivative (Acr-9Tb) was a more efficient inhibitor of translation in rabbit reticulocyte lysate than the homologous conventional 9-mer, 9Tb, or even than an unmodified 12-mer (Verspieren *et al.*, 1987). The inhibition was specific: this oligomer had no effect on translation of Brome Mosaic Virus mRNA which do not contain the complementary sequence (Verspieren *et al.*, 1988). In contrast, a complementary acridine-linked 6-mer (Acr-6Tb) induced no significant decrease of *T. brucei* protein synthesis.

Trypanocidal activity. Addition of 100 μM Acr-9Tb to procyclic *T. brucei* induced drastic changes in both the morphology and the motility of the parasites, ultimately leading to cell death. This effect was not seen either with Acr-6Tb or with non-complementary acridine-linked oligomers. The unmodified 9Tb had no effect (Verspieren *et al.*, 1987). Therefore, the trypanocidal activity of Acr-9Tb is due to the presence of a complementary sequence and of the acridine residue. This suggests that this activity arises from the formation of a specific complex with the mini-exon sequence. Besides increasing the affinity of the oligo for the target RNA, acridine was responsible for a longer lifetime of the molecule in the growth medium because it prevented it being degraded by 3' exonucleases. Moreover, the kinetics of uptake of Acr-9Tb were quicker than that of 9Tb (J.J. Toulmé, unpublished results).

Optimization of the target

The inhibitory properties of antisense oligomers depend on the concentration of the oligo/RNA complex. This will be affected by chemical and physical characteristics of the oligomer (affinity, stability, uptake; see Table 1). It will also be affected by factors concerned with the RNA itself. Due to peculiarities (access and structure), not all the targets are expected to lead to equivalent inhibitory effects. We performed a detailed study on a series of unmodified oligos, 9 to 35 nucleotides long, complementary to *T. brucei* or *T. vivax* mini-exon sequences (Verspieren *et al.*, manuscript in preparation). On the one hand, we measured their affinity for RNA by thermal elution of filter-bound complexes, characterized by Tc, the temperature corresponding to the mid-point transition. On the other hand, we determined their effect on protein synthesis in cell-free systems. For most of the oligos, Tc was related to their length, l, and to their G + C content, x. The amplitude of the protein synthesis inhibition, at a given oligonucleotide concentration, increased with (l + x) (Figure 1). However, a few oligomers had a low Tc as compared to ones for which the value of (1 + x) was similar. This resulted in a reduced antisense effect (Verspieren *et al.*, unpublished results). All these oligomers were targeted to the 5' end of the mini-exon sequence, that is, a region containing modified nucleic acid bases (Freistadt *et al.*, 1987). In particular, the presence of a modified adenine residue in the sixth position of the *T. brucei* mini-exon sequence did not allow efficient pairing of the opposite thymine, resulting in a weak complex. This was also



10 20 30

$^{5?}$AACUAACGCUAUUAUUAGAACAGUUUCUGUACUAUAUUG ... *T. brucei*

ATAATAATCTT 11 Tb

TAATAATCTTGT 12 Tb mid

GTCAAAGACATGA 13 MEX

CGATAATAATCTTGTC 16 Tb

TTGCGATAATAATCTTGTCAAAGACATGATATAA 34 Tb

FIGURE 1. Effect of complementary oligodeoxynucleotides on *in vitro* translation of *Trypanosoma brucei* RNA. Total RNAs, extracted from parasites (MITat 1.5; clone 118) grown in mice, were translated in wheat germ extract containing ^{35}S-methionine in the presence of the indicated oligonucleotide (synthesized on an Applied Biosystems synthesizer, USA, and purified by high-performance liquid chromatography). Protein synthesis was quantitated by trichloroacetic acid precipitation and liquid scintillation counting. Oligonucleotide sequences are given at the bottom of the figure.

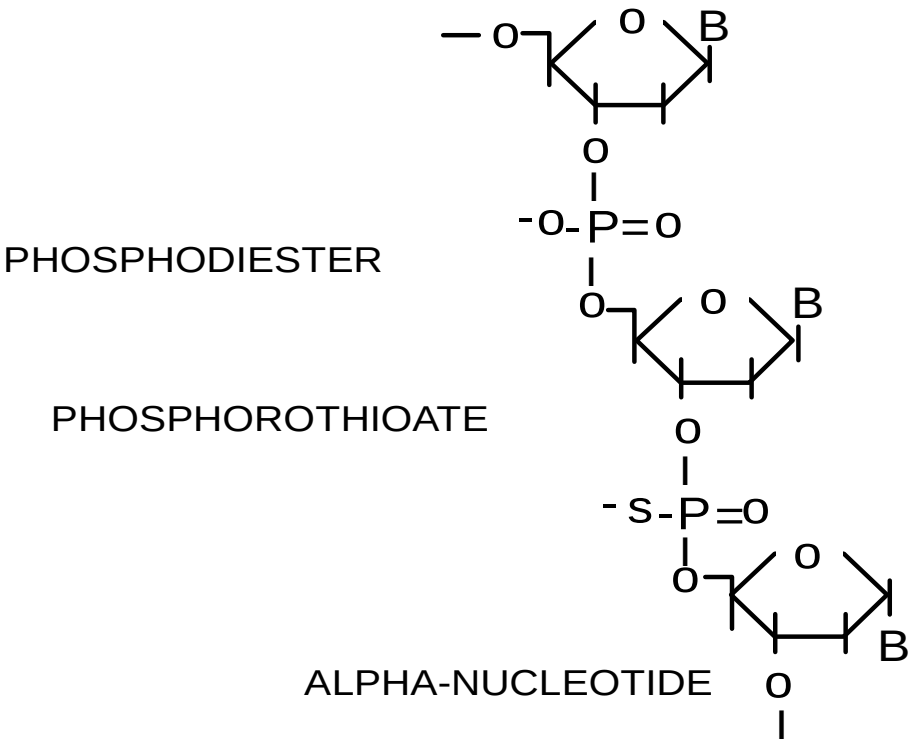


FIGURE 2. Chemical structure of phosphodiester (top), phosphorothioate (middle) and α-nucleotide units (bottom).

observed with *T. vivax* RNA, suggesting the presence of modified bases in the mini-exon of this parasite as well.

TOWARDS ANTISENSE OLIGONUCLEOTIDES OF IMPROVED EFFICIENCY

The trypanocidal activity of Acr-9Tb was observed only at concentrations higher than 80 μM. Although the acridine derivative prevented attack by 3' exonucleases, the phosphodiester backbone could be cleaved by endonucleases. Efficiency of antisense oligos would be improved if analogues resistant to nuclease degradation were used. For this purpose, we focused on two different chemical modifications: phosphorothioate derivatives and α-oligomers (Figure 2). A 17-mer targeted to the coding region of the rabbit ß-globin gene did not block elongation of the polypeptide chain when the α-analogue (17α) was used, whereas the phosphorothioate congener (17PS) was as efficient as the unmodified 17-mer (17PO) in wheat germ extracts and more efficient in micro-injected *Xenopus* oocytes (Cazenave *et al.*, 1989). These results were explained by the

long life-time of 17PS as compared to 17PO and the failure of 17α to induce RNase-H activity.

Modified oligos (α- and phosphorothioate) complementary to the mini-exon of *T. brucei* were synthesized. An α-11-mer did not inhibit *in vitro* translation. This oligo might bind very weakly, as we failed to detect any signal by thermal elution of filter-bound complexes. Preliminary experiments performed with phosphorothioate derivatives (12- and 24-mers) did not indicate a trypanocidal activity of these oligos below 30 μM, the concentration at which a non-specific toxicity was observed. Therefore, these modifications did not result in the expected improvement.

However, recently we demonstrated that an α-16-mer, linked to an acridine derivative, complementary to the AUG region of the rabbit β-globin gene, induced a specific inhibition of globin synthesis by a mechanism that remains to be determined (Boiziau *et al.*, unpublished results). Therefore, it seems that more efficient antisense oligos could be obtained by introduction of multiple chemical modifications within the same molecule. In particular, linkage of cleaving (Verspieren *et al.*, 1988) or photo-crosslinking reagents (Kean *et al.*, 1988) to antisense oligos could lead to molecules that will permanently block translation of the target gene.

ACKNOWLEDGEMENTS

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COUNTRY/COLLABORATIVE REPORTS

C.V.L. AND SOKOINE UNIVERSITY, TANZANIA, AND C.T.V.M., SCOTLAND:

Chemotherapy for trypanosomiasis

R.S. Silayo

Faculty of Veterinary Medicine
Sokoine University of Agriculture
P.O. Box 3019
Morogoro, Tanzania

Animal trypanosomiasis is one of the most important diseases hindering livestock production in Tanzania, where 60% of the country is occupied by savannah tsetse flies. Control methods include attack on the vector by insecticide application and/or selective bush clearance, and use of trypanocidal drugs for curative and prophylactic purposes. However, increasing costs of vector-control methods and fear of the deleterious environmental impact as a result of using these methods have led to increasing reliance on chemotherapy for the control of trypanosomiasis.

Drug resistance is one of the most important problems when using chemotherapy to combat trypanosomiasis, since the number of drugs available for field use has remained limited. Monitoring the development of drug resistance is therefore important in trypanosomiasis control strategies relying on chemotherapy.

The author has been involved in studies on chemotherapy for trypanosomiasis at the Central Veterinary Laboratory, Temeke, Dar es Salaam; the Centre for Tropical Veterinary Medicine, Edinburgh, Scotland; and Sokoine University of Agriculture, Morogoro, Tanzania.

At the Central Veterinary Laboratory we are interested in determining whether diminazene aceturate can retain efficacy when used over a prolonged period. This is a continuation of work started by E. Wiesenhutter on dairy farms in and around Dar es Salaam during the late 1960s. We found that diminazene aceturate appeared to induce no drug resistance even after many years of regular use on one particular dairy farm. However, surveillance of drug resistance in other areas of the country revealed cases of diminazene resistance.

At the Centre for Tropical Veterinary Medicine, studies were carried out using rabbits to determine the efficacy of treating animals with diminazene aceturate at different stages of cyclically transmitted *Trypanosoma congolense* infections. It was shown that relapse infections occurred in some animals treated 8–10 days post-infection, when trypanosomal chancres were at their largest. The reason for the relapses and the relevance of this finding to bovine trypanosomiasis in the field need to be determined. Studies were also carried out on isometamidium

prophylaxis in rabbits. These studies showed that the duration of prophylaxis appeared not to depend on the number of infective tsetse bites.

The chemotherapy studies at Sokoine University have two goals: to isolate putative drug-resistant *T. congolense* stocks from the field and establish their sensitivities to diminazene aceturate and isometamidium chloride, and their pathogenicity. Studies have also been conducted with certain stocks on the stability of resistance. We found that drug resistance can be stable. For example, *T. congolense* ADRI was resistant to 70 mg/kg diminazene aceturate in mice and maintained this level of resistance after 50 passages in mice.

AHMADU BELLO UNIVERSITY, NIGERIA,
AND UNIVERSITY OF GUELPH, CANADA:

Trypanocidal action of polyunsaturated fatty acids and lysophosphatidyl-choline derivatives

A.I. Ukoha

Department of Biochemistry
Ahmadu Bello University
Zaria, Nigeria

Current address:
Department of Chemistry and Biochemistry
College of Physical Science
University of Guelph
Ontario NIG 2W1, Canada

Reports have shown that trypanosomes readily take up exogenous 1-acyl-*sn*-lyso-phosphatidylcholine (LPHChO) and rapidly hydrolyze it through endogenous phospholipase A_1 to form free fatty acids and glycerophosphocholine. At the same time there is a rapid transacetylation of the LPHChO by trypanosomal acyltransferase to form PHChO, which is incorporated into the membrane of the parasite. These two enzyme systems enable the trypanosome to acquire the bulk of its fatty acids and choline requirements by exogenous LPHChO. Thus, if an unusual fatty acid can be incorporated into the LPHChO, the effect on the trypanosome may be therapeutically beneficial.

Trypanosoma brucei brucei (5×10^6/ml) were preincubated in filter-sterilized modified Eagle's 199 culture medium supplemented with 0.2 mM mercaptoethanol, 2 mM pyruvate, 0.01 mM hypoxanthine and 1% essential amino acids, in an atmosphere of 5% CO_2 - 95% air at 37 ⌈C for 1 hour. Various concentrations of the fatty acids were added to the medium and incubated for another 1 hour. Trypanosome viability was determined by counting the number of motile parasites using a Neubauer haemocytometer. The EC_{50} for each compound, defined as the concentration of the drug that reduces parasite motility to 50% after one hour's exposure, was calculated. In a further study using the more toxic fatty acids and LPHChO derivatives, time-dependent experiments were performed using the medium supplemented with 15% v/v horse serum.

Among the C_{20} polyunsaturated fatty acids, arachidonate (C_{20}:4[n-6]) was the least toxic tested. The acetylenic analogue, eicosatraynoate, which is a potent inhibitor of arachidonate metabolism at the level of cyclo-oxygenase and lypoxygenase, had less effect than arachidonate. On the other hand, eicosapentaenoic acid (C_{20}:5[n-3]), which is also an inhibitor of arachidonate metabolism

via the cyclo-oxygenase pathway, showed a marked effect on trypanosome viability. A similar trypanocidal effect was seen for C_{20}:3(n-3) against C_{20}:3(n-6). Trypanocidal effects of the n-3 polyunsaturated fatty acids were also observed for the C_{22} unsaturated fatty acids. Similar toxicities were observed for the corresponding methyl esters, thus suggesting that the trypanocidal action could not be accounted for by the detergent-like nature of the fatty acids since the methyl esters do not have polar heads. The pattern of toxicity may be related to preferential uptake of polyunsaturated fatty acids between the n-3 and n-6 series. The results with the n-3 LPHChO compounds showed that they also were potent trypanocides. When the LPHChO of the n-3 series were tested in medium supplemented with horse serum, the parasites died.

UNIVERSITY OF ADDIS ABABA, ETHIOPIA:

Animal health and production management in a peasant farming community of southwestern Ethiopia

E. Mamo

Institute of Pathobiology
Addis Ababa University
P.O. Box 1176
Addis Ababa, Ethiopia

Large numbers of families have been moved from northern Ethiopia to tsetse-infested areas of southwestern Ethiopia. Most of the new settlements, as well as the resident population, cannot undertake viable animal production, mainly due to health problems. Meat and milk supply is severely short.

The principal animal health problems are trypanosomiasis, tick-borne diseases, fascioliasis and gastrointestinal parasites. There is, in addition, a serious shortage of feed during the dry season (7–8 months per year), during which productivity is reported to fall sharply. Hence there is a need to tackle this aspect along with health-related activities.

The objectives of a proposed project involving Ethiopian universities, the Ministry of Agriculture and the Institute of Agricultural Research include controlling major health problems of farm animals, tackling the dry-season shortage of feed and improving the general productivity of the animals. The general strategy that will be adopted is to select communities for new settlers or local population settlements and to take full responsibility in these communities for animal health and production parameters.

A source of funds has not yet been identified for this project but there is a possibility of obtaining financial support from the government and/or one or more non-governmental agencies. Involvement of bilateral and multilateral organizations as well as other outside sources would help project members obtain materials and supplies not available within the country. We hope to commence the project within the coming year.

SOKOINE UNIVERSITY, TANZANIA,
AND GLASGOW UNIVERSITY, SCOTLAND

Pharmacology of anti-trypanosomal drugs

L.D.B. Kinabo

Department of Pharmacology
Faculty of Veterinary Medicine
Sokoine University of Agriculture
P.O. Box 3019
Morogoro, Tanzania

Current address:
Department of Veterinary Pharmacology
Glasgow University Veterinary School
Bearsden Road
Glasgow G61 1QH, UK

Over the last four years, research on anti-trypanosomal drugs in the Department of Veterinary Pharmacology at Glasgow University has focused primarily on isometamidium chloride (Samorin®). This is due to the drug's apparent superiority in both therapeutic and prophylactic treatments for bovine trypanosomiasis. The aspects that we have examined include:

- pharmacokinetics of isometamidium in cattle using high-performance liquid chromatographic and radioimmunoassay techniques
- adverse reactions, particularly tissue damage in cattle
- toxicological potential of isometamidium residues in edible tissues

The data we have obtained are not sufficient to confirm that isometamidium residues in edible tissues are not toxic in humans. To obtain more information, particularly on the molecular mechanisms of toxicity, we are evaluating the mutagenic potential of isometamidium and other anti-trypanosomal drugs currently used. We are using bacteria/mammalian microsome tests, incorporating four strains of *Salmonella typhimurium* and one strain of *Escherichia coli*. We are also evaluating the extent to which isometamidium is excreted in milk of treated goats and hope to extend these experiments to cattle. In the light of a recent report that the drug was effective in controlling an outbreak of trypanosomiasis in pigs at a dose that in other animal species would be lethal (15 mg/kg), we are examining the kinetics of isometamidium in pigs. Finally, we are attempting to improve the sensitivity and specificity of the current analytical methods for use in detailed pharmacokinetic and toxicological studies. We hope that the kinetic and toxicological data obtained will help researchers improve dosing regimens and assess the risk associated with isometamidium residues in edible tissues.

UNIVERSITY OF IBADAN, NIGERIA:

Chemotherapy for animal trypanosomiasis

R.O.A. Arowolo

Department of Veterinary Physiology and Pharmacology
Faculty of Veterinary Medicine
University of Ibadan
Ibadan, Nigeria

Over the last fifteen years, members of the Department of Veterinary Physiology and Pharmacology at the University of Ibadan have been studying aspects of chemotherapy for animal trypanosomiasis caused by *Trypanosoma vivax*, *T. congolense* and *T. brucei*. We have carried out studies on the pharmacology of the currently used veterinary trypanocides, on drug combinations and on the physiological and trypanocidal effects of local herbal extracts and other agents.

Results of one study showed that the responses to trypanocidal therapy of two strains of *T. vivax* (Y58 and Y486) isolated by Leeflang *et al.* (1976) differed between themselves (Arowolo and Ikede, 1977, 1980; Arowolo, 1988) and differed from the response of *T. brucei* 8/18 in sheep and laboratory rodents. The use of isometamidium chloride or diminazene aceturate in combination with α-chlorohydrin (an anti-fertility agent) or its analogue was less efficacious than the use of trypanocides alone. Similar results were produced using a combination of salicylhydroxamic acid or glycerol with the above trypanocides (Arowolo and Uche, 1988). In a few cases these combinations produced toxic manifestations in the host. Alpha-chlorohydrin, although effective *in vitro* against *T. vivax* Y58, was ineffective *in vivo* in trypanosome-infected animals (Arowolo and Heath, 1979).

A study on the chemoprophylactic effects of isometamidium chloride and its dextran complex in goats experimentally infected with *T. congolense* indicated that at a dose of 0.2 mg/kg body weight, no significant prophylaxis was produced by either preparation. However, use of the dextran complex resulted in the longest prepatent period and a relatively insignificant parasitaemia. Results from this chemoprophylactic study differed from those of a study by James using rats (1978).

Working on the principle that a chemotherapeutic study is incomplete without a pharmacodynamic study (Albert, 1963), the pharmacodynamic activities of diminazene, homidium and isometamidium were assessed in animal tissues *in vivo* and *in vitro*. Results from *in vitro* studies showed that diminazene, homidium and low concentrations of isometamidium were anti-cholinergic and anti-histaminic while higher concentrations of isometamidium augmented cholinergic and anti-histaminic activities. Both homidium and isometamidium showed anti-adrenaline and anti-serotonin effects while diminazene enhanced both adrenaline and serotonin (Arowolo and Eyre, 1984). The three trypano-

cides blocked neuromuscular transmission in rats while diminazene (0.4–0.8 mg/ml) depressed rat blood pressure in a dose-dependent manner. *In vivo*, at a dose of 3.5 mg/kg body weight, diminazene prolonged the recovery of dogs and laboratory rodents from thiopental anaesthesia. The same dose of diminazene also cured and protected infected rats from the deleterious and fluctuating effects of the parasites on blood pressure. Therapeutic doses of diminazene also improved depressed liver function in rabbits experimentally infected with *T. brucei* (Arowolo *et al.*, 1988).

Further pharmacological studies on currently available drugs and on use of existing anti-parasitic agents are in progress. Work on herbal extracts is still preliminary. Epidemiological studies on causes of drug resistance are being proposed. Our laboratory seeks international and external support and collaboration in its various research activities.

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KENYA TRYPANOSOMIASIS RESEARCH INSTITUTE:

Chemotherapy at the Kenya Trypanosomiasis Research Institute

A.R. Njogu and P.D. Sayer

Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu, Kenya

The treatment of trypanosomiasis. both in domestic stock and in human patients, has been central to the development of the Kenya Trypanosomiasis Research Institute (KETRI) during the first ten years of its existence. Improved methods of use for the established human and animal trypanocides, none of which is less than 35 years old, revised treatment protocols and novel compounds have each received attention. This program was initiated by a small number of Kenyan staff who had been compelled to leave the East African Trypanosomiasis Research Organisation, in Tororo, Uganda, when the responsibility for research institutes, previously held by the East African Community, devolved to the individual states of Kenya, Uganda and Tanzania in 1977. This group set out to create an institute from scratch for which finance, physical facilities, equipment and staff would be needed. Now, thirty Kenyan scientists work in a modem purpose-built institute at Muguga, Kenya.

A field station, consisting of staff housing and a laboratory, to study and test practical and economically viable systems of prophylaxis and treatment in animals, especially in cattle, camels and goats, was set up for KETRI with encouragement and assistance from Galana Game and Ranching Ltd. This station has been staffed for more than ten years by a KETRI team composed of a senior field officer, an entomologist, a veterinarian, senior supervisory scientists and support staff. The vast area of the ranch (6000 km*), the large cattle population (25,000 head), a large camel herd (200 head) and the varied ecological zones and weather patterns provided an excellent opportunity to investigate large-scale livestock management under different levels of tsetse and trypanosomiasis challenge. Also, in addition to a long-term assessment of tsetse fly dynamics on the ranch, it was possible to determine the prophylactic needs of cattle kept in areas in which tsetse fly populations were reduced by odour-baited insecticide-impregnated targets, or by pour-on insecticides applied to cattle. The numerous supporters of this long-term series of programs include the Ranch itself, the Kenya Government, the Overseas Development Administration (ODA) of the United Kingdom, RMB (Rhone Poulenc Kenya), Bayer East Africa, Camco and Wellcome Kenya.

Through the Galana connection, KETRI has identified a race of East African Zebu, the Orma Boran, that exhibit valuable qualities of trypanotolerance. Their

prophylactic needs under medium to heavy trypanosome challenge were found to be half of those required to protect the improved Boran cattle.

Under these ideal conditions for field experiments, strategies for prophylactic treatment of cattle exposed to trypanosome challenge were developed and tested over many years at Galana Ranch, where management standards were high. A similar system is now under scrutiny at an area of pastoral management and group ranching systems at Nguruman, funded and staffed by ODA. Such methods have resulted in considerable savings on trypanocidal drugs for the livestock producer. (For Galana Ranch, at 1984 prices, the savings were estimated to be US$ 60,000 per year.) A similar approach has resulted in the development of treatment and prophylactic strategies applicable to camels infected with *Trypanosoma evansi.* This research was conducted on camel herds in northern Kenya in association with a program of the Kenya Arid Lands Research Station and was funded by RMB (May & Baker, UK). Such systems are now being disseminated by the use of residential demonstration herds under the auspices of a non-governmental charity organization (FARM Africa).

A laboratory has been set up to permit research and development of trypanocidal drugs by Rhone Merieux. A new product, cymelarsan, to treat *T. evansi* in camels is in the final stages of development. New formulations of isometamidium are also being tested.

The fate of trypanocides in body fluids and in tissues can be examined by both radiolabelled and non-radioactive methods. These methods have been developed to investigate whether drug use can be improved through more detailed understanding of the drug's pharmacokinetics, and also to determine the safety of these compounds in food products. For these studies, KETRI's metabolic stanchions can accommodate up to sixteen adult cattle. These first-class facilities have been built with funds from the Italian Government through a division run jointly by the Food and Agriculture Organization and the International Atomic Energy Agency. Studies are also conducted on other animal species and on cattle in the field under tsetse challenge. In addition, new compounds have been tested in cattle under experimental conditions in the laboratory and in the field.

The human sleeping sickness treatment centre at Alupe Hospital provides facilities for patients and conducts surveillance and long-term follow-up of treated cases that originate from the endemic areas around Lake Victoria in Kenya and from neighbouring countries. There has been a constant flow of cases, occasionally reaching epidemic proportions. At Alupe there are staff and equipment to collect detailed clinical data before, during and after treatment. New therapeutic agents and regimens are tested in clinical trials at this centre when patients do not respond to treatment with melarsoprol.

Screening novel compounds for trypanocidal activity and combinations of the established drugs is conducted in culture, rodents and in monkey models of the human disease. This program incorporates aspects of work from different units within KETRI and functions in collaboration with other institutions, both in Kenya and overseas.

The United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Dis-

eases has concentrated on the development and use of animal models at KETRI for screening some 100 compounds in mice infected with *T. brucei.* It has also concentrated on treatment trials in a vervet monkey model of *T. rhodesiense* infection, which closely mimics the disease in man. Two hundred and forty treatment courses have now been administered to primates, the majority of which were in the late encephalitic stage of the disease. A monkey model of ***T.*** *gambiense* is being developed.

Trypanosome culture systems have been established with funds from ODA to enable researchers to study drug activity and to screen *T. congolense* isolates from the field for their level of drug resistance. Similar culture systems have also been developed for *T. brucei, T. evansi* and *T. simiae.* The latter program is backed by the Free University of Berlin. *T. rhodesiense* culture has also been established in collaboration with New York University and Pace University, funded by a PSTC project of the United States Agency for International Development. Combined therapies involving DL-a-difluoromethylomithine (DEMO), melarsoprol, suramin and nifurtimox are being studied in culture, mice and monkeys inoculated with *T. rhodesiense.* From the primate studies, the value of combination therapy was confirmed and the difficulty of treating certain isolates of *T. rhodesiense* with orally administered DFMO became apparent. Apart from drug evaluation and screening procedures, these systems enable researchers to investigate the mechanisms of action and the metabolic pathways of certain drugs and their metabolites. Thus, a group of new compounds was found to be active against *T. brucei* at the Walter Reed Army Institute for Research. Five of these were evaluated at KETRI with funding from the United States Medical Research Unit in Kenya and have shown promise in mice. They will later be tested in primates.

AHMADU BELL0 UNIVERSITY, NIGERIA,
AND ILRAD, KENYA:

Pharmacokinetics and therapeutic efficacy of diminazene in *Trypanosoma congolense*-infected Zebu cattle

Y.O. Aliu

Department of Physiology and Pharmacology
Faculty of Veterinary Medicine
Ahmadu Bello University
Zaria, Nigeria

Diminazene is a widely used anti-trypanosomal and anti-babesial drug for which pharmacokinetic data have been determined in goats (Aliu et *al.*, 1984) and sheep (Aliu and Odegaard, 1985). The range of therapeutic or trypanocidal plasma levels of diminazene is not known, but doses of 1.0-3.5 mg/kg are considered curative, while an *in vitro* concentration of 0.5 µg/ml is trypanocidal and a feed containing 0.1 µg/ml will eliminate trypanosome infections in tsetse flies (Hawking, 1963).

Results of pharmacokinetic studies of drugs with half-lives greater than 9 hours suggested to Baggot (1978) that relatively low multiple doses at appropriate time intervals are more likely than singlelarge doses to maintain therapeutic blood levels for a relatively long period and to yield a higher degree of clinical effectiveness. In goats, diminazene aceturate (Berenil) was found to have a systemic availability of 50%; a half-life of 21 hours following intramuscular administration of 3.5 mg/kg; and to maintain a trypanocidal plasma concentration for 35.3 hours. Renal clearance, 0.072 ml/kg/min, accounted for only 11% of total body clearance and approximately 0.14% was excreted unchanged in milk following an intravenous dose of 2.0 mg./kg. In sheep, however, after an intramuscular dose of 3.5 mg/kg of diminazene diaceturate (Ganaseg®), the drug had a systemic availability of 95%. half-life was 11.5 hours and the total body clearance was 0.89 ml/kg/min.

The objectives of studies to be carried out soon at the International Laboratory for Research on Animal Diseases (Nairobi) include determination of the pharmacokinetics and urinary excretion of diminazene aceturate in Zebu cattle and the efficacy of various Berenil® therapeutic protocols in treating *Trypanosoma Congolense* infections in goats that are resistant to diminazene. -

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RESEARCH INSTITUTE FOR VETERINARY SCIENCE AND INSTITUT PERTANIAN BOGOR, INDONESIA:

Chemotherapy of *Trypanosoma evansi*

I.P. Sukanto*, R. Agustini*, P. Stevenson†, A. Day‡ and R.C. Payne*

*Research Institute for Veterinary Science
J1.R.E. Martadinata 30
P.O. Box 52
Bogor, West Java, Indonesia

†Kenya Trypanosomiasis Research Institute
P.O. Box 362
Muguga, Kenya

‡Institut Pertanian Bogor
Bogor, Indonesia

An experimental study was conducted in Indonesia using mice to determine the responses of 27 *Trypanosoma evansi* stocks, isolated in different parts of Indonesia, to suramin, isometamidium chloride, diminazene aceturate and quinapyramine sulphate. Mice were inoculated with 10 trypanosomes and treatments administered 24 hours later. Suramin was the most effective of these drugs: parasites disappeared from the circulation within 48 hours following treatment. Isometamidium chloride did not effectively control the infections and most of the mice died with fulminating parasitaemias. Diminazene aceturate and quinapyramine sulphate were only partially effective. Although not all isolates of *T. evansi* tested were susceptible to suramin, this drug probably remains the treatment of choice in Indonesia.

WORKSHOP SUMMARY

Workshop summary

S.L. Croft

London School of Hygiene and Tropical Medicine
Keppel Street
London WClE 7HT, UK

To summarize effectively a meeting in which 20 papers have reviewed widely different areas of the chemotherapy of African trypanosomes, several other parasitic protozoa and some aspects of cancer is not possible without greatly detracting from the substance of the many excellent presentations. A few general impressions remain.

The first part of the workshop, on trypanosome metabolism, clearly indicated how our improved understanding of biochemical pathways can lead to the identification of new targets and the design of new drugs. Some selectively toxic inhibitors have already been identified, although the new tools for rational chemotherapy-molecular biology, which can be harnessed to produce pure parasite protein, and X-ray crystallography and NMR (nuclear magnetic resonance), which can define protein structure-have yet to be fully exploited. Many of the current lead compounds, for example the pyrazolopyrimidines and sterol synthesis inhibitors, were identified by a mixture of serendipity, intuition and an awareness of research in other fields, where an observation on the activity of one compound prompted a question which in turn led to a rational examination of related compounds. Because of differences in enzyme structure (e.g., dihydrofolate reductase), rate of enzyme turnover (e.g., omithine decarboxylase), subcellular localization (e.g., glycosome) or molecule (e.g., trypanothione), the targets for selective toxicity may vary. Yet these targets are frequently on pathways that interlink, suggesting possibilities for combination therapy, a strategy important for enhancing drug potency as well as circumventing resistance. It is evident from the workshop and from the current scientific literature that most of these studies have concentrated upon *Trypanosoma brucei* species and that knowledge of similar pathways in *T. vivax, T. congolense* and other trypanosome species pathogenic for domestic livestock lags behind.

The mechanisms of drug resistance and cross-resistance in African trypanosomes are poorly understood. However, the use of molecular and biochemical techniques to study drug resistance in tumour cells and *Plusmodium* species has demonstrated the potential of these approaches. The workshop presentations on *Leishmania* and *Plasmodium* have shown that studies on resistance can also lead to an improved understanding of the mode of drug action and uptake, leading to improved drug design. This area of research is developing fast, although at present experiments appear to produce more questions than answers.

The presence of a therapeutic level of a drug at the site of infection is a reflection of the pharmacological properties of the compound. The last section of the workshop discussed drug-delivery systems that are used to improve the

in vivo activity of drugs with poor pharmacological properties. Regardless of whether the drug-delivery system was most useful for improving targeting to the infected tissue, to the parasites or to an intracellular biochemical target, it is clear that in relation to trypanosomiasis, this area of research is in the early stages and not all systems are suitable to the problems posed by this disease.

The long-term objective of much of the research discussed at the workshop is the identification of a novel trypanocide. No doubt the rational/semi-rational approach will identify a selectively toxic compound with good pharmacological characteristics. However, the cost of drug development for most tropical diseases is not an economic proposition and progress will certainly require close cooperation among scientists, the pharmaceutical industry and international organizations. The identification of a drug with broader applications, such as 9-deazainosine, to treat African trypanosomiasis, Chagas' disease and leishmaniasis may prove a more attractive proposition for development. If a drug is already used clinically or has potential against a more profitable disease, the attraction would be even greater. Several drugs discussed at the workshop, including allopurinol, ketoconazole and difluoromethylomithine, fall into this category. It will be interesting to see whether the research on dihydrofolate reductase, trypanothione and DNA topoisomerase II, discussed at the workshop, will identify compounds with this broad anti-trypanosomatid spectrum of activity. Meanwhile, short- to medium-term advances are possible. Our understanding of the mode of action of new and established drugs should suggest combinations for clinical trial, and new formulations of established drugs should improve therapeutic levels and reduce local toxicity.

The mood of the meeting was one of optimism and determination, reflecting the commitment of the participants. It provided an effective forum for exchange of information, ideas and addresses. Let us hope that the next decade fulfils the hopes raised by the advances of the last decade.

APPENDIX:
LIST OF PARTICIPANTS

List of participants

Professor Y .O. Aliu
Department of Physiology and Pharmacology
Faculty of Veterinary Medicine
Ahmadu Bello University
Zaria
Nigeria

Dr. R.O.A. Arowolo
Department of Veterinary Physiology and Pharmacology
Faculty of Veterinary Medicine
University of Ibadan
Ibadan
Nigeria

Dr. C.J. Bacchi
Biology Department of Haskins Laboratories
Pace University
41 Park Row
New York
New York 10038
USA

Dr. S.M. Beverley
Department of Biological Chemistry and Molecular Pharmacology
Harvard Medical School
250 Longwood Avenue
Boston
Massachusetts 02 115
USA

Dr. E.J. Bienen
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. A.B. Clarkson, Jr.
Department of Medical and Molecular Parasitology
New York University Medical Center
550 First Avenue
New York
New York 10016
USA

Dr. V. Codjia

Current address:
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Permanent address:
Division Trypanosomiase Animale
Direction de 1'Elevage et des Industries Animales
B.P. 03-2036
Cotonou
Benin

Dr. S.L. Croft
London School of Hygiene and Tropical Medicine
University of London
Keppel Street
London WC1E 7HT
UK

Dr. G. d'Ieteren
International Livestock Centre for Africa
P.O. Box 46847
Nairobi
Kenya

Dr. S.J. Doxsey
Department of Biochemistry and Biophysics
School of Medicine
University of California
San Francisco
California 94 143
USA

Dr. J.J. Doyle
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Ms. Intisar Elamin

Current address:
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Permanent address:
Department of Preventive Medicine and Veterinary Public Health
Faculty of Veterinary Science
University of Khartoum
Box 32
Khartoum North
Sudan

Dr. A.H. Fairlamb
Department of Medical Parasitology
London School of Hygiene and Tropical Medicine
Keppel Street
London W 1 CE 7HT
UK

Dr. W.R. Fish
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. P.R. Gardiner
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. L.J. Goad
Department of Biochemistry
University of Liverpool
P.O. Box 147
Liverpool L69 3BX
UK

Dr. J. Goodchild
Worcester Foundation for Experimental Biology
222 Maple Avenue
Shrewsbury
Massachusetts 01545
USA

Dr. A.R. Gray
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Ms. M.A. Gray
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Dr. R.M. Injairu
Director
National Veterinary Research Institute
P.O. Kabete
Kenya

Dr. J. Inselburg
Department of Microbiology
Dartmouth Medical School
Hanover
New Hampshire 03756
USA

Ms. R. Jamnadass

Current address:
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Permanent address:
Department of Zoology
Kenyatta University
P.O. Box 43844
Nairobi
Kenya

Dr. D. Jitla
Department of Zoology
Kenyatta University
P.O. Box 43844
Nairobi
Kenya

Dr. R. Kaminsky
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Mr. J.K. Kiaira
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. L.D.B. Kinabo

Current address:
Department of Veterinary Pharmacology
Glasgow University Veterinary School
Bearsden Road
Glasgow G61 1QH
UK

Permanent address:
Department of Veterinary Physiology, Biochemistry, Pharmacology and Toxicology
Sokoine University of Agriculture
P.O. Box 3017
Morogoro
Tanzania

Dr. D.S. Kitosi

Current address:
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Permanent address:
Faculty of Veterinary Medicine
University of Nairobi
P.O. Box 30197
Nairobi
Kenya

Dr. Kofi-Tsekpo
Kenya Medical Research Institute
P.O. Box 54840
Nairobi
Kenya

Mr. V.N. Konji
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. R. Kratzer
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Dr. D.J. Krogstad
Washington University School of Medicine
P.O. Box 8118
St. Louis
Missouri 63 110
USA

Dr. S. Maloo
International Livestock Centre for Africa
P.O. Box 80147
Nairobi
Kenya

Dr. M. Mamman

Current address:
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Permanent address:
Department of Physiology and Pharmacology
Faculty of Veterinary Medicine
Ahmadu Bello University
Zaria
Nigeria

Dr. E. Mamo
Institute of Pathobiology
Addis Ababa University
P.O. Box 1176
Addis Ababa
Ethiopia

Dr. S. Meshnick
The City College of New York
138th Street and Convent Avenue
New York
New York 10031
USA

Dr. P.A.M. Michels
Research Unit for Tropical Diseases
International Institute of Cellular and Molecular Pathology
Avenue Hippocrate 74
B-1200 Brussels
Belgium

Dr. J.B. Mitchell
School of Biology and Tropical Diseases
Chemotherapy Research Unit
Sunderland Polytechnic
Sunderland SR 1 3SD
UK

Mr. K.M. Mizinga

Current address:
Department of Physiology and Pharmacology
College of Veterinary Medicine
University of Georgia
Athens
Georgia 30602
USA

Permanent address:
Department of Biomedical Sciences
Samora Machel School of Veterinary Medicine
University of Zambia
P.O. Box 32379
Lusaka
Zambia

Dr. Amna El Amin Mohammed
Department of Preventative Medicine and Public Health
Faculty of Veterinary Science
University of Khartoum
Khartoum
Sudan

Dr. S.K. Moloo
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Professor G. Mugera
Dean
Faculty of Veterinary Medicine
University of Nairobi
P.O. Box 30197
Nairobi
Kenya

Dr. N.B. Murphy
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. L. Mutharia
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. M.J. Mutinga
Programme Leader
Medical Vectors Research Programme
International Centre of Insect Physiology and Ecology
P.O. Box 30772
Nairobi
Kenya

Dr. V.M. Nantulya
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. Njagi
Chairman
Department of Botany
Kenyatta University
P.O. Box 43844
Nairobi
Kenya

Dr. A.R. Njogu
Director
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Professor R.M. Njogu
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. N.K. Olembo
Chairman
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. J. Omuse
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Dr. J.D. Onyango
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Dr. L.H. Otieno
Programme Leader
Tsetse Research Programme
International Centre of Insect Physiology and Ecology
P.O. Box 30772
Nairobi
Kenya

Dr. A.S. Peregrine
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Dr. I.B. Roninson
Center for Genetics (M/C 669)
University of Illinois College of Medicine at Chicago
808 South Wood Street
Chicago
Illinois 606 12
USA

Dr. B.T. Rouse
Department of Microbiology
University of Tennessee
Knoxville
Tennessee 37996
USA

Dr. P.D. Sayer
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya

Dr. T.A. Shapiro
Department of Biological Chemistry
School of Medicine
Johns Hopkins University
725 North Wolfe Street
Baltimore
Maryland 21205
USA

Dr. R.S. Silayo
Department of Veterinary Microbiology and Parasitology
Faculty of Veterinary Medicine
Sokoine University of Agriculture
P.O. Box 3019
Morogoro
Tanzania

Dr. K.A. Sinei
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. R.K. Sones
Rhone Poulenc Kenya Ltd.
P.O. Box 30438
Nairobi
Kenya

Dr. W.P. Sukanto
Baltivet
P.O. Box 52
Bogor
West Java
Indonesia

Dr. I.A. Sutherland
Department of Veterinary Physiology
University of Glasgow Veterinary School
Bearsden Road
Glasgow G61 1QH
Scotland
UK

Dr. J.J. Toulmé
Laboratoire de Biophysique
Museum National d'Histoire Naturelle
INSERM U201
CNRS UA481
43 rue Cuvier
75231 Paris Cedex 05
France

Dr. M. Touré
International Laboratory for Research on Animal Diseases
P.O. Box 30709
Nairobi
Kenya

Professor A. Tumbo-Oeri
Department of Biochemistry
Nairobi University
P.O. Box 30197
Nairobi
Kenya

Dr. J.F. Turrens
Department of Biochemistry
College of Medicine
University of South Alabama
Mobile
Alabama 36688
USA

Dr. A.I. Ukoha

Current address:
Department of Chemistry and Biochemistry
College of Physical Science
University of Guelph
Ontario NIG 2W 1
Canada

Permanent address:
Department of Biochemistry
Ahmadu Bello University
Zaria
Nigeria

Dr. S. Van Nieuwenhove
c/o Embassy of Belgium
P.O. Box 30461
Nairobi
Kenya

Dr. F.M.D. Vellieux
BIOSON Research Institute
Department of Chemistry
University of Groningen
Nijenborgh 16
9747 AG Groningen
The Netherlands

Dr. J.B.O. Were
Director
Clinical Research Centre
Kenya Medical Research Institute
P.O. Box 54840
Nairobi
Kenya

Dr. E. Zwegarth
Kenya Trypanosomiasis Research Institute
P.O. Box 362
Kikuyu
Kenya