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13. ABSTRACT (Maximum 200) Leishmaniasis, a disease caused by protozoan parasites of the <i>Leishmania spp.</i> , is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease are either ineffective or toxic. Malaria is considered to be the most important infectious disease of humans on a world-wide scale. It is estimated that 300 million persons are infected at any one time in the world. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis and malaria. The objective of this research was to: 1) complete testing of potential WRAIR compounds on <i>L. mexicana</i> DNA synthetic enzymes; 2) establish <i>in vitro</i> cultivation of <i>Plasmodium falciparum</i> ; and 3) develop an antimalarial drug-evaluation system that incorporates both <i>in vitro P. falciparum</i> viability and DNA synthetic enzyme inhibitor protocols.			
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FOREWORD

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Linda Nolan 11/24/96
PI - Signature Date

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Part 1: Biochemistry and Chemotherapy of Leishmaniasis

Introduction

Leishmaniasis remains a serious world health problem with an estimated 2 to 3 million people affected by this disease annually (Iwu et al., 1994). Since the introduction of the pentavalent antimonials over 50 years ago, no new, effective antileishmanial drugs have been developed.

Creation of a rapid, drug-evaluation system for the screening of potential antileishmanial agents is needed and has been the focus of our previous work. By incorporating *in vitro* viability tests utilizing cultures of *Leishmania mexicana*, HeLa cells, and human T4 cells with assays utilizing isolated enzymes from *L. mexicana* (DNA polymerase, topoisomerase types I and II), we have developed an effective screening protocol.

The data presented here represent the continuation of that research and document the effects of the Walter Reed compound WR238605 on the activities of topoisomerase types I and II isolated from *L. mexicana*.

I. The Effect of WR238605 on the Catenation Activity of Topoisomerase Type II Isolated from *Leishmania mexicana*

Materials

Compound WR238605 was obtained from the Walter Reed Army Institute of Research (Washington, DC). Partially purified *Leishmania mexicana* topoisomerase type II was previously isolated in our laboratory (Khan et al., In Prep.). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Methods

Topoisomerase type II catenation activity: Topoisomerase type II catenation assays were conducted using supercoiled pBR322 DNA as the substrate. Reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 15 µg/ml BSA, 1 mM ATP, 5 mM MgCl₂, 1% PEG, 50 ng pBR322 DNA, and 5 µl enzyme in a total volume of 30 µl, were incubated at 30°C for 30 minutes in the presence or absence of WR238605 (10 µM or 100 µM in DMSO; LC50 and 10 X LC50 concentrations, respectively). Heat-inactivated (10 minutes at 50°C) *L. mexicana* topoisomerase type II was used as the enzyme control. A reaction mixture containing 0.5% DMSO was used as the WR238605 diluent control. Reaction mixtures were stopped by the addition of 5 µl of stop buffer containing 0.05% bromphenol blue, 0.05% xylene cyanole in 40% sucrose. Twenty µl samples were electrophoresed through 0.9% agarose gels for 2.5 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.2). Replicate experiments were

conducted on two different days.

Results

Results are presented in Figure 1. WR238605, at concentrations of 10 μM or 100 μM , did not inhibit the catenation activity of *L. mexicana* topoisomerase type II (Lanes 4 and 6). In the presence of the heat-inactivated enzyme catenation activity was not observed, with or without WR238605 (Lanes 3, 5, and 7). Catenation activity was not inhibited by 0.5% DMSO (Lane 8).

1 2 3 4 5 6 7 8

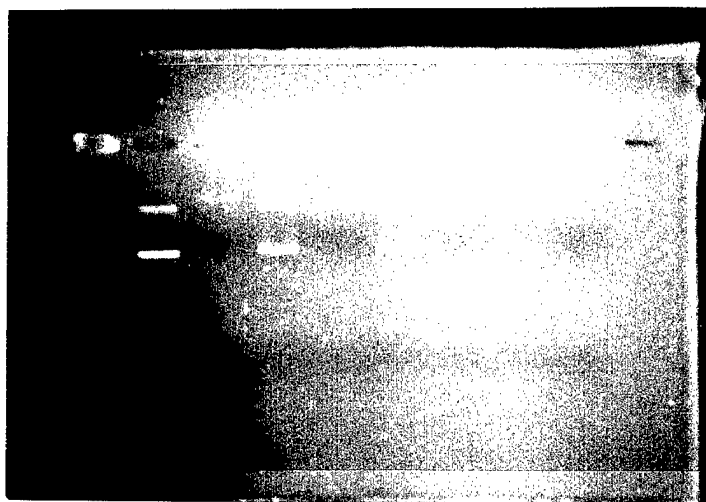


Figure 1: Results of topoisomerase type II catenation assay using partially purified topoisomerase type II from *Leishmania mexicana* and Walter Reed compound WR238605. Lane 1: pBR322 DNA control in reaction mixture without the enzyme; Lane 2: catenation activity of enzyme in reaction mixture; Lane 3: heat-inactivated enzyme control in reaction mixture; Lane 4: catenation activity of enzyme in presence of 10 μM WR238605; Lane 5: heat-inactivated enzyme control in the presence of 10 μM WR238605; Lane 6: catenation activity of enzyme in presence of 100 μM WR238605; Lane 7: heat-inactivated enzyme control in the presence of 100 μM WR238605; Lane 8: catenation activity of enzyme in the presence of 0.5% DMSO.

II. The Effect of WR238605 on the Catenation Activity of Topoisomerase Type I Isolated from *Leishmania mexicana*

Materials

Compound WR238605 was obtained from the Walter Reed Army Institute of Research (Washington, DC). Partially purified *Leishmania mexicana* topoisomerase type I was previously isolated in our laboratory. All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Topoisomerase type I catenation activity: Topoisomerase type I catenation assays were conducted using PhiX174 RF II DNA (the nicked circular form of bacteriophage PhiX174) as the substrate. Reaction mixtures containing 10 mM Tris-HCl, pH 7.4, 90 mM MgCl₂, 0.1 mM EDTA, 1% PEG, 5 mM spermidine, 50 ng PhiX174 RF II DNA, and 5 µl enzyme in a total volume of 30 µl, were incubated at 30°C for 30 minutes in the presence or absence of WR238605 (10 µM or 100 µM in DMSO; LC50 and 10 X LC50 concentrations, respectively). Heat-inactivated (10 minutes at 50°C) *L. mexicana* topoisomerase type I was used as the enzyme control. A reaction mixture containing 0.5% DMSO was used as the WR238605 diluent control. Reaction mixtures were stopped by adding 5 µl of stop buffer containing 0.05% bromphenol blue, 0.05% xylene cyanole in 40% sucrose. Twenty µl samples were electrophoresed through 0.9% agarose gels for 2.5 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE. The experiment was replicated and run on two different gels.

Results

Results are presented in Figure 2. Topoisomerase type I from *L. mexicana* catenates PhiX174 RF II (Lane 2). No catenation activity was observed, in the presence of the heat-inactivated enzyme with or without WR238605 (Lanes 3, 5, and 7) or in the presence of the diluent, 0.5% DMSO (Lane 9). The diluent, 0.5% DMSO, however, did show an effect on the catenation activity of the active *L. mexicana* topoisomerase type II (Lane 8). A similar effect was observed with 10 and 100 µM WR238605 (Lanes 4 and 6).

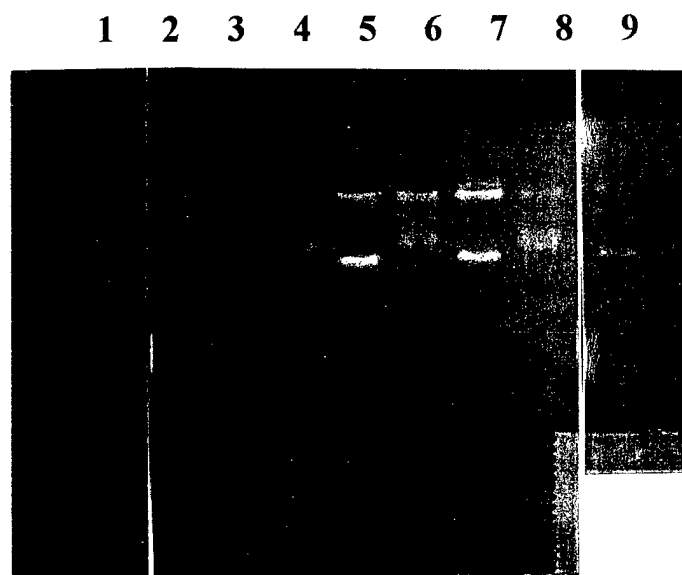


Figure 2: Results of topoisomerase type I catenation assay using partially purified topoisomerase type I from *Leishmania mexicana* and Walter Reed compound WR238605. Lane 1: PhiX174 RF II DNA control in reaction mixture without the enzyme; Lane 2: catenation activity of enzyme in reaction mixture; Lane 3: heat-inactivated enzyme control in reaction mixture; Lane 4: catenation activity of enzyme in presence of 10 μ M WR238605; Lane 5: heat-inactivated enzyme control in the presence of 10 μ M WR238605; Lane 6: catenation activity of enzyme in presence of 100 μ M WR238605; Lane 7: heat-inactivated enzyme control in the presence of 100 μ M WR238605; Lane 8: catenation activity of enzyme in the presence of 0.5% DMSO; Lane 9: heat-inactivated enzyme in the presence of 0.5% DMSO.

III. The Effect of WR238605 on the Decatenation Activity of Topoisomerase Type II Isolated from *Leishmania mexicana*

Materials

Compound WR238605 was obtained from the Walter Reed Army Institute of Research (Washington, DC). Partially purified *Leishmania mexicana* topoisomerase type II was previously isolated in our laboratory. Kinetoplast DNA, topoisomerase type II assay buffer (10X), gel loading buffer (10X), etoposide, human topoisomerase type II, and linear marker pRYG DNA were obtained from TopoGEN, Inc. (Columbus, OH).

Topoisomerase type II decatenation activity: Topoisomerase type II decatenation assays were conducted using kinetoplast DNA (kDNA), the mitochondrial DNA of *Crithidia fasciculata*, as the DNA substrate. Reaction mixtures containing assay buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 µg/ml nuclease free BSA), 275 ng kDNA, and 5 µl enzyme in a total volume of 20 µl, were incubated at 37°C for 60 minutes in the presence or absence of WR238605 (10 µM or 100 µM in DMSO; LC50 and 10 X LC50 concentrations, respectively). Heat-inactivated (10 minutes at 50°C) *L. mexicana* topoisomerase type II was used as the enzyme control. Etoposide (100 µM or 500 µM in DMSO) was used as a topoisomerase type II inhibitor control. To verify the inhibitory capability of etoposide under our assay conditions, human topoisomerase type II decatenation activity was assayed in the presence or absence of etoposide (100 µM in DMSO). A reaction mixture containing 0.5% DMSO was used as the diluent control. Reaction mixtures were stopped by the addition of 2 µl of gel loading buffer containing 0.25% bromophenol blue in 50% glycerol. Twenty µl samples were electrophoresed through 0.9% agarose gels for 2 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE. Replicate experiments were conducted on two different days.

Results

Results are presented in Figure 3. Decatenation activity was not inhibited by 0.5% DMSO (Lane 4). WR238605, at concentrations of 10 µM or 100 µM (lanes 5 and 6), and etoposide, at concentrations of 100 µM or 500 µM (Lanes 7 and 8), also did not inhibit the decatenation activity of *L. mexicana*. Decatenation activity was not observed in the presence of the heat-inactivated enzyme (Lane 9). Etoposide, at a concentration of 100 µM, did inhibit the decatenation activity of human topoisomerase type II (Lane 11).

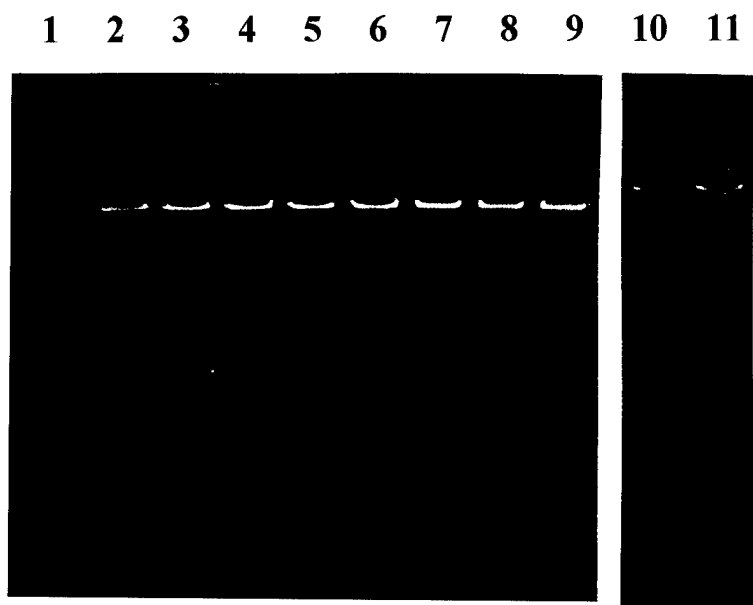


Figure 3: Results of topoisomerase type II decatenation assay using partially purified topoisomerase type II from *Leishmania mexicana* and Walter Reed compound WR238605. Lane 1: linear marker pRYG DNA; Lane 2: kDNA control in reaction mixture without enzyme; Lane 3: decatenation activity of enzyme in reaction mixture; Lane 4: decatenation activity of enzyme in the presence of 0.5% DMSO; Lane 5: decatenation activity of enzyme in the presence of 10 μM WR238605; Lane 6: decatenation activity of enzyme in the presence of 100 μM WR238605; Lane 7: decatenation activity of enzyme in the presence of 100 μM etoposide; Lane 8: decatenation activity of enzyme in the presence of 500 μM etoposide; Lane 9: heat-inactivated enzyme control in reaction mixture; Lane 10: decatenation activity of human topoisomerase type II in reaction mixture; Lane 11: the effect of etoposide (100 μM) on decatenation activity of human topoisomerase type II.

Discussion

Walter Reed compound WR238605 was previously shown to inhibit the viability of *L. mexicana* *in vitro*. In an attempt to clarify its mechanism of action, enzyme assays utilizing topoisomerase types I and II isolated from *L. mexicana* were performed. WR238605 at LC50 and 10 X LC50 concentrations (i.e., 10 and 100 μ M, respectively) did not effect *L. mexicana* topoisomerase type II catenation or decatenation activities. Since a solvent effect on topoisomerase type I catenation activity was observed, no statement can be made regarding the effect of WR238605 in this assay. However, it was previously shown in this laboratory that WR238605 did not effect topoisomerase type I relaxation activity which suggests that its mechanism of action is not inhibition of this enzyme activity.

It is interesting to note that etoposide, a known poison of human topoisomerase type II, did not effect the decatenation activity of *L. mexicana* even at a concentration 5 times the inhibitory concentration observed with human topoisomerase type II.

Conclusions

The mechanism of action of the Water Reed compound WR238605 against *Leishmania mexicana* cultures does not appear to be through inhibition of the enzymatic activity of topoisomerase type II. Analysis of WR238605 and *L. mexicana* topoisomerase type I catenation activity was hindered by an observed solvent effect, but previous data suggest that WR238605 does not inhibit this enzyme activity.

Biochemistry and Chemotherapy of Malaria

Introduction

Despite decades of research and applied management, malaria still ranks as one of the most infectious diseases in the world (Gamage et al., 1994). Chemotherapeutic approaches to malaria treatment are hindered by the widespread emergence of drug-resistant strains of *Plasmodium falciparum*, the species responsible for the most virulent form of human malaria (Berman et al., 1991).

Topoisomerases are essential enzymes that catalyze the concerted breaking and rejoining of the DNA backbone, processes involved in DNA replication, transcription, and recombination. These enzymes are present in high levels in rapidly dividing cells, such as cancer cells and late blood-stage malaria parasites (Cheesman et al., 1994). Since the growth rate (and DNA synthesis rate) of the late blood stage parasites resembles that of a malignant more than a normal cell, it has been suggested that the parasites may be more sensitive to DNA-compromising drugs than normal human cells (Lee and Inselburg, 1993; Riou et al., 1986). Numerous antibacterial and antitumor agents are already known to inhibit topoisomerase type II (Huff and Kreuzer, 1990). Topoisomerases, therefore, have become targets for antimalarial chemotherapy.

As in the case of leishmaniasis, development of a rapid drug-evaluation system for screening purposes is essential. Our laboratory currently maintains two strains of *P. falciparum* of different origin and drug sensitivity: strain HB3 (Honduras) is pyrimethamine resistant and strain W2 (Indochina) is chloroquine resistant.

The following report is divided into 3 main sections. Section I describes our laboratory's cultivation techniques. Section II focuses on the development and evaluation of various *in vitro* methodologies for use as potential screening protocols. Section III describes the development of a crude extract preparation for *P. falciparum* topoisomerase types I and II assays to be used for enzyme inhibition studies.

I. *In Vitro* Cultivation of *Plasmodium falciparum*

A. Establishment of Cultures

Materials

Plasmodium falciparum strains HB3 (South America) and W2 (Indochina) were generously provided by Dr. D. Wirth, School of Public Health, Harvard Medical School, Boston, MA. RPMI 1640 medium was obtained from Gibco (Grand Island, NY). Glucose, N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), pyruvate, glutamine, hypoxanthine, gentamicin, and human AB serum were purchased from Sigma Chemical Company (St. Louis, MO). Fresh frozen human plasma, type A positive, and transfusion grade packed red blood cells, type A positive, were obtained from the American Red Cross (Boston, MA). Tissue culture flasks with canted necks and plug seals were

purchased from Fisher Scientific (Pittsburgh, PA).

Methods and Results

RPMI 1640 medium was supplemented with 1.1% glucose, 1.1% TES, 1.1% pyruvate, 3% glutamine, 0.05% hypoxanthine, 25 µg/ml gentamicin, and 10% type A positive human plasma. Packed red blood cells (RBCs) were washed twice with unsupplemented RPMI 1640 medium containing 25 µg/ml gentamicin prior to use in culturing. Parasites were cultured in 5% hematocrit in a 5% CO₂, 1% O₂, 94% N₂ environment created by passing gas over cultures then storing with their caps tightly closed in a 37°C incubator. Table 1 presents the guidelines used for establishing cultures in various sized flasks.

Table 1: Guidelines for establishing *P. falciparum* cultures in various sized flasks.

	25 cm ²	Flask Size 75 cm ²	150 cm ²
Medium	5 ml	25 ml	50 ml
Washed RBCs	0.5 ml	2.5 ml	5 ml
Parasitemia	1%	1%	1%
Gas	20 sec (3-4 Psi)	40 sec (6-8 Psi)	60 sec (6-8 Psi)

B. Maintenance of Cultures

Materials

See Part A of this section. Diff-Quik stain kit was obtained from Baxter-VWR (Philadelphia, PA).

Methods and Results

The optimal range of parasitemia was determined to be 1-10% for the long-term culturing of *P. falciparum*. Healthy parasite cultures were found to grow from 1% to 10% in two days, with the ring-to-ring cycles determined to be approximately 44 and 41 hours for *P. falciparum* strains HB3 and W2, respectively. Table 2 presents the guidelines used for maintaining cultures in various sized flasks. Table 3 presents guidelines for subculturing *P. falciparum* grown in 25 cm² flasks. Cultures

were evaluated for health and parasitemia by microscopic examination of thin layer slides. Slides were stained using the Diff-Quik stain kit as follows: 5 seconds in fixative, 20 seconds in solution I, and 30 seconds in solution II. Slides were then washed in running tap water and air dried. Parasitemia was determined by counting the number of parasites in 1000 RBCs. For assay purposes, parasitemia was determined and categorized by the following stages: ring, late ring, trophozoite, and schizont.

Table 2: Guidelines for maintaining *P. falciparum* cultures in various sized flasks.

	25 cm ²	Flask Size 75 cm ²	150 cm ²
Medium	5 ml	25 ml	50 ml
Washed RBCs ¹	0 ml	0 ml	0 ml
Parasitemia	1-2%	1-2%	1-2%
Gas	20 sec (3-4 Psi)	40 sec (6-8 Psi)	60 sec (6-8 Psi)

¹ Every other day sufficient RBCs were added to maintain 5% hematocrit, if necessary.

Table 3: Guidelines for subculturing *P. falciparum* grown in 25 cm² flasks.

Percent Parasitemia	Split Ratio	Volume (ml)		
		Parasitized RBCs ¹	Fresh Medium ²	Fresh RBCs ³
1-2	None			0.1 if needed
3-4	1:3	1.5	3.5	0.5
5-8	1:5	1.0	4.0	0.5
8-12	1:10	0.5	4.5	0.5
> 12	1:20	0.025	4.75	0.5

¹ Volume of parasitized RBCs/media left in culture flask.

² Volume of fresh, supplemented medium added to culture flask.

³ Volume of fresh, washed RBCs added to culture flask.

II. In Vitro Drug Screening Protocols

Non-radiolabel and radiolabel methods for drug screening viability protocols were evaluated since both are reported in the literature (Basco and Le Bras, 1992; Hoon et al., 1995; Kotecka and Rieckmann, 1993; Lee and Inselburg, 1993), with some studies utilizing both (Chen et al., 1994; Peterson et al., 1990). Etoposide, a known antitumor agent, was used as the test drug for the pilot non-radiolabel experiment.

A. Non-Radiolabel Viability Studies - Preliminary Study Based on the Work of Pouvelle et al. (1994)

Materials

Etoposide was purchased from Sigma Chemical Company (St. Louis, MO).

Methods

Culture conditions: Asynchronous cultures of *Plasmodium falciparum* strain W2 were set up in 24-well tissue culture plates (Fisher Scientific, Pittsburgh, PA). Cultures were incubated at 37°C in a candle jar based on the methodology of Jensen and Trager (1977).

Drug treatment: One hundred µl of etoposide, dissolved in DMSO, were added to each culture for final concentrations of 20 or 200 µg/ml. The concentration of DMSO in the cultures did not exceed 0.17%. Solvent controls received DMSO for a final concentration of 0.17%. Additional control cultures were set up that received medium alone. Treatments and controls were set up in duplicate in 24-well tissue culture plates. Cultures were incubated at 37°C in candle jars through one life cycle (42 hr). At the end of that time, slides were made and the percentage parasitemia was determined for each culture by counting the number of parasites in 1000 RBCs.

Results

Preliminary results are presented in Table 4. Cultures treated with 200 µg/ml etoposide showed a reduced overall percent parasitemia (0.3%) compared to control cultures (2.5% for DMSO control and 3.7% for media control). Cultures treated with 20 µg/ml etoposide had an overall percent parasitemia of 3.2%.

Table 4: Results of pilot study with etoposide and *Plasmodium falciparum* strain W2.

Treatment	Percent Parasitemia
Media Control	3.7
DMSO Control	2.5
20 µg/ml Etoposide	3.2
200 µg/ml Etoposide	0.3

Conclusion

This methodology can be used as a protocol for non-radiolabel drug screening viability tests. Drug screening protocols should include more than 2 treatment concentrations. Data on etoposide should be repeated with additional concentrations.

B. Radiolabel Studies - Preliminary Study Utilizing a Microcentrifuge-Based ^3H -Hypoxanthine Assay

This methodology was based on the work of Dutta et al. (1990).

Materials

Sorbitol was obtained from Sigma Chemical Company (St. Louis, MO). ^3H -hypoxanthine (1 mCi/ml in sterile water) was purchased from Moravek (Brea, CA). ScintiVerse was obtained from Fisher Scientific (Pittsburgh, PA).

Methods

Synchronization of cultures: Cultures of *P. falciparum* strain W2 and HB3 were resuspended in medium, transferred to centrifuge tubes, and centrifuged for 10 min at 800 X g. The supernatant was removed and 5% sorbitol was added at a ratio of 1 part pellet to 4 parts 5% sorbitol. The pellet was resuspended, incubated at room temperature for 5 min with occasional gentle mixing, then centrifuged for 10 min at 800 X g. The supernatant was removed, the pellet resuspended in unsupplemented medium containing gentamicin, and centrifuged for 10 min at 800 X g. The washing was repeated two more times. After the last wash, a slide was made and the percent parasitemia and stages present were determined. Supplemented medium was added and cultures were established as previously described in this report.

Radiolabelling of cultures: Synchronized cultures (in medium without hypoxanthine, total volume = 200 μl) were established in sterile microcentrifuge tubes, covered with parafilm wiped with 70% ethanol, and incubated at 37°C in a candle jar for 18 hr. Five cultures were set up for each strain of parasite (W2 and HB3), and 2 cultures were set up containing non-parasitized RBCs as controls. At the end of this time, 25 μl (containing 5 μCi) ^3H -hypoxanthine were added to each culture. Cultures were returned to candle jar incubation for an additional 27 hr. Following incubation, cultures were centrifuged for 5 min at 1500 X g, the supernatants were removed, and pellets washed twice with 0.5 ml ice cold normal saline (0.9% NaCl). At the end of the second wash, 500 μl deionized/distilled water were added to each pellet to lyse the cells and each tube was vortexed until the solution turned a clear, pale red. Two hundred μl of the hemolysate were added to 5 ml scintillation fluid, vortexed vigorously into solution, and stored in a dark refrigerator for 30 min prior to counting. Samples were counted for 2 min.

Results

Results of the pilot study are presented in Table 5. RBCs parasitized with *P. falciparum* strains W2 and HB3 showed a mean 4.6-fold increase in ^3H -hypoxanthine uptake compared to non-parasitized control RBCs.

Table 5: Results of pilot study with ^3H -hypoxanthine and *P. falciparum* strains W2 and HB3.

Culture	Mean CPM
Strain W2	8411
Strain HB3	8011
RBC Contro l	1771

Conclusion

This methodology can be used as a protocol for radiolabel drug screening viability tests.

III. Crude Extract Preparation for *P. falciparum* Topoisomerase Types I and II Assays

This section describes the studies involved in developing a crude enzyme extraction procedure for *P. falciparum* topoisomerase types I and II. Since some investigators (e.g., Lee and Inselburg, 1993) worked with isolated populations of parasitized RBCs containing late stages (trophozoites and schizonts) for the purpose of obtaining enhanced enzyme activities, we conducted one pilot study (Part A) as a preliminary evaluation of this technique for future use.

A. Method for Concentration of Viable Trophozoite- and Schizont-Infected Red Blood Cells

Materials

Plasmagel was purchased from Cellular Products, Inc. (Buffalo, NY).

Methods

Cultures were suspended in medium, transferred to centrifuge tubes, and centrifuged for 5 min at 800 X g. The pellet was resuspended in unsupplemented medium containing 25 $\mu\text{g}/\text{ml}$ gentamicin and centrifuged for 5 min at 800 X g. Supernatant was removed and unsupplemented medium containing 25 $\mu\text{g}/\text{ml}$ gentamicin was added to reach a final hematocrit of approximately 40%. Plasmagel was added to the cell suspension at a ratio of 2:1 cell suspension:plasmagel and gently mixed. The

suspension was incubated for 15 min at 37°C. The upper phase was transferred to a centrifuge tube and centrifuged for 10 min at 800 X g. The pellet containing the concentrated late stage parasites was resuspended in supplemented medium, slides were made, and parasite stages counted.

Results

Results of a typical plasmagel separation of parasite stages for *P. falciparum* strain W2 are presented in Table 6. The initial culture had a relatively uniform distribution of parasite stages with a combined trophozoite + schizont percentage of 43.0. The plasmagel methodology resulted in the isolation of a subpopulation of parasites with a combined trophozoite + schizont percentage of 82.0.

Table 6: Results of a typical plasmagel separation of parasite stages for *P. falciparum* strain W2.

	% of Total Parasitized RBCs			
	Ring	Late Ring	Trophozoite	Schizont
Initial culture	28.5	28.0	21.0	22.0
Upper Phase	2.0	16.0	45.0	37.0

Conclusion

This method provided a quick, simple separation and concentration of late stage parasites, but produced a low overall yield.

B. Pilot study #1: Preliminary Study to Investigate Hemoglobin Removal from Parasitized RBCs

Materials

Ethanol, chloroform, and isoamyl alcohol were obtained from Fisher Scientific (Pittsburgh, PA). Hanks Balanced Salt Solution was purchased from Sigma Chemical Company (St. Louis, MO). The dye reagent for protein assay was obtained from Bio-Rad Laboratories (Hercules, CA). Kinetoplast DNA, topoisomerase type II assay buffer (10X), and human topoisomerase type II were purchased from TopoGEN, Inc. (Columbus, OH).

Methods

Preparation of parasite extracts: Preliminary experiments were conducted on asynchronous parasite cultures of *Plasmodium falciparum* strains HB3 and W2 (Table 7). Populations of parasitized RBCs were suspended in culture medium, centrifuged for 5 min at 800 X g, and washed once with Hanks Balanced Salt Solution. After the supernatant was removed, the cells were immediately frozen in liquid nitrogen and transferred directly to a -80°C freezer for storage. RBCs not infected with parasites were also processed in the same manner to serve as experimental controls.

Removal of hemoglobin: The following procedure is a modification of the methods of Yohida and Watanabe (1972) and Colowick and Kaplan (1955). Frozen RBC pellets were allowed to thaw on ice. Pellets collected on different dates were combined into one sample for each strain, total wet weights were determined (Table 8). An equal volume of 0.01 M phosphate buffer, pH 7.5, was added to each sample. Samples were vortexed and aliquots were removed for protein analysis. 24 µl of ethanol-chloroform (2:1, v/v), cooled at -60°C, was added to each 100 µl of sample. Samples were shaken vigorously on ice for 20 minutes, then centrifuged at 13,000 X g for 30 minutes. The hemoglobin precipitates were discarded and aliquots of the supernatants were removed for protein analysis. The supernatants were then divided into two aliquots. One was used directly in the enzyme assay, the other was sonicated 3 X 2 seconds on ice, prior to enzyme analysis.

Protein analysis: Protein was determined according to a modified Bio-Rad dye-binding method. Assays were conducted in Falcon 3071 microplates (Fisher Scientific, Pittsburgh, PA) and analyzed by a Molecular Devices' THERMOmax microplate reader (Menlo Park, CA) at 590 nm.

Topoisomerase type II decatenation activity: The topoisomerase type II decatenation assay was used as an initial screen for enzyme activity due to its sensitivity for detection of low enzyme titers (TopoGEN, 1995). Topoisomerase type II decatenation assays were conducted using kinetoplast DNA (kDNA), the mitochondrial DNA of *Crithidia fasciculata*, as the DNA substrate. Reaction mixtures containing assay buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 0.5 mM ATP, 0.5 mM dithiothreitol, 30 µg/ml nuclease free BSA), 275 ng kDNA, and 5 µl extract in a total volume of 20 µl, were incubated at 37°C for 60 minutes. Human topoisomerase type II was used as the positive control. At the end of 60 minutes, 1 µl (containing 1.8 µg) proteinase K was added for a final concentration of 50 µg/ml. Samples were incubated at 37°C for 15 minutes. Reaction mixtures were stopped by the addition of 2 µl of gel loading buffer containing 0.25% bromophenol blue in 50% glycerol. Twenty µl of chloroform:isoamyl alcohol (24:1) were added, samples vortexed briefly, then centrifuged for 5 seconds in a microfuge. The chloroform:isoamyl alcohol extraction was repeated. Twenty µl of the blue colored upper phase were electrophoresed through 0.9% agarose gels for 2 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.2).

Results

Total protein values for the crude extracts are presented in Table 9. The protein content of the crude

extracts used in the enzyme assay (for extract volumes of 5 and 10 μ l, respectively) are: 530 and 1060 μ g for strain HB3; 155 and 310 μ g for strain W2; and 300 and 600 μ g for control RBCs.

Decatenation activity was not observed in any of the crude preparations. Decatenation of kDNA was observed in the presence of human topoisomerase type II.

Conclusion

Hemoglobin was still present in high quantity which inhibited extraction and detection of *P. falciparum* topoisomerase type II activity. A different methodology is required.

Table 7: Percents parasitemia and life stages of *Plasmodium falciparum* strains HB3 and W2 cultures used in crude extract preparations.

Strain	% Total Parasitemia	% Rings	% Later Stages (Trophozoites + Schizonts)
HB3	10-15	50	50
W2	22	40	60

Table 8: Total wet weights of combined pellets used in crude extract preparations of *Plasmodium falciparum* strains.

Wet Weight (grams)	
Strain HB3	0.7750
Strain W2	0.8610
Control RBCs	0.1289

Table 9: Total protein values obtained for steps in the crude extraction preparation.

Extraction Step	Total Protein (mg)		
	HB3	W2	Control RBCs
Resuspended Pellet	287	277	27
Hemoglobin Removed	26	1.2	4.1

C. Pilot study #2: Preliminary Study Based on the Work of Inselburg et al. (1987)

Materials

Saponin, benzamidin, leupeptin, pepstatin, aprotinin and soybean trypsin inhibitor were obtained from Sigma Chemical Company (St. Louis, MO).

Methods

Preparation of parasite extracts: The following is a modification of the methodology of Inselburg et al. (1987). Preliminary experiments were conducted on asynchronous parasite cultures of *Plasmodium falciparum* strain HB3 (Table 10). Populations of parasitized red blood cells (RBCs) were suspended in culture medium, centrifuged for 10 min at 800 X g, and resuspended in 3 ml phosphate buffered saline (PBS; 0.01M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 0.14 M NaCl, pH 7.4). Small aliquots were removed for cell counting and protein analysis. Additional PBS was added and the cell suspension was centrifuged at for 10 min at 800 X g. Supernatant was removed and volume of pellet was recorded. Pellet was resuspended in 20 volumes of (room temp.) 0.015% saponin in PBS, incubated at room temperature for 10 minutes with occasional mixing, then centrifuged for 10 min at 5000 X g at 4°C. Supernatant was removed and the pellet was washed 2 X with cold PBS. Parasites were resuspended at a concentration of 1×10^9 parasites/ml in TEK buffer (50 mM Tris, 1 mM EDTA, 0.3 M KCl, pH 7.4) containing 10 mM benzamidin, 20 µg/ml leupeptin, 20 µg/ml pepstatin, 50 µg/ml aprotinin and 50 µg/ml soybean trypsin inhibitor, sonicated in an ice bath with 5 equally spaced 10 second bursts at full power during a 4 min period, then centrifuged for 30 min at 29,900 X g at 4°C. The supernatant was stored at -80°C.

Topoisomerase type II decatenation activity: Topoisomerase type II decatenation assay was previously described in Pilot Study #1, Part B of this section.

Results

UV fluorescent contaminants were present in the crude extract. Topoisomerase type II decatenation activity was not observed in the crude preparation. Decatenation of kDNA was observed in the presence of human topoisomerase type II.

Due to the presence of UV fluorescent contaminants and absence of decatenation activity, protein determination of the crude extract was not performed.

Conclusion

Further modification of the current methodology is required and should include removal of the high salt (0.3 M KCl) in the final parasite suspension buffer.

Table 10: Percentage parasitemia and life stages of *Plasmodium falciparum* strain HB3 used in crude extract preparation.

Culture	% of Total Parasitized Cells				Overall % Parasitemia
	Ring	Late Ring	Trophozoite	Schizont	
A	51	17	18	14	8.4
B	36	24	25	15	9.6
Mean	43.5	22	27.5	14.5	9.0

D. Finalized Crude Extract Preparation for Enzyme Assays

Materials

See Pilot Study #2, Part C of this section. Supercoiled (form I) plasmid DNA, topoisomerase type I assay buffer (10X), and human topoisomerase type I were obtained from TopoGEN, Inc. (Columbus, OH).

Methods

Preparation of parasite extracts: Preliminary experiments were conducted on asynchronous parasite cultures of *Plasmodium falciparum* strain HB3 (Table 11). Pilot Study #2 methodology was followed except: 1) parasites were resuspended at a higher final concentration of 1.7×10^9 parasites/ml (instead of 1.0×10^9 parasites/ml); and 2) parasites were resuspended in TE buffer (50 mM Tris, 1 mM EDTA, pH 7.4) (instead of TEK buffer with the high salt concentration of 0.3 M KCl) containing 10 mM benzamidine, 20 μ g/ml leupeptin, 20 μ g/ml pepstatin, 50 μ g/ml aprotinin and 50 μ g/ml soybean trypsin inhibitor.

Protein analysis: Protein was determined as previously described in Pilot Study #1, Part B of this section.

Topoisomerase type II decatenation activity: Topoisomerase type II decatenation assay was previously described in Pilot Study #1, Part B of this section.

Topoisomerase type I relaxation activity: Topoisomerase type I relaxation assays were conducted using supercoiled (form I) plasmid DNA as the DNA substrate. Reaction mixtures containing assay buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol), 0.25 μ g DNA, and 5 μ l extract in a total volume of 20 μ l, were incubated at 37°C for 60 minutes. Human topoisomerase type I was used as the positive control. At the end of 60 minutes, reaction mixtures were stopped by the addition of 2 μ l of gel loading buffer containing 0.25% bromophenol blue in 50% glycerol. Twenty μ l of chloroform:isoamyl alcohol (24:1) were added, samples vortexed briefly, then centrifuged for 5 seconds in a microfuge. Eighteen μ l of the blue colored upper phase were electrophoresed through 0.9% agarose gels for 2 hours at 25 mA current with a forward pulse time of 6 seconds, a reverse pulse time of 2 seconds, and a ramp setting of 1. The running buffer was 0.5 X TBE.

Results

Topoisomerase type II decatenation activity was observed in the *P. falciparum* strain HB3 crude extract preparation (Figure 4). Topoisomerase type I relaxation activity was also observed in the strain HB3 crude extract preparation (Figure 5). Similar results were obtained for *P. falciparum* strain W2 crude extracts prepared using this methodology.

Conclusion

This protocol provides a rapid extraction of crude enzymes that is sufficient for detection of topoisomerase types I and II. Preliminary isolation of subpopulations of late stage parasitized RBCs (see Part A of this section) does not appear necessary.

Table 11: Percentage parasitemia and life stages of *Plasmodium falciparum* strain HB3 used in crude extract preparation

Culture	% of Total Parasitized RBCs Trophozoites + Schizonts	Overall % Parasitemia
A	84	8.6
B	92	4.8
C	88	8.5
D	97	3.5

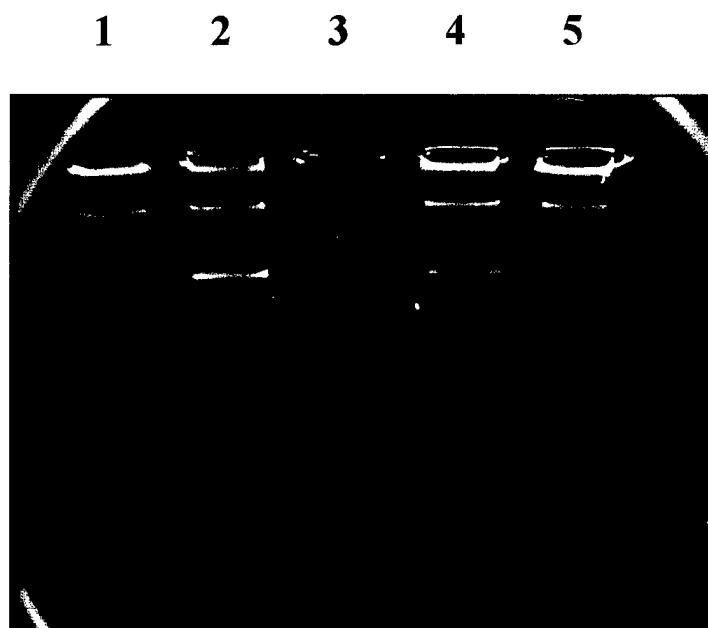


Figure 4: Results of topoisomerase type II decatenation assay using crude extract of *P. falciparum* strain HB3. Lane 1: kDNA control in reaction mixture without enzyme; Lane 2: decatenation activity of human topoisomerase type II; Lane 3: *P. falciparum* extract in reaction mixture without kDNA substrate; Lane 4: decatenation activity of *P. falciparum* extract; Lane 5: heat-inactivated (10 min at 50°C) *P. falciparum* extract with kDNA substrate.

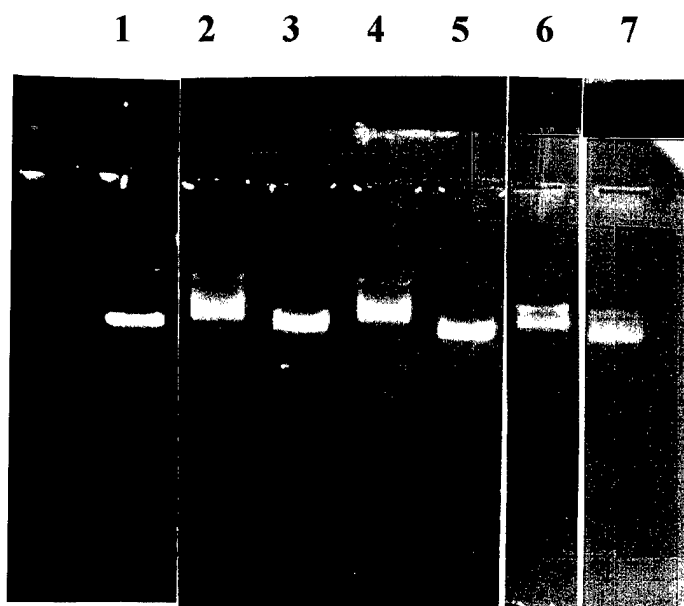


Figure 5: Results of topoisomerase type I relaxation assay using crude extracts of *P. falciparum* strains HB3 and W2. Lane 1: supercoiled plasmid DNA control; Lane 2: relaxation activity of *P. falciparum* strain HB3 extract; Lane 3: heat-inactivated *P. falciparum* strain HB3 extract with supercoiled DNA substrate; Lane 4: relaxation activity of *P. falciparum* strain W2 extract; Lane 5: heat-inactivated *P. falciparum* strain W2 extract with supercoiled DNA substrate; Lane 6: relaxation activity of human topoisomerase type I; Lane 7: relaxed marker DNA.

Discussion

The creation of an effective, antimalarial drug-evaluation system requires the development and incorporation of a variety of different methodologies. Our laboratory has established and routinely maintains two strains of *Plasmodium falciparum* of different origin and drug sensitivity, the pyrimethamine-resistant strain HB3 from Honduras and the chloroquine-resistant strain W2 from Indochina.

Utilizing these two strains of *P. falciparum*, *in vitro* viability methodologies have been established using both radiolabel and non-radiolabel protocols. In addition, a rapid, crude enzyme extraction preparation has also been developed that does not require the preliminary isolation of late-stage subpopulations of parasitized red blood cells. Both topoisomerase type I and II activities are present in these extracts, which, when incorporated into the overall screening protocol, provide a tool for drug inhibition studies.

Conclusions

An antimalarial drug evaluation system has been developed utilizing *Plasmodium falciparum* strains HB3 and W2. This system incorporates *in vitro* viability tests with topoisomerase type I and II drug screening protocols.

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Appendix: Publications Resulting from Contract for 1996

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