IN VITRO SENSITIVITY OF TRICHOMONAS VAGINALIS TO DNA TOPOISOMERASE II INHIBITORS

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Abstract. Vaginal trichomoniasis is a highly prevalent sexually transmitted disease caused by a microaerophilic protozoan *Trichomonas vaginalis*. The disease is one of the most common sexually transmitted disease and can augment the predisposition of individuals to human immunodeficiency virus (HIV) infection. Although the disease can be treated with metronidazole and related 5-nitroimidazole, cases of trichomonal vaginitis which are refractory to standard treatment seems to be increasing. Clearly, new antitrichomonad agents are needed and DNA topoisomerase II may acts as a new target for antitrichomonad agents. In this study, *in vitro* sensitivity of *T. vaginalis* to DNA topoisomerase II was investigated. Axenic culture of local strain of *T. vaginalis* was performed. Both eukaryotic and proksayotic DNA topoisomerase II inhibitors such as ellipticine, amsacrine and fluoroquinolones were tested for effectiveness against *T. vaginalis in vitro* compared to metronidazole. *T. vaginalis* was sensitive to metronidazole under aerobic conditions. Minimal inhibitory concentrations (MICs) of eukaryotic DNA topoisomerase II inhibitors, ellipticine and amsacrine, were 6.4 mM and 64 mM, respectively. The MICs of prokaryotic DNA topoisomerase II or DNA gyrase inhibitors; ciprofloxacin, ofloxacin and norfloxacin were 64, 960 and 1,280 mM, respectively. Based on the results, among DNA topoisomerase II inhibitors ellipticine was the most effective drug against *T. vaginalis in vitro* whereas fluoroquinolones did not show high antitrichomonad activity.

INTRODUCTION

Trichomonas vaginalis is a protozoan commonly found in the human genitourinary tract and transmitted primarily by sexual intercourse. This organism causes vaginitis in women and non-gonococcal urethritis in men. *T. vaginalis* vaginitis is one of the most common sexually transmitted diseases (STD), with around 120 million worldwide estimated to suffer from trichomoniasis every year. The disease is highly prevalent throughout the world including developing countries (Brabin *et al*, 1995).

More disturbing is the number of asymptomatic cases that are not treated. In North America alone more than 8 million new cases are reported yearly (WHO, 1995) with an estimated rate of asymptomatic cases as high as 50% (Fouts and Kraus, 1980; Wolner-Hanssen *et al*, 1989). This disease has important medical, social and economic implications. Women who are infected during pregnancy are predisposed to premature rupture of the placental membranes, premature labor and low-birth-weight infants (Hardy *et al*, 1984; Minkoff *et al*, 1984). Also linked to the disease are cervical cancer (Gram *et al*, 1992; Kharsany *et al*, 1993; Zhang and Begg, 1994), atypical pelvic inflammatory disease (Heine and McGregor, 1993) and infertility (Grodstein *et al*, 1993).

As with other STDs, *T. vaginalis* infection can augment the predisposition of individuals to human immunodeficiency virus (HIV) infection (Cameron and Padian, 1990). Laga *et al* (1994) found that HIV seroconversion in female prostitutes was significantly associated with the occurrence of other nonulcerative STDs, such as trichomoniasis. It is thought that trichomoniasis may increase the transmission of HIV by causing local accumulations of HIV-infected or HIV-susceptible cells such as lymphocytes and macrophages.

The disease caused by *T. vaginalis* can be effectively treated with metronidazole and related 5-nitroimidazole drugs (Lossick, 1986). Metronidazole has been used successfully during the past 30 years in the treatment of a broad range of infections caused by anaerobic protozoa and bacteria. This drug is used extensively to treat a number of human infections, including trichomoniasis, giardiasis and amebiasis.

Although the drug is effective in most patients with trichomoniasis, about 8% of cases of trichomonal vaginitis are refractory to standard treatment. Most of the treatment failures are caused by *T. vaginalis* strains with lowered sus-

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ceptibility to metronidazole. Typically, the treatment-resistant isolates show low susceptibility to the drug *in vivo* and under aerobic conditions *in vitro*. The prevalence of metronidazole-resistant *T. vaginalis* seems to be increasing (Muller, 1983; Lossick, 1982). Furthermore, concern about mutagenic and carcinogenic potential of metronidazole has been raising (Johnson, 1993). On the basis of this information, it is clear that research for development of new antitrichomonad drugs should be given higher priority.

DNA topoisomerases are a unique class of enzymes that change the topological state of DNA by breaking and rejoining the phosphodiester backbone of DNA. DNA topoisomerases are involved in various DNA transactions such as replication, transcription, and recombination in both prokaryotes and eukaryotes (Wang and Wang, 1985; Osheroff, 1989). Based on their differences in their reaction mechanisms, DNA topoisomerases are classified into two types. Type I DNA topoisomerase changes the topological state of DNA by transiently breaking one strand of the DNA double helix and therefore characteristically changing the linking number in multiples of one. Type II DNA topoisomerase catalyses the strand-passing reaction by making a transient, enzyme-bridge, double strand breaks and consequently changing the linking number of DNA in multiples of two (Liu, 1989). As these processes are essential for multiplication of all cells, inhibition of these reactions will stop cell division and cell growth (Liu, 1989). DNA topoisomerases have been identified as the targets of chemotherapeutic compounds and it is proposed that they may also be possible targets for antitrichomonad drugs.

DNA topoisomerase II inhibitors were tested in the present study to determine the minimal inhibition concentration (MIC) against *T. vaginalis* in culture. The tested drugs are ellipticine, amsacrine (m-AMSA), fluoroquinolones, and metronidazole as a standard drug. Ellipticine, amsacrine (m-AMSA) are eukaryotic DNA topoisomerase II inhibitors. Flouroquinolones are inhibitors of DNA gyrase, a bacterial DNA topoisomerase II.

MATERIALS AND METHODS

Parasite

A local strain of *Trichomonas vaginalis* employed for this study was taken from a patient at Rajavithi Hospital, Bangkok, Thailand.

Cultivation of *Trichomonas vaginalis*

Trypticase yeast extract maltose (TYM) medium was used for culturing *Trichomonas vaginalis*. The medium consisted of trypticase (BBL) 4 g, yeast extract 2 g, maltose 1 g, ascorbic acid 0.2 g, KCl 0.2 g, $KHCO_3$ 0.2 g, K_2HPO_4 0.1 g, KH_2PO_4 0.2 g, agar (Bacto agar) 0.1 g. These ingredients were mixed with 180 ml H_2O, dissolved with heat and aliquot into screw-capped tube containing 7 ml of medium then all tubes were autoclaved.

Axenic cultures of *Trichomonas vaginalis*

A single colony of *T. vaginalis* was obtained aseptically by using agar plate culture technique (Hollander, 1976) and transferred to a screw-capped tube containing 7 ml TYM medium. Before using, 1 ml inactivated human serum was added to the medium as nutrient supplement. To prevent bacterial contamination of the culture, antibiotics (1,000 U/ml penicillin G potassium salt and 500 mg/ml streptomycin sulfate) were used. The cultures were then incubated at 37ºC. Subcultures were usually done every 2-3 days.

Drug preparation

m-AMSA and ellipticine were initially dissolved in absolute DMSO to give a concentration of 9 mM. Metronidazole was dissolved in sterile distilled water to give a concentration of 1mM. Fluoroquinolones (ofloxacin, norfloxacin and ciprofloxacin) were dissolved in distilled water to give a concentration of 10 mM. Drug solutions were freshly prepared before use by diluting with TYM medium to appropriate concentration.

Effects of DNA topoisomerase II inhibitors on *T. vaginalis*

Standard microtiter plates with 8 rows of 12 flat-bottom wells were used in the test. Various concentrations of the drug added in the wells in 50 ml aliquots. A 200 µl of suspension containing 5 x 10^4/ml *T. vaginalis* was placed into each well (Tachezy *et al*, 1993). All of the drug concentrations and controls were tested in duplicate. The plates were incubated at 37ºC. The plates were examined after 24 hours with an inverted microscope at x25 to search for motile trichomonads. The minimal inhibitory concentration (MIC) is defined as the lowest concentration of the drugs in which no motile organism was seen.

In order to observe the effect of DMSO on the parasite, an experiment was performed expos-

ing *T. vaginalis* to DMSO in ten-fold dilution for 24 hours. It was observed that DMSO 20% did not effect the motility of the parasite.

RESULTS AND DISCUSSION

A local strain of *T. vaginalis* was cultured axenically in TYM Diamond's Medium (Diamond, 1957) supplemented with 1% human serum. After culturing 2-3 days, *T. vaginalis* were observed under binocular microscope. In a freshly wet-mounted preparation, it can be observed that *T. vaginalis* were actively motile and usually pear-shaped. The organisms vary somewhat in size and shape and are most easily identified by their twitching motility. Physiochemical conditions do alter the appearance of the parasite. In axenic culture the shape of the protozoan tends to be uniform, *ie* pear shaped or oval (Fig 1). Under certain conditions, *T. vaginalis* can round up and internalize the flagellum. Some believe these forms to be pseudocysts, but it is more likely that they are degenerate forms of *T. vaginalis*, since they have not been reported to give rise to normal motile forms.

Giemsa staining demonstrates morphological characteristics of *T. vaginalis*. There are four free anterior flagella and the fifth posterior flagellum. Undulating membrane that extends about half way across the organism. A slender hyaline, rod-like structure, called an axostyle commences at the nucleus and bisects the protozoan longitudinally. It protrudes through the posterior of the parasite terminating in a sharp point. A prominent nucleus in *T. vaginalis* is located in its anterior portion.

Table 1 shows the minimal inhibitory concentrations (MIC) of m-AMSA, ellipticine, flouroquinolone antibiotics and metronidazole; Fig 2 shows the morphological effect of ellipticine. The local strain of *T. vaginalis* which was employed throughout this study is sensitive to metronidazole under aerobic conditions (MIC=0.096 µM). Aerobic resistant strains of *T. vaginalis* can attain MIC >100 µM *in vitro* (Lossick *et al*, 1986). Aerobic resistance to metronidazole can be developed *in vitro* under low drug pressure applied to the parasite for a long period of time. This fact is of concern with regard to the possibility of appearance of drug-resistant *T. vaginali*s due to inappropriate metronidazole regimens in patients (Tachezy *et al*, 1993).

Cosar and Julou (1959) first demonstrated

Table 1

Minimal inhibitory concentrations (MICs) of ellipticine, amsacrine, flouroquinolones and metronidazole against *Trichomonas vaginalis* in cultures.

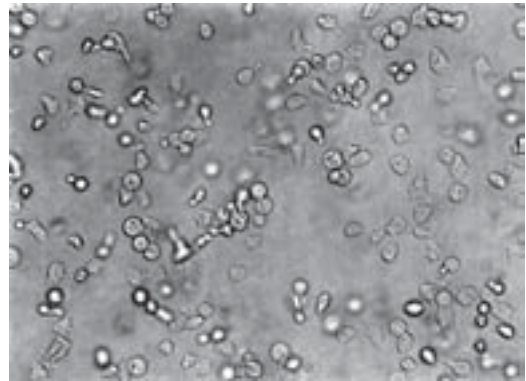| Drugs	Minimal inhibitory concentration (µM)
Ellipticine	6.4
Amsacrine	64
Ofloxacin	960
Norfloxacin	1,280
Ciprofloxacin	64
Metronidazole	0.096

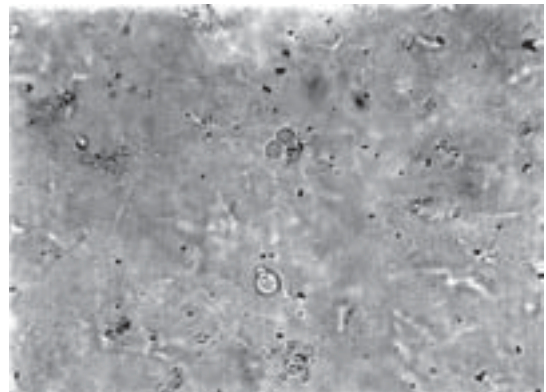Fig 1–The appearance of *Trichomonas vaginalis* in control well after incubation for 24 hours (x25).

Fig 2–The appearance of *Trichomonas vaginalis* exposed to minimal inhibitory concentration of ellipticine (6.4 mM) for 24 hours (x25).

antitrichomonad activity of metronidazole. When metronidazole was first introduced, cure rates approximated 95%, but within 2 years of its introduction, the first case of metronidazole resistance was reported in Canada. Nitroimidazole resistance has been reported in most areas of the world and, in recent years, the prevalence of metronidazole resistance seems to be increasing (Lossick, 1986; Muller, 1988).

It is therefore wise to consider other potential drugs for the treatment of trichomoniasis. Among DNA topoisomerase II inhibitors tested, ellipticine, a eukaryotic DNA topoisomerase II inhibitor, showed the lowest MIC against *T. vaginalis* (MIC=6.4 µM). The appearance of parasites in culture wells treated with MIC of ellipticine is demonstrated in Fig 2. Most of parasites were lysed and disappeared, only a few rounded parasites were observed. Amsacrine (m-AMSA), another eukaryotic DNA topoisomerase II inhibitor containing strong antitumor activity (Wilson *et al*, 1981) showed lower activity (MIC=64 µM) against *T. vaginalis.* Ellipticine is non intercalating agent whereas amsacrine is DNA intercalator (Pommier, 1993). However, as DNA topoisomerase II inhibitors, ellipticine and amsacrine are able to stimulate topoisomerase II-mediated DNA cleavage and induced fragmentation of chromosomal DNA in cells (Furlong *et al*, 1978; Ralph, 1980; Nelson *et al*, 1984). In addition, amsacrine also demonstrated antimalarial activity: the IC_{50} against *Plasmodium falciparum* K1 strain was 0.5 µM (Chavalitshewinkoon *et al*, 1993).

The effects of prokaryotic DNA topoisomerase II inhibitors, including ofloxacin, norfloxacin and ciprofloxacin, were also determined. MICs of ofloxacin, norfloxacin, and ciprofloxacin were 960 µM, 1,280 µM and 64 µM, respectively (Table 1). Ciprofloxacin was the most effective fluoroquinolone against *T. vaginalis in vitro*. However, its MIC was 10 times higher than that of ellipticine. Since the principal target of fluoroquinolones is prokaryotic DNA topoisomerase II (DNA gyrase), an essential bacterial enzyme which catalyses the introduction of a negative superhelical twist into closed covalently circular chromosomal and plasmid DNA within the bacterial cell (Drlica, 1984). In fact, *T. vaginalis* does not contain mitochondria but has a special organelle, hydrogenosome (Muller, 1980), and DNA is not present in this organelle (Johnson *et al*, 1993). Therefore, it seems that these fluoroquinolones do not have an appropriate target in *T. vaginalis* compared with *P. falciparum* which is sensitive to fluoroquinolones, and the mitochon-

drial DNA topoisomerase II of malaria parasite has been suggested as a drug target. However, further work needs to be done to show whether DNA topoio`smerase I or II possibly act as targets for trichomoniasis chemotherapy.

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