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Research paper

Role of disulfide linkage in action of bis(dialkylaminethiocarbonyl) disulfides as potent double-Edged microbicidal spermicide: Design, synthesis and biology☆

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ABSTRACT

Trichomoniasis and candidiasis are amongst the most common morbidity-causing reproductive tract infections, generally treated by Metronidazole and Fluconazole respectively. Poor vaginal efficacy, drug-resistance and non-spermicidal nature limit their use as topical microbicidal contraceptives. Bis(dialkylaminethiocarbonyl)disulfides (**4**–**38**) were designed as dually active, non-surfactant molecules capable of eliminating *Trichomonas vaginalis* and *Candida* strains as well as irreversibly immobilizing 100% human sperm instantly, at doses non-cytotoxic to human cervical epithelial cells and vaginal microflora *in vitro*. Compounds **12, 16, 17** were fifty times more active than nonoxynol-9, OTC vaginal spermicide, and compounds **12** and **17** have shown remarkable *in vivo* activity in rabbit model. Most promising compound **17** has shown promise for further development as a double-edged vaginal microbicide due to their improved activity and safety along with notable *in vivo* trichomonicidal activity. Role of disulfide group was established by loss of spermicidal activity on chemical modifications (**39**–**56**). These disulfides might be targeting thiol groups present over cell membrane of human sperm and *Trichomonas* as shown by fluorescence labeling of free thiols.

1. Introduction

Increasing sexually transmitted infections [1] (STIs) along with the population explosion is a global challenge [2–4] that cannot be overlooked. Growing drug-resistance of *Trichomonas* to Metronidazole (MTZ), and fungal strains to Fluconazole, is a cause of serious concern [5–6]. Furthermore, nonoxynol-9 (N-9), the OTC vaginal spermicide does not protect against STIs and HIV in clinical situations but may in fact enhances their incidences due to its non-specific surfactant action [7–9]. Most heterosexual women would like to reduce the risk of acquiring STIs [10] and control their fertility. Trichomoniasis, the most prevalent, non-viral STI, affects 250–350 million people worldwide every year causing serious discomfort to women along with associated problems of adverse pregnancy outcomes, pre-term delivery, low-birth weight infants, infertility, and cervical cancer.[11] It is now well established that trichomoniasis [12] extensively raises the vulnerability to HIV [13,14] and therefore controlling trichomoniasis alone could significantly reduce the incidence of new HIV infections. Similarly,

Abbreviations: STIs, sexually transmitted infections; N-9, nonoxynol-9; DTC, dithiocarbamate; DSF, disulfiram; SAR, structure activity relationship; MTZ, metronidazole; MOPS, [3-(N-morpholino)propanesulfonic acid]; LDH, lactate dehydrogenase; DMEM, dulbecco's modified eagle's medium; HCG, human chorionic gonadotrophin; TLC, thin layer chromatography.

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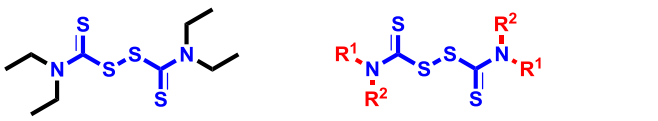
candidiasis caused by the fungus *Candida albicans*, is strongly associated with HIV-AIDS [15]. In spite of increased 'weapon store' for antifungal agents, currently available drugs do not suffice the growing demand of managing infections in complex patient populations [16, 17]. One of the major problems is increased drug resistance mainly due to chronic antimycotic therapy in HIV-infected and other immuno-compromised patients [18]. Usually, the female partner shoulders the primary responsibility of STI and pregnancy protection during most of the heterosexual contacts, including 'vulnerable' contacts amongst adolescents and promiscuous adults [19]. As prevention of pregnancy and infection is better than abortion and cure later, the best strategy would be to arrest the infection along with sperm in vagina during transmission, and therefore there is a need to develop a topically active medication against STIs (trichomoniasis and candidiasis) and sperm. Free thiol groups critically control the survival of predominantly anaerobic cells like *Trichomonas vaginalis* [20], *C. albicans* [21] and spermatozoa [22]. For example, the cleavage of disulfide bonds of sperm cell-specific hexokinase type 1 is associated with increased hexokinase activity and initiation of sperm motility [24]. The unique redox properties of protein thiols play an important role in enzyme catalysis, protein folding, and redox signaling, making it a key residue for chemical intervention [21,23] for manipulating cellular energy metabolism, motility and subsistence of *Trichomonas*, *Candida* and sperm cells. Consequently, sulfhydryl binding agents can impede sperm, *Trichomonas* and *Candida* cells to achieve prophylactic contraception as exemplified by *N*-ethyl maleimide [25], a specific sulfhydryl alkylating agent, acrylophenone [26], quinolines [27] and a variety of other thiol agents [28].

Dithiocarbamate (DTC) is a desirable pharmacophore in various medicinally significant compounds and is widely exploited in microbicidal spermicides [29–36], fungicides [37,38] and anti-HIV [39,40] agents. Thus, it was hypothesized that incorporating DTC and disulfide in a single chemical entity can make it to interact with multiple targets (*Trichomonas*, fungi and sperm) simultaneously. While investigating the DTC-disulfide hybrid framework, Disulfiram (DSF) molecule (Fig. 1) was found to be the most appropriate structure. Disulfiram is a FDA approved deterrent to alcohol abuse, which is current in clinical use [41]. DSF was synthesized and as expected it exhibited sperm immobilizing activity, which was mild. Encouragedby this observation it was thought worthwhile to modify DSF framework and to synthesize bis(N-substituted piperazinethiocarbonyl) disulfides (Fig. 1) as safe, multi-targeting microbicidal contraceptives.

2. Results and discussion

2.1. Chemistry

The compounds **4**–**38** have been synthesized [42] according to Scheme 1 using different sodium salts of dithiocarbamic acid (**3**). Secondary amine was reacted with carbon disulfide under alkaline condition to furnish sodium dialkylcarbamodithioate (**3**) which was further treated with sodium nitrite and hydrochloric acid at 0–5 °C in water to provide corresponding bis(dialkylaminethiocarbonyl) disulfides (**4**–**38**, Table 1).



Disulfiram (DSF) **General structure of compounds synthesized**

Fig. 1. Structures of Disulfiram and synthesized compounds (**4**–**38**).

2.2. Biological evaluation

2.2.1. Spermicidal activity

The spermicidal activity of compounds (**4**–**38**, Table 1) was evaluated in comparison to N-9. Twenty-five compounds (**4**, **7**–**10**, **12**–**18**, **20**–**25**, **28**, **31** and **33**–**37**) irreversibly immobilized 100% human sperm at concentration ranging from 1 to 0.001% (MEC) within 30 sec. Six compounds (**4**, **12**, **16**–**18** and **35**) were found to be more potent than commercially available spermicide N-9.

The results of the effect on sperm motility of compounds (**4**–**38**, Table 1) propose that if -NR^1R^2 was dimethyl amine (**4**) the compound showed moderate spermicidal activity (MEC, 0.01%). If methyl group was replaced by cyclohexyl (**5**) or benzyl (**6**), the activity was completely lost, while introduction of cyclic amine (**7**–**11**) further decreases spermicidal activity. Among amines with single nitrogen like pyrrolidine (**7**), piperidine (**8**), 4-methylpiperidine (**10**) and azepane (**11**) activity remains unchanged in five (**7**) and six (**8**, **10**) membered ring while decreases when ring size increases to seven (**11**). Incorporation of one oxygen atom (**9**) into compound **8** increases activity by 10 folds. Whereas introducing additional nitrogen atom in amino residue remarkably increased spermicidal activity (**12**–**18**, MEC 1–0.001%). Among the alkyl substituted piperazines (**12**–**15**) chain length determine the spermicidal activity i.e., bulkier the alkyl group lesser the activity. While a substitution of the alkyl group with allyl (**16**), butyronitrile (**17**) and morpholino alkyl (**18**) retained high activity. Furthermore presence of aryl/heteroaryl group at NR^1R^2in this framework decreased the spermicidal action (**19**–**23**, MEC 0.1 and 1%) while a benzoyl group imparted mild activity (**24**, MEC 0.5%). A decrease or complete loss of spermicidal effect was observed when carboxylate (**25**–**27**), mesyl (**28**), tosyl (**29**), alkyl/benzyl carbodithioate (**30**–**34** and **38**) groups were introduced at NR^1R^2 (Table 1). Interestingly replacement of alkyl carbodithioate group with alkyl amino carbodithioate increased the spermicidal action (**35**–**37**, MEC, 0.5–0.002%).

A close look at structure activity relationship (SAR) of bis(dialkylaminethiocarbonyl) disulfide (**4**–**38**) revealed that small alkyl group at N^4 position of piperazine (**12**, **16**, **17**, MEC 0.001%) is desirable for sperm immobilization activity.

2.2.2. Anti-Trichomonas activity

Seventeen compounds (**4**, **6**–**13**, **16**–**18**, **23**, **25**, **33**, **35** and **38**; Table 2) showed anti-trichomonal activity against Metronidazole (MTZ) susceptible strains with MIC ranging from 3.12 to 100 μg/mL (MTZ = 2.0 μg/mL), while fourteen compounds (**4**, **6**–**13**, **16**–**18**, **25** and **38**) among these exhibited trichomonicidal action against resistant strain at MIC 3.12–100 μg/mL (MTZ = 50.0 μg/mL). It is evident from the results (Table 2) that eleven compounds (**4**, **6**, **7**, **9**–**12**, **16**–**18** and **25**) illustrated better activity than MTZ against *Trichomonas* resistant strain. The comparison of anti-*Trichomonas* activity against susceptible and resistant strains revealed that MTZ lost its activity by 25 times against resistant strain while compounds (**4**, **7**, **9**, **10**, **17** and **25**) had better profile as there was no loss of activity.

The results of trichomonacidal activity against MTZ susceptible strain revealed that if -NR^1R^2 was alkyl substituted acyclic amines (**4**–**6**), dimethylamine (**4**) and benzylmethyl amine (**6**) were more preferred as their activity was comparable to standard drug MTZ. Whereas with cyclic amines (**7**–**11**) the activity was significant and pyrrolidine (**7**) and morpholine (**9**) seemed to be more desirable (MIC, 3.125 μg/mL) and an enhancement in ring size (**8**, **11**) resulted in decreased activity. An addition of a methyl group (**10**) in compound **8** at position 4 enhanced the activity by four fold. While a